Elucidation of metabolites in coloured wheat grains (*Triticum aestivum L.*) by HPLC-Orbitrap-MS/MS

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Á minha família, pelo amor e apoio incondicional.
Aos meus amigos, pela compreensão e motivação nos momentos mais difíceis.
Esta conquista é tão minha quanto vossa!

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Abstract

Recently, coloured-grain wheat varieties have garnered interest due to their potential benefits. Compared to common wheat, these varieties are richer in phytochemicals like anthocyanins, flavonoids, carotenoids, and other phenolic compounds, which offer important antioxidant and anti-inflammatory properties. In this study, we compared the phenolic/metabolic profile of four varieties of coloured wheat (*i.e.* blue, purple, dark and yellow colour) with conventional amber wheat using a high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) metabolomics approach. More than 100 metabolites were identified, revealing differences among samples. The quantification of anthocyanins via SRM (*Single Reaction Monitoring)* showed that purple, blue, and dark grain wheat varieties had higher levels of anthocyanins compared to red and yellow varieties, particularly cyanidin-, delphinidin-, and petunidin-3-O-glucoside. In general, the most significant differences were found in flavonoids, suggesting that the activation of the biosynthesis pathway of these compounds plays an important role in the grain colouring process. This study establishes a foundation for understanding flavonoid accumulation and the mechanisms behind grain coloration in coloured wheat, while also highlighting the potential benefits these varieties can offer.

Keywords: Coloured-grain wheat, high-resolution mass spectrometry, cerealomics.

Resumo

Recentemente, as variedades de trigo de grão colorido têm suscitado interesse devido aos seus potenciais benefícios. Em comparação com o trigo comum, estas variedades são mais ricas em fitoquímicos tais como antocianinas, flavonóides, carotenóides e outros compostos fenólicos, que possuem importantes propriedades antioxidantes e anti-inflamatórias. Neste estudo, comparámos o perfil fenólico/metabólico de quatro variedades de trigo colorido (nomeadamente, de cor azul, roxa, preta e amarela) com o trigo convencional de cor âmbar, utilizando uma abordagem baseada em cromatografia líquida de alta eficiência acoplada a espetrometria de massa (HPLC-ESI-MS/MS). Foram identificados mais de 100 metabolitos, observando-se diferenças entre as amostras. A quantificação de antocianinas através de SRM (*Single Reaction Monitoring)* mostrou que as variedades de trigo de grão roxo, azul e preto apresentam níveis mais elevados de antocianinas em comparação com as variedades vermelha e amarela, particularmente na cianidina-, delfinidina- e petunidina-3-O-glicosídeo. Em geral, as diferenças mais significativas foram encontradas ao nível dos flavonóides, sugerindo que a ativação da via de biossíntese destes compostos desempenha um papel importante no processo de coloração do grão. Este estudo estabelece uma base para a compreensão da acumulação de flavonóides e dos mecanismos subjacentes à coloração do grão no trigo colorido, ao mesmo tempo que realça os potenciais benefícios que estas variedades podem oferecer.

Palavras-chave: Trigo de grão colorido, espetrometria de massa de alta resolução, cerealómica.

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List of abbreviations

Abs	Absorbance
AGC	Automatic Gain Control
ChEBI	Chemical Entities of Biological Interest
FDA UNII – NLM	Food and Drug Administration – Unique Ingredient Identifier – National Library of Medicine
GA	Gallic acid
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MS	Mass Spectrometry
NIST	National Institute of Standards and Technology
ROS	Reactive Oxygen Species

1. Introduction

1.1. Coloured wheat

Wheat (*Triticum aestivum*) plays an important role in the human diet. It is used in the production of various food products (pasta, cookies, noodles, etc.) and its grain is considered a significant source of starch, protein, minerals, B-group vitamins and dietary fibres [1]. Wheat is thought to have been cultivated for the first time around 10 000 years ago, as part of the agricultural revolution. For around 8 000 years, it has been the main source of food for some of the world's major civilizations and today it remains the most common crop in the world, with around 772 million tons produced in more than 120 countries [2].

In general, amber-coloured wheat varieties continue to be consumed daily around the world. However, new sources of natural food are increasingly being explored, including naturally coloured foods that are rich in antioxidants. Thus, coloured wheat varieties have become increasingly popular due to the potential benefits they can bring to human health. These species are richer in phytochemicals such as anthocyanins, flavonoids, carotenoids and other phenolic compounds which, in addition to giving vibrant colours to the grains, are associated with various health benefits [2]. Coloured wheat varieties emerged in the 19th century by crossbreeding interspecies crosses of *Triticum aestivum* with different wild wheat species (*Triticum monococcum L. spp. aegilopoides*, *Thinopyrum ponticum* and *Thinopyrum bessarabicum*) [2].

Carotenoids and anthocyanins are the primary pigments responsible for wheat grain colour. For example, yellow wheat derives its colours from carotenoids expressed in the endosperm, while purple and blue hues result from anthocyanin accumulation in the pericarp and aleurone layers, respectively. In black wheat, which is the result of crossing purple wheat with blue wheat varieties, anthocyanins are present in both the pericarp and aleurone layers (*Figure 1*). It is important to note, however, that the phytochemical profile and, consequently, grain colour is highly dependent on genetic and environmental factors, growing conditions and technology used [2]. In particular, the anthocyanin content in coloured wheat grains is greatly influenced by various factors, including environmental conditions (such as climate, soil composition, sunlight exposure, and mycotoxin infections), agricultural practices (like nitrogen fertilization), technological

processes, and genotype. This underscores the need for evaluations across multiple harvests in the same location and a broader consideration of pigments [3, 4].

Phytochemicals, especially polyphenols, not only provide antioxidant benefits but also aid plant growth and reproduction, protecting crops from herbivores, microorganisms, and environmental stressors like cold, drought, and UV radiation [5]. Thus, coloured wheat varieties, because they are richer in polyphenols, like be more resistant to unfavourable environmental conditions or microorganisms, for example. As such, the study of these varieties is important from an agronomic perspective. The main challenge, however, lies in large-scale cultivation, as their yield is lower than that of commonly grown non-coloured varieties. This reduced yield is directly linked to the cultivation techniques, environmental conditions, and other factors that significantly impact the phytochemical profile of coloured wheat varieties [4].



Figure 1 - Wheat varieties under study: Bohemia (red grain), Bona Vita (yellow endosperm), Jumiko (purple pericarp), Oxana (blue aleurone layer) and Zora (black grain).

1.2. Polyphenols

Polyphenols are secondary metabolites found in plants, originating from the shikimate and phenylpropanoid pathways and characterized by the presence of one or more phenolic units, with no nitrogen-containing functions [6].

In plants, polyphenols serve various essential roles, including promoting growth and reproduction, defending against herbivores and pathogens, and protecting tissues from

stressors such as cold, drought, and UV irradiation. They also contribute to tissue pigmentation in flowers, grains, and fruits. In food products, polyphenols influence key sensory attributes like flavour, colour, aroma and oxidative stability [7]. Additionally, with growing interest in foods that offer functional and health benefits, polyphenols have garnered significant attention for their potential in preventing chronic conditions such as diabetes, cardiometabolic diseases, neurodegenerative disorders and cancer [8, 9].

1.2.1. Classification of polyphenols

Polyphenols are a large group of natural products, with more than 8 000 structural variants identified [6]. They are generally divided into four classes (namely phenolic acids, flavonoids, stilbenes and lignans), according to the number of phenolic rings they have and the elements that link these rings [10] (*Figure 2*). Note that polyphenols can exist either in free form (aglycone) or conjugated with sugar units, for example. To simplify, the classification of polyphenols is done according to the chemical structures of the aglycones [6].

Phenolic acids and flavonoids are the most abundant classes of phenolic compounds in wheat [5].

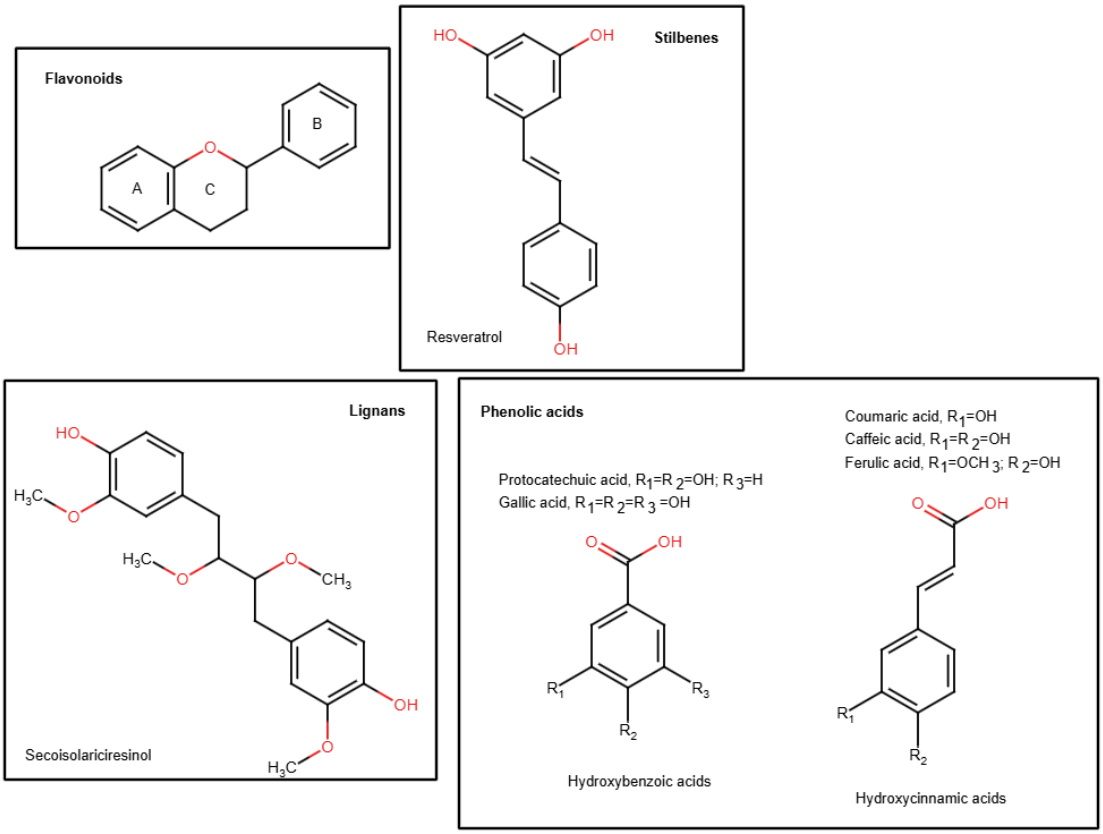


Figure 2 - The major classes of polyphenols.

Phenolic acids can be divided into two main types (hydroxybenzoic and hydroxycinnamic acid derivatives) based on their C1–C6 and C3–C6 backbones (*Figure 2*) [6].

According to previous studies, in wheat and especially in coloured wheat varieties, ferulic acid was found as the most abundant phenolic acid. Other phenolic acids identified are gallic, vanillic, syringic, protocatechuic, *p*-coumaric and caffeic acids [11, 12].

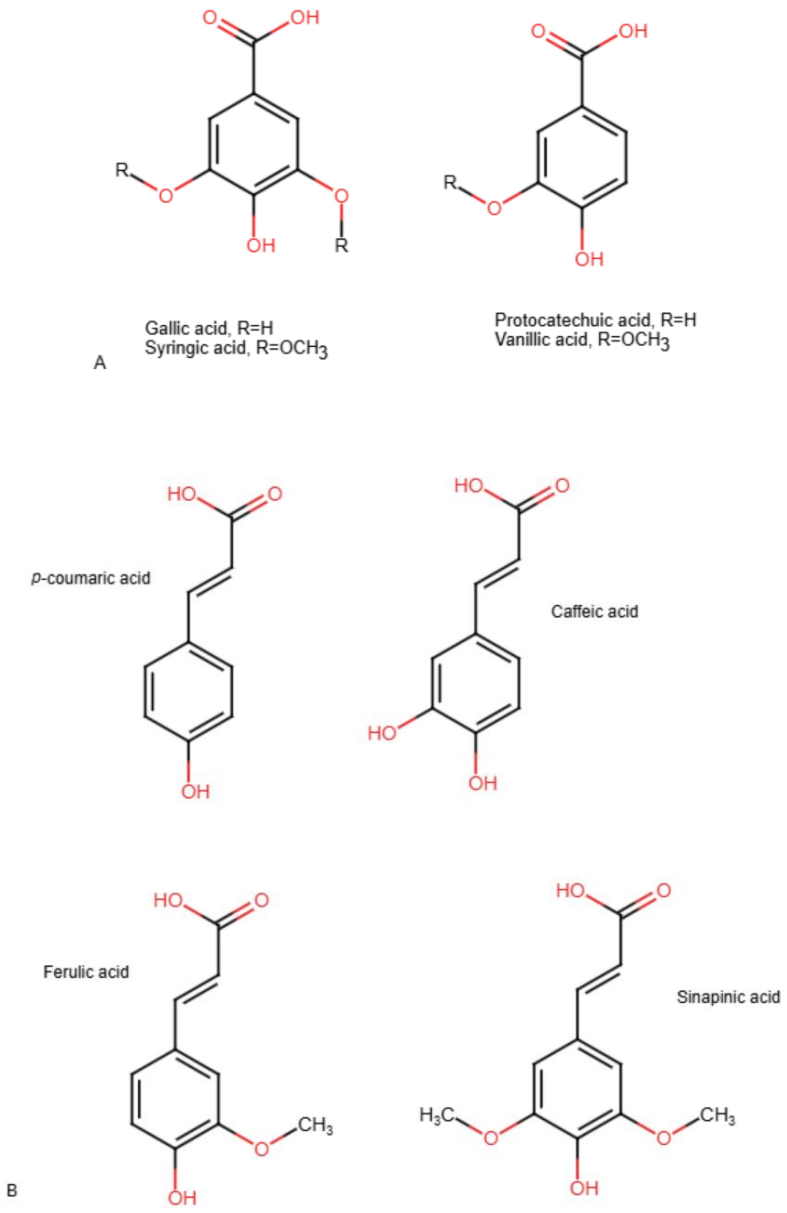


Figure 3 - Structures of typical phenolic acids in food. A- Benzoic acids, B-Cinnamic acids.

As for flavonoids, these are one of the most important classes of compounds in higher plants and is responsible for most of the blue, purple and emerald tones found in flowers, leaves, fruit and vegetables. Also, most of the yellow, orange and red colours that are not carotenoids belong to the flavonoid family [13, 14]. The general structure of these compounds includes two phenolic units (Ring A and B) linked by an oxygenated heterocycle (Ring C) (*Figure 2*). According to the type of heterocycle involved and its

substitution pattern, the flavonoid group can be divided into six subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols [15-18]. Other flavonoid subclasses of lesser dietary importance include chalcones, dihydrochalcones, dihydroflavonols, flavan-3,4-diols, coumarins, and aurones [19]. Within each subclass, compounds can differ in the position and number of hydroxyl, methoxyl and glycosyl groups and can also be substituted (e.g. glycosylated or acylated) [20].

Flavones, flavonols, flavanones and flavanonols are the most common flavonoid subgroups in the plant kingdom. More specifically, flavones and flavonols and their glycosides, methoxides and other acylated products constitute the major subgroup of all polyphenols [6]. Quercetin and kaempferol (*Figure 4*) are the most common flavonol aglycones and hundreds of different glycosidic combinations of these compounds are already known. The number of flavanones and their 3-hydroxylated derivatives (flavanonols, also known as dihydroflavonols) identified has also increased significantly in recent years [6]. Finally, some flavanones have unique substitution patterns, such as prenylated flavanones, furanoflavanones, pyranoflavanones and benzylated flavanones.

Examples of flavonoids already identified by Liu et al., 2010 in different coloured wheat varieties are glycosylated derivatives of apigenin, chrysoeriol, kaempferol, luteolin and quercetin [12]. Note that, this study, like other studies mentioned, focus only on specific compounds and analyse wheat varieties that have a particularly genetic blackground and are subject to determinate environmental conditions which, as already mentioned, influences its phytochemical profile.

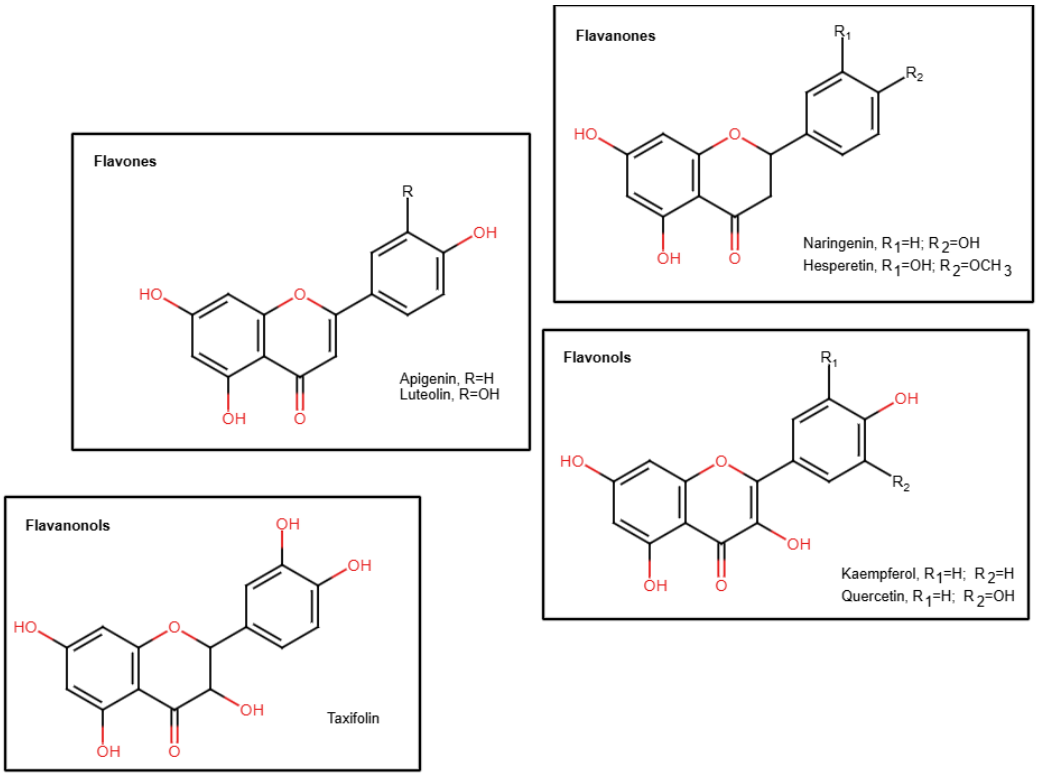


Figure 4 - Examples of flavones, flavonols, flavanones and flavanonols.

On the other hand, flavanols or flavan-3-ols are often referred to as catechins and, differently from most flavonoids, they don't have a double bond between C2 and C3, or carbonyl group in C4 [6]. This, combined with hydroxylation at C3, means that flavanols have two chiral centres (at C2 and C3), and therefore four possible diastereoisomers. Catechin is the isomer with the *trans* configuration and epicatechin is the isomer with the *cis* configuration and each of these two configurations has two stereoisomers, i.e. (+)-catechin, (-)-catechin, (+)-epicatechin and (-)-epicatechin [6]. The isomers most often found in food plants are (+)-catechin and (-)-epicatechin (*Figure 5*).

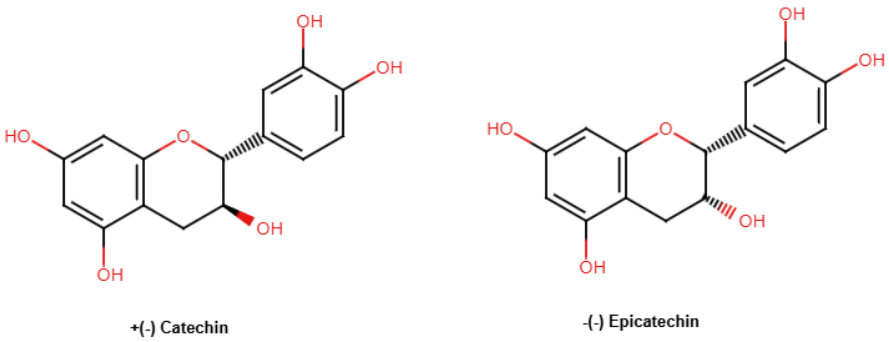


Figure 5 - Examples of flavanols.

Note that catechin and epicatechin are able of forming polymers (oligomeric/polymeric proanthocyanidins or condensed tannins). The degree of polymerization of proanthocyanidins can vary from dimers to around 200 monomeric flavanol units. Proanthocyanidins composed exclusively of (epi)catechin units are called procyanidins and are the most abundant type of proanthocyanidin in plants. In contrast, proanthocyanidins containing subunits of (epi)afzelechin or (epi)gallocatechin are the least common and are called propelargonidins and prodelphinidins, respectively [21]. Proanthocyanidins and flavanols can sometimes be esterified by gallic acid or, although rarely, by sugars.

Catechin, epicatechin and gallocatechin are examples of flavanols already identified in coloured wheat varieties and procyanidin A1 is an example of a proanthocyanidin [5, 22] (*Figure 6*).

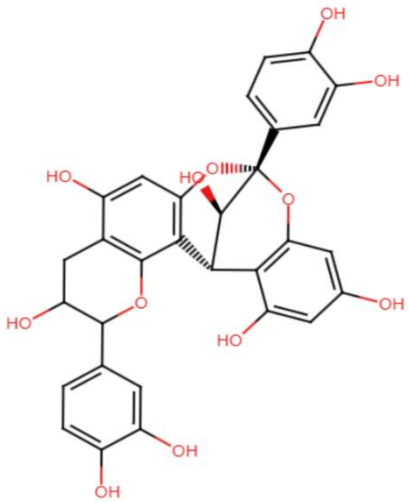
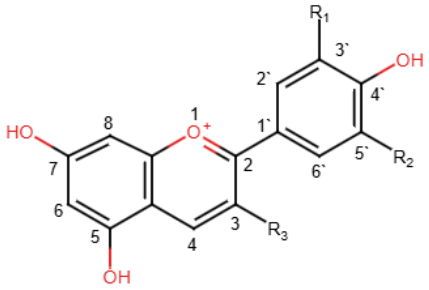


Figure 6 - Example of a proanthocyanidin (procyanidin A1).

On to another subclass of flavonoids, anthocyanidins are responsible for most of the red, blue and purple colours found in flower petals, fruits and vegetables, and certain special varieties of grains. In plants, anthocyanidins exist mainly in glycosidic forms which are frequently mentioned to as anthocyanins (*Figure 7*). A total of more than 500 anthocyanins are known which differ in their hydroxylation and methoxylation patterns in the Ring B and glycosylation with different sugar units [6, 23]. Most of these compounds are based on cyanidin, delphinidin and pelargonidin and their methylated derivates. Anthocyanins are chemically stable in acidic solutions and their colour is pH-dependent, *i.e.*, red in acidic and blue in basic aqueous media.

In the specific case of wheat, anthocyanins are expressed in the outer layers of the grain and play an important role in the development of its colour, as previously mentioned. In coloured wheat varieties, the anthocyanins already identified are essentially glycosylated derivatives of cyanidin, delphinidin, peonidin, petunidin, malvidin and pelargonidin [5]. The anthocyanins present in largest quantities in purple wheat are cyanidin 3-glucoside and peonidin 3-glucoside, with cyanidin 3-glucoside being the predominant anthocyanin. In blue wheat, the predominant anthocyanins are delphinidin-3-rutinoside, delphinidin-3-glucoside and malvidin-3-glucoside. The grain colour of black wheat is due to the anthocyanins present in the pericarp and aleurone layer [5]. This variety has a higher anthocyanin content compared to purple and blue wheat. Studies have concluded that: the total anthocyanin content of black wheat varies from 56 to 198 mg kg^{-1} dry kernel weight; for blue wheat anthocyanin content ranges between 17 and

211 mg kg^{-1} dry kernel weight; and in purple wheat it varies between 10 and 305 mg kg^{-1} dry wheat kernel [23].



Aglycone	R_1	R_2	R_3
Delphinidin	OH	OH	OH
Cyanidin	OH	H	OH
Petunidin	OCH_3	OH	OH
Peonidin	OCH_3	H	OH
Malvidin	OCH_3	OCH_3	OH
Pelargonidin	H	H	OH

Figure 7 - Major anthocyanidins.

In addition to the subclasses already mentioned, there are others that are not so relevant in the case of wheat, such as isoflavones, neoflavonoids and chalcones. Structurally, isoflavones have their ring B bounded to the C3 position of ring C. Isoflavones such as genistein and daidzein are mainly found in the form of 7-O-glucosides and 6”-O-malonyl-7-O-glucosides [6]. Neoflavonoids are not often found in plant-derived food products, the most common neoflavone being dalbergin. Open ring chalcones are most found in fruits such as apples and cherries [6].

Isoflavones such as formononetin and glucosylated genistein derivatives have already been identified in coloured wheat varieties [7] (*Figure 8*).

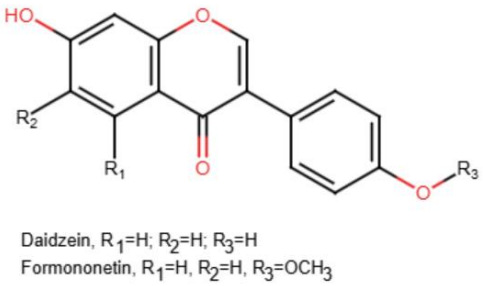


Figure 8 - Examples of isoflavones found in wheat.

1.2.2. Biosynthesis

All phenolic compounds are synthesized by plants from aromatic aminoacids derived from the shikimate pathway [6]. The aromatic ring B and chromane ring of flavonoids originate from the aminoacid phenylalanine, while the ring A originates from three malonyl-coA units. These units are added within sequential condensation and decarboxylation reactions, which mark the start of the flavonoid biosynthesis pathway [6].

In more detail, phenylalanine ammonia lyase catalyzes the conversion of phenylalanine into cinnamic acid, which leads to C6-C3 structures. Chalcone synthase then catalyzes the condensation of the final intermediate coumaroyl-CoA with three molecules of malonyl-coA, forming the first flavonoid structure, the naringenin chalcone. This molecule is then isomerized by chalcone flavanone isomerase into a flavanone (naringenin). All flavonoids are then formed from this flavanone (*Figure 9*). It should be noted that the various compounds are also formed from the naringenin chalcone. For example, intermediate flavanones (2S)-flavanones are converted by flavanone 3-hydroxylase into dihydroflavonols which, in turn, are reduced by dihydroflavonol reductase into flavan-3,4-diols (leucoanthocyanins). Finally, these compounds are converted into anthocyanidins by anthocyanidin synthase. The glycosylation of flavonoids is catalyzed by glucosyltransferase [6].

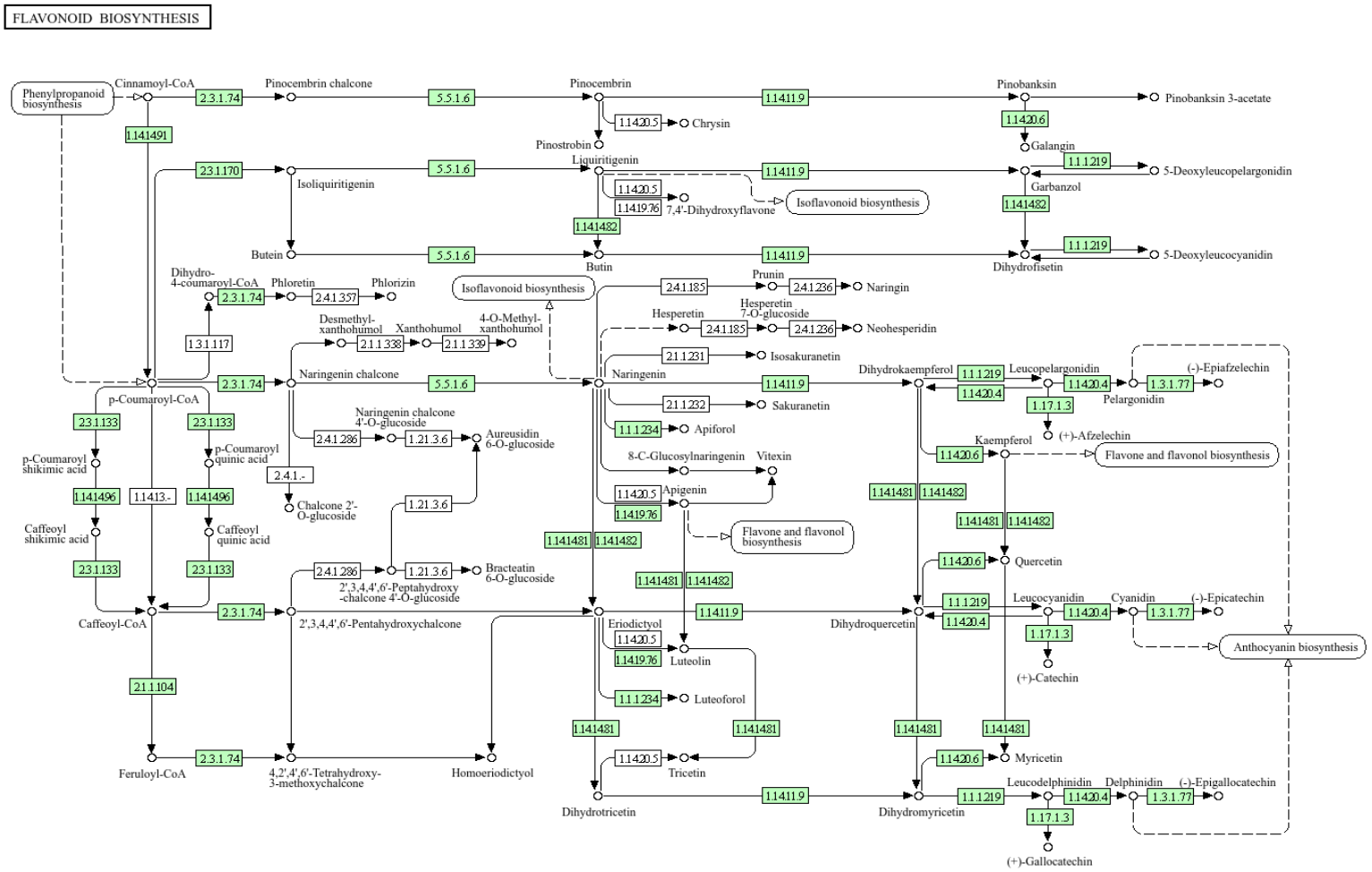


Figure 9 - The flavonoid biosynthetic pathway in plants. Adapted from *Kyoto Encyclopedia of Genes and Genomes* (KEGG).

Phenolic acids, on the other hand, are synthesized from either phenylalanine or tyrosine (*Figure 10*). The process begins with the deamination of phenylalanine and/or tyrosine, resulting in the formation of cinnamic acid and/or *p*-coumaric acid, respectively [24]. These aromatic rings are subsequently hydroxylated and methylated, producing derivatives such as ferulic and caffeic acids. The three key reactions involved in phenolic acid formation are deamination, hydroxylation, and methylation. Additionally, benzoic acid can be formed through the degradation of the cinnamic acid side chain. Similar hydroxylation and methylation reactions can occur on the aromatic ring of benzoic acid, leading to derivatives like *p*-hydroxybenzoic acids [24].

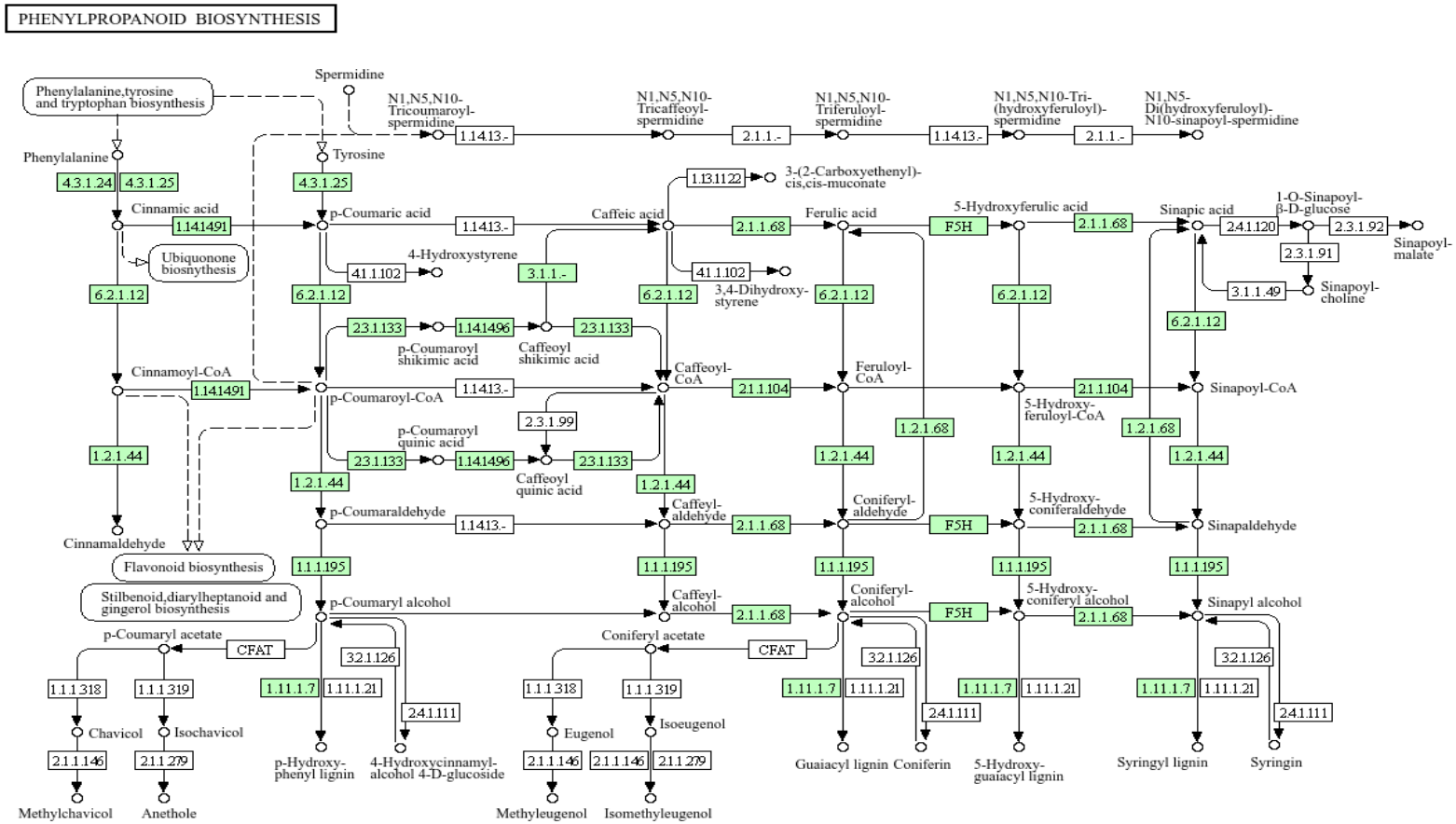


Figure 10 - The phenylpropanoid biosynthetic pathway in plants. Adapted from *Kyoto Encyclopedia of Genes and Genomes* (KEGG).

1.2.3. Potential human health benefits

As already mentioned, polyphenols are secondary metabolites that plants produce to protect themselves from other organisms/ abiotic stressors. Studies already carried out suggest that these compounds could also play an important role in human health. Eating high amounts of fruit, vegetables and whole grains, which are foods rich in polyphenols, has been associated with a reduced risk of developing many chronic diseases, such as cancer, cardiovascular disease, chronic inflammation and degenerative diseases [6]. According to recent studies, many of these diseases are related to oxidative stress caused by reactive oxygen and nitrogen species and phytochemicals, especially polyphenols, are strong antioxidants that can neutralize free radicals by donating an electron or a hydrogen atom. Polyphenols are also able of acting as metal ion chelators. The chelation of transition metals, such as Fe^{2+}, has the effect of reducing the rate of the Fenton reaction (oxidation of Fe^{2+} to Fe^{3+} by H_2O_2, which generates hydroxyl radicals), thus preventing oxidation caused by hydroxyl radicals, which are very reactive [25, 26].

In addition, in recent decades it has been realized that polyphenols are also able of acting as modifiers of signal transduction pathways, with implication in cell survival and death under both normal and pathological conditions (*Figure 11*) [27-30]. They are associated with the inhibition of certain enzymes involved in the production of ROS, such as xanthine oxidase (XO), NADPH oxidase (NOX) and nitric oxide synthase (iNOS) and enzymes involved in the metabolism of arachidonic acid, such as phospholipase A2 (PLA2), cyclooxygenase (COX) and lipoxygenase (LOX). By inhibiting the latter enzymes, polyphenols reduce the release of arachidonic acid, prostaglandins (PGs) and leukotrienes (LTs), which is one of their most important anti-inflammatory mechanisms [31, 32]. Polyphenols have also been shown to modulate the NF-κB and MAPKs signalling cascades and up-regulate phase II antioxidant enzymes such as hemeoxygenase-1 (HO-1), glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST), catalase and superoxide dismutase (SOD), targeting the common nuclear redox factor Nrf2 [32-34]. Activation of the transcription factor Nrf2 and the electrophilic response element EpRE (also called the antioxidant response element or ARE) to which Nrf2 binds are key protective mechanisms against chemically induced oxidative/electrophilic stress [35]. The beneficial effects that polyphenols can have on our health (including their antioxidant and anti-inflammatory activities), however, are always very dependent on the quantity consumed and bioavailability of these compounds. A compound can only exert its biological effect if it is absorbed [36].

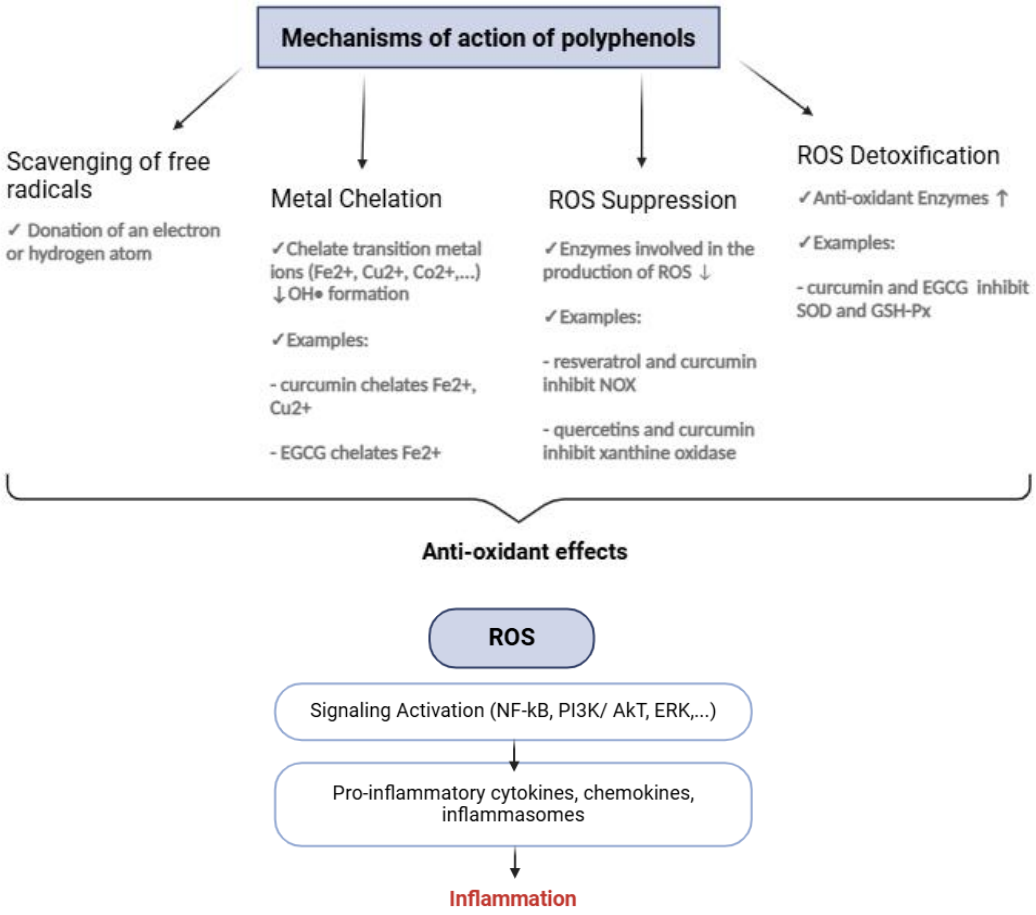


Figure 11 - Key polyphenolic actions. ROS, reactive oxygen species; NOX, NADPH oxidase; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase; ERK, extra-cellular signal regulated kinases; PI3K/AkT, phosphatidylinositide 3-kinases/protein kinase B; EGCG, epigallactocatechine gallate; NF-kB, factor nuclear kappa B.

1.3. Other important phytochemicals

1.3.1. Carotenoids

Carotenoids are pigments responsible for the yellow, orange and red colours found in fruits, vegetables and cereals, for example. Structurally, these compounds are 40-carbon isoprenoids with long conjugated polyene chains and, in cereals, whether they are in conjugated or free form is determined by genotype [1, 2].

In the case of yellow wheat, the identity of carotenoids is wide, such as lutein, alpha-carotene, beta-carotene, beta-apocarotenal, antheraxanthin, beta-cryptoxanthin, zeaxanthin, triticoxanthin, taraxanthin (lutein 5, 6-epoxide), and flavoxanthin. In general, lutein is the most plentiful among any remaining carotenoids, followed by zeaxanthin, antheraxanthin, alpha-carotene, and beta-carotene. In contrast, beta-cryptoxanthin is a minor part or not recognized. Some carotenoids, like alpha-carotene, beta-carotene, gamma-carotene, and beta-cryptoxanthin, are converted into retinol (vitamin A), while others, such as lutein and zeaxanthin, primarily act as antioxidants. The biological activity of these carotenoids, however, is affected by temperature and light [2].

This carotenoid profile is similar across blue, purple and black wheat varieties. There are some differences, however, in terms of the concentration at which these compounds are present. The blue wheat varieties, in studies already carried out, achieved a lower total carotenoid content compared to the red wheat. On the other hand, purple wheat has shown to have a total carotenoid content equivalent to or slightly higher than that common wheat varieties, thus suggesting that purple wheat varieties are similar in total carotenoid content to traditional wheat. Finally, black wheat has been described to show major total carotenoid content than red wheat varieties [37, 38]. To conclude, the most important differences between common wheat varieties and coloured wheat varieties will be in the profile of anthocyanins and other phenolic compounds and not in carotenoid content.

1.4. An overview of Metabolomics

Metabolomics is characterized by the identification and quantification of the metabolites present in a biological system [39]. Within metabolomics, a targeted or untargeted approaches can be adopted. In an untargeted approach, the objective is to detect as many metabolites as possible present in a sample, while in targeted metabolomics, the focus is limited to a predefined set of metabolites [40].

A metabolomics experiment involves different steps, including sample acquisition and preparation, the separation and detection of analytes, data extraction and mining, and comprehensive data analysis. In more detail, the identification and quantification of the metabolites will depend, in first, on sample preparation, also known as sample pre-

treatment or clean-up. The main objectives of this step are to reduce or remove interferences and undesirable compounds and concentrate the samples to increase sensitivity. In liquid chromatography-mass spectrometry (LC-MS)-based metabolite analyses, for example, one of the most widely used techniques for sample clean-up is the solid-phase extraction (SPE) [41].

There are many analytical methods that can be used to detect a diverse range of classes of molecules, each of which will be associated with specific limitations related to detection, sensitivity, dynamic range and quantification. In metabolomics, mass spectrometry (MS) and nuclear magnetic resonance (NMR) are the primary tools for analysing small molecules [42]. Note that, for separating target compounds, chromatographic methods like liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE), typically used in combination with MS, are commonly employed [41]. Reversed-phase high performance liquid chromatography (HPLC) coupled with a mass spectrometric detector (LC-MS) is the most widely use analytical technique for analysis of polyphenols.

Mass spectrometry (MS) is a highly sensitive technique capable of detecting, quantifying, and analyzing the structures of hundreds of metabolites in a single measurement. MS detection sensitivity and precision are, however, influenced by experimental parameters and instrument settings, including the methods used for metabolite extraction, separation, and ionization [41].

Key ionization techniques that enable compound detection and measurement include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and matrix-assisted laser desorption ionization (MALDI) (*Figure 12*). Through ionization, neutral molecules are given a charge, allowing them to be manipulated via electric, magnetic, or radio-frequency fields based on their mass-to-charge (m/z) ratio [41].

The most widely used technique for fragmenting ions is collision-induced dissociation (CID), although there are also other methods such as surface-induced dissociation (SID) and infrared multiphoton dissociation (IRMPD). Achieving high resolution and sensitivity for target compound detection often requires multiple MS detection modes, as increased sensitivity can reduce resolution, and vice versa [41].

Mass analyzers with a single configuration include quadrupole (Q), linear ion trap (LIT), quadrupole ion trap (QIT), time of flight (TOF), Fourier transform ion cyclotron resonance (FTICR), and Orbitrap. By utilizing hybrid configurations, metabolite identification can be improved through the generation of high-resolution, precise MS/MS spectra. For both

qualitative and quantitative metabolomics analysis, common tandem (MS/MS) analyzers include triple-quadrupole ion trap (QTrap), triple quadrupole (QqQ), quadrupole-time-of-flight (Q-TOF), and linear-quadrupole ion trap-Orbitrap (LTQ-Orbitrap) [41].

In targeted analysis, data processing and analysis are simplified by the availability of standards, which help refine the analytical method. This access to standards enables precise calibration and tailored strategies for accurately identifying and measuring specific compounds [41]. On the other hand, untargeted metabolomic analysis produces complex data sets that need to be processed using specialized software for accurate interpretation. Ideally, these software programs should be able to perform background spectral filtering, correctly assign peaks to the same compound (identifying matching m/z values and properly assigning adducts), align peaks across multiple samples, and, in the end, normalize these peaks (adjusting intensities and minimizing analytical drift) [40].

There are already many publicly available databases that can help identify compounds in the absence of standards such as the mass spectral libraries MassBank [43], mzCloud (https://www.mzcloud.org) and NIST (http://chemdata.nist.gov), the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) (https://www.kegg.jp/) or the chemical database ChemSpider (http://www.chemspider.com).

Finally, metabolomics has been applied in diverse areas, such as clinical analysis, environment, food and nutrition, forensic toxicology, among others.

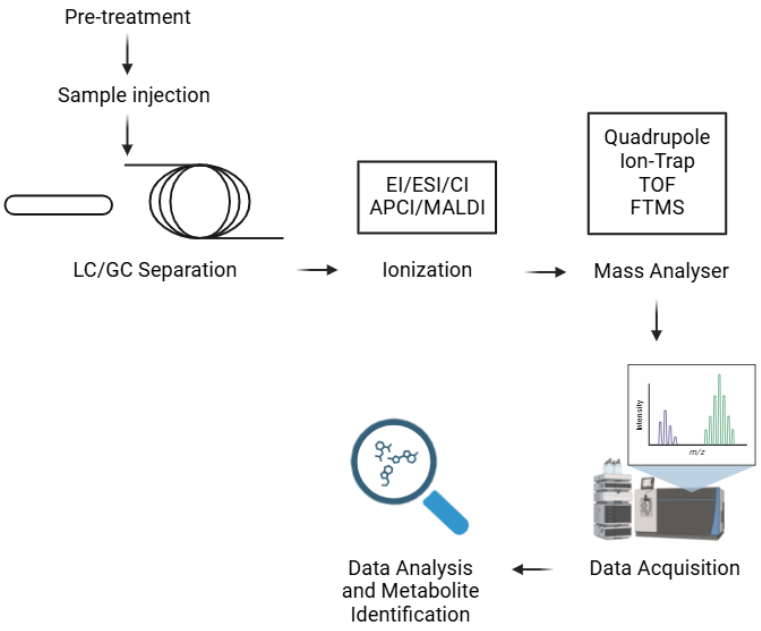


Figure 12 - General workflow for mass spectrometry based metabolomic analysis. GC, gas chromatography; LC, liquid chromatography; EI, electron impact ionization; CI, chemical ionization; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; MALDI, matrix assisted laser desorption ionization; TOF, time of flight; FTMS, Fourier transform mass spectrometry.

1.5. Objective of the study

Given the benefits that polyphenols can bring, and since it has already been proven in previous studies that coloured wheat varieties have a higher amount of phenolic compounds (especially anthocyanins) compared to more common wheat varieties, in this work we compared the phenolic/metabolic profile of different wheat genotypes grown in Czech Republic using a modern metabolomic workflow based on high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS). As mentioned above, most of the studies already carried out focus on specific compounds, and few analyse the metabolic differences between different varieties. A more comprehensive analysis, *i.e.* a large-scale detection, identification and quantification of the metabolites present in the different varieties of coloured wheat, will therefore be necessary to elucidate the metabolic differences that exist between them and clarify the potential benefits they could bring to our health and to industry.

2. Material and methods

2.1. Plant materials

During 2020/2021, five wheat genotypes were cultivated at the *Agricultural Research Institute* in Kroměříž, Czech Republic (49.2851172N, 17.3646269E). The experimental locate, positioned at 235 m above sea level, is characterized by Luvic Chernozem (Loamic) soil. The region experiences an average annual temperature of 9.2 °C, with mild winters, and receives approximately 576 mm of rainfall each year. Each genotype was grown on five separate plots, each measuring 10 m², following conventional growing

methods. The plots were harvested individually, and 1 kg of grain from each plot was collected to form a sample. These grains were stored at 22 °C in cloth bags, protected from light, until they were processed. Detailed characteristics of the wheat genotypes studied are presented in *Table 1*.

Table 1 - Characteristics of wheat (*Triticum aestivum L.*) genotypes. CZE, Czech Republic; SVK, Slovakia.

Cultivar	**Growth type**	**Country of origin**	**Cultivar status**	**Pedigree**	**Grain colour**
Bohemia	Winter	CZE	Released	(540i-92 × 6192a-92) × (540i-92 × Kontrast)	Amber
Bona Vita	Winter	SVK	Released	(SO-690 × Arida) × Arida	Yellow endosperm
AF Jumiko	Winter	CZE	Released (2018)	ANK-28A × Meritto	Purple pericarp
AF Oxana	Winter	CZE	Released (2019)	RU 440–6 × Ludwig	Blue aleurone
AF Zora	Winter	CZE	Released (2021)	(Skorpion × Bohemia) × (Indigo x Bohemia)	Black

2.2. Wheat flour milling

Wheat flours were produced using an Ultra Centrifugal Mill ZM 200, with a rotor speed of 15 000 rpm and screen aperture size of 250 µm (*Retsch Inc., Germany*). Note that, before the milling process, the grains were cleaned by sieving through a 2 mm sieve, followed by the manual removal of any impurities. To protect the integrity of the bioactive components, the flours were placed at -20 ºC immediately after milling and remained at this temperature until analysis.

2.3. Extraction of free phenolic compounds

The extraction of free phenolic compounds from wheat flour was carried out in triplicate and used a combination of solvent extraction, centrifugation and solid phase extraction (SPE). In detail, free phenolic compounds were extracted from 1 g of wheat flour using 15 mL of 80% ethanol containing 0.5% HCl (v/v). The suspension was stirred at room

temperature for 45–60 min and then centrifuged at 3 000 g for 10 min (4 ºC). This extraction was carried out two more times: first with the same acidified ethanol solution and then with 15 mL of 70% acetone (v/v). The supernatants from all extractions were combined and concentrated by evaporating the solvents (acetone and ethanol) using a rotary evaporator set at 37 ºC.

Following evaporation, the extracts were stored at 4 ºC overnight and centrifuged at 15 000 g for 10 min (4 ºC), for protein removal. The subsequent supernatant was filtered through 0.45 µm CHROMAFIL®PET syringe filters (MACHEREY-NAGEL) and subjected to solid-phase extraction using 35 cc Oasis HLB cartridges (Waters), according to manufacturer's protocol. The SPE cartridges were activated with 50 mL of methanol and equilibrated with 50 mL of ultrapure water. The sample was diluted to a final volume of 100 mL, washed with 40 mL of ultrapure water, and the phenolic compounds were then eluted using 60 mL of a 50:50 (v/v) methanol: acetonitrile solution. Finally, the eluted fraction was evaporated, freeze-dried, and stored at -20 ºC until analysis.

2.4. Extraction of conjugated phenolic compounds

For extraction of conjugated phenolic compounds, the residues resulting from the extraction of free phenolic compounds were submitted to alkaline and acid hydrolyses, respectively. In more detail, the pellets (~1 g) were resuspended with 5 mL of 10M NaOH and 12 mL of ultrapure water. The mixture was hydrolysed for 16 hours at room temperature and under stirring. Then, the pH was adjusted to 2.0 with 7 mL of 20M HCl and re-extracted with 15 mL of ethyl acetate for 3 times (centrifugations at 2 500 g for 10 min at 4 ºC). The final supernatants were, after, pooled, evaporated and reconstituted to 2 mL with methanol. In order hand, the pellets resulting from alkaline hydrolyse were resuspended with 2.5 mL of concentrated HCl. The mixture was hydrolysed for 30 min at 85 ºC and then, after cooling, 3 extractions were carried out with 20 mL of ethyl acetate (centrifugations at 2 500 g for 10 min at 4 ºC). The supernatants resulting from the acid hydrolysis were also pooled, evaporated and reconstituted to 2 mL with methanol. Finally, the products of alkaline and acid hydrolysis were filtered with a 0.45 µm pore size membrane (MACHEREY-NAGEL) and stored at –20 ºC until analysis.

2.5. Determination of total phenolic content

The Folin-Ciocalteu method, previously described by Kim et al., 2006 [44], was used to determine the total phenolic content in the extracts. Firstly, samples were prepared at a concentration of 5 mg/mL in 10% methanol. Then, 50 µL of the sample was combined with 50 µL of Folin-Ciocalteu's reagent (1:5), followed by 100 µL of 6% sodium carbonate. The plate was kept at room temperature in the dark for 30 minutes. Afterward, the absorbance was measure at 765 nm using a *FlexStation™ 3 Multi-Mode Microplate Reader* (Molecular Devices), and the phenolic content was calculated using gallic acid as the reference standard.

2.6. LC-MS/MS analysis of phenolic compounds

A Vanquish HPLC system, contained an Accucore™ C18 HPLC column (particle size 2.6 µm, 2.1 × 150 mm), and linked to a hybrid high-resolution Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific) was used to LC-MS/MS analysis. The gradient elution was executed according to the following program: 0 – 60 min 0 –100% water/acetonitrile/acetic acid (79:20:1, v/v/v), 70 – 85 min 95% acetronitrile, and 87–105 min 100% water/acetic acid (99:1, v/v). The column was kept at 25 °C, the flow rate was set at 0.3 mL/min, and 25 µL (250 µg) of sample was injected. Samples were left at 5 °C in the autosampler.

The resolution of full scans was set to 60 000, AGC target was set to standard, and the maximum injection time was set to automatic for mass spectrometry data acquisition. The electrospray ionization (ESI) capillary voltage was 2 500 V in negative ion mode and 3 500 V in positive ion mode, with the tube lens set at 70%. Capillary temperature was maintained at 300 ºC and the sheath, auxiliary, and sweep gas flow rates were 50, 10, and 1, respectively. Spectra were recorded over a mass range of 150 to 1000 m/z.

For confirmation purposes, in addition to the full scan acquisition method, a data-dependent MS/MS analysis was performed using higher-energy collisional dissociation (HCD) with a stepped collision energy mode of 30, 45, and 70% and an isolation window of 2 m/z. The resolution and AGC target for MS/MS were set to 15 000 and standard, respectively. Two scan filters were applied between each full MS and MS/MS event: an

intensity threshold filter of 1 x 10^6, and a charge state filter that excluded multiply charged ions.

To specifically target anthocyanins in positive ion mode, Single Reaction Monitoring (SRM) experiments were performed, with an Orbitrap resolution of 120 000, a stepped collision energy mode of 45%, and an isolation window of 0.8 m/z. The following transitions were monitored: cyanidin 3-O-glucoside m/z 449.108 → 287.06, cyanidin 3-O-rutinoside m/z 595.166 → 287.06, cyanidin O-malonyl-hexoside m/z 535.109 → 287.06, delphinidin 3-O-glucoside m/z 465.103 → 303.05, delphinidin O-rutinoside m/z 611.161 → 303.05, peonidin 3-O-glucoside m/z 463.124 → 301.07, peonidin O-rutinoside m/z 609.182 → 301.07, peonidin O-malonyl-hexoside m/z 549.124 → 301.07, petunidin 3-O-glucoside m/z 479.119 → 317.07, petunidin O-rutinoside m/z 625.177 → 317.07, malvidin 3-O-glucoside m/z 493.134 → 331.08, pelargonidin 3-O-glucoside m/z 433.113 → 271.06, pelargonidin O-rutinoside m/z 579.171 → 271.06, and pelargonidin O-malonyl-hexoside m/z 519.113 → 271.06 [3]. External standards of cyanidin 3-O-glucoside, delphinidin 3-O-glucoside and petunidin 3-O-glucoside were used to establish calibration curves for the quantification of these analytes and their derivatives. The concentrations of the standards considered for the construction of the calibration curves were 0.001, 0.01, 0.1, 1, 5, 10 and 50 µg/mL.

2.7. Wheat phenolics profiling

Compound Discoverer software (*v. 3.3.1.11*, Thermo Scientific) was used to processed MS/MS data from real and blank samples with a workflow composed by various steps: a) spectrum processing nodes which extract the mass spectral data from input set files and align retention times of the compounds in the sequence, based on an adaptive curve and maximum shift of 1.5 min; b) compound detection nodes to detect and group compounds by molecular weight, retention time and chromatographic S/N ration, with a tolerance of 10 ppm, minimum number of 3 scans per peak, peak rating threshold of 4 in at least 2 replicate files, retention time tolerance of 1 min and maximum element count of C100 H150 O50 N10; c) compound identification and scoring nodes (mzLogic) based on mzCloud, ChemSpider (BioCyc, ChEBI, FDA UNII - NLM, KEGG, MassBank, NIST), and *in-house*-built mass libraries; and d) peak area refinement and fill

gap nodes which apply QC (quality control) correction, manage missing values and remove background compounds.

2.7.1. Creation of a wheat metabolite library

Although databases like mzCloud or MassBank have been created for metabolite annotation using MS/MS data, plant metabolomics still encounters major hurdles. These include the underrepresentation of plant metabolites and the risk of misidentifying them as non-plant compounds [45]. Thus, and before the profiling process described above, a wheat metabolite library was created for untargeted metabolomics. To begin, an exhaustive literature search was carried out and the results of various studies were compiled in which many compounds present in wheat and other related species (*e.g. Oryza sativa*, *Hordeum vulgare*, *Passiflora incarnata*, *Passiflora caerulea* and *Passiflora alata*) were identified by LC-MS/MS. In more detail, a mass list was manually created containing the name, chemical formula, molecular weight and chemical structure of the compounds identified by Cerrato et al., 2020 [46], Tais et al., 2021 [47], Ed Nignpense et al., 2022 [48], Ozarowski et al., 2018 [49], Lee et al., 2023 [50], Barros Santos et al., 2022 [51], Wang et al., 2020 [5], Razgonova et al., 2021 [20], Dinelli et al., 2011 [52], Wojakowska et al., 2013 [53] and Shahidi et al., 2022 [54].

In total, the mass list contained 359 compounds, namely 300 phenolic compounds, 20 organic acids, 32 peptides and aminoacids, 4 carbohydrates, 1 essential nutrient (choline) and 2 phospholipids. As it can be seen from *Figure S1* in appendix, phenolic compounds made up most of the compounds. From these, 239 flavonoids were identified, 48 phenolic acids and 13 other phenolic compounds. To validate the mass list built *in-house*, the extracts from coloured wheat grains were used for untargeted metabolomics analyses. The obtained raw data files were submitted to Compound Discoverer for database searching using the library created in this study and mzCloud. The identification was based on MS/MS spectral matching, and, in the end, the spectral library was consolidated in the mzVault format. In conclusion, we obtained an mzVault spectral library with 82 compounds, specifically 66 phenolic compounds, 7 organic acids and 9 peptides and aminoacids. Of the phenolic compounds identified, 40 were grouped as flavonoids, 23 as phenolic acids and 3 as others (*Figure S2,* in appendix).

2.8. Statistical analysis

Single Reaction Monitoring (SRM) experiments for the analysis of anthocyanins were performed in duplicate. On par with the determination of the general anthocyanin profiles (which gave rise to a heatmap which shows the logarithmic value of the average areas for each anthocyanin and for each wheat variety), the absolute concentration of these compounds was determined for each sample by establishing calibration curves. These calibration curves were constructed using external standards of cyanidin 3-O-glucoside, delphinidin 3-O-glucoside and petunidin 3-O-glucoside, as mentioned above. The concentrations of cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside and cyanidin O-malonyl-hexoside were determined for each sample considering the calibration curve of cyanidin 3-O-glucoside. On the other hand, the concentrations of dephinidin 3-O-glucoside, dephinidin O-rutinoside, peonidin 3-O-glucoside, peonidin O-rutinoside and peonidin O-malonyl-hexoside were determined through the calibration curve of delphinidin 3-O-glucoside. Finally, the calibration curve for petunidin 3-O-glucoside was used to determine the concentration of petunidin 3-O-glucoside, petunidin O-rutinoside, malvidin 3-O-glucoside, pelargonidin 3-O-glucoside, pelargonidin O-rutinoside and pelargonidin O-malonyl-hexoside. In the end, concentration values were converted to µg/Kg dry weight. Note that the areas of the peaks were determined using the Xcalibur™ software (Thermo Scientific).

The full scan + ddMS^2 analysis was carried out in triplicate, and the data were processed using the Compound Discoverer software (*v. 3.3.1.11*, Thermo Scientific), as previously described. The compounds identified were then grouped according to the classes to which they belonged, and their relative abundance was determined for each wheat variety using the ratio between the peak area (average of the areas obtained in the three replicas) and the TIC (*Total Ion Current*). Note that TIC is the sum of the relative abundances of all the ions in each mass spectrum and, thus, by dividing the area of an individual peak by this parameter, we normalize the results. This normalization was important to eliminate matrix effects and correct for technical variations (such as fluctuations in ion flow or variations in the efficiency of the ionization system), which made the results comparable. In the end, the *t*-test was used to determine whether the differences between coloured varieties (Bona Vita, Jumiko, Oxana and Zora) and non-coloured variety (Bohemia) were statistically significant or not. They were considered

statistically relevant when *p*-value < 0.05. GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was used for graph drawing.

3. Results and discussion

3.1. Total phenolic content

Results from the determination of free, conjugated and total phenolic content in wheat extracts can be found in *Table 2*. Bohemia was found to be the variety with the highest content in free phenolics, followed by Zora and Jumiko. Note that, in general, higher free phenolic contents correlated with higher extraction yields (calculated by the ratio between the mass of flour from which the free phenolic compounds were extracted and the mass of extract obtained after freeze-drying). As to bound phenolic content, it was found to be higher than free phenolic content in all varieties. Note that, in plants, phenolic compounds can be present in a free form (aglycone), esterified (with sugar units, for example) or in an insoluble form (conjugated to macromolecules like polysaccharides found in cell walls). Thus, it was found that most of the phenolic compounds in the varieties under study were complexed with other biomolecules, what is common in plants for structural and defence functions. The highest bound phenolic content and total phenolic content (sum of bound and free phenolics content) was found in Jumiko and Zora.

These results show that there are differences in polyphenol content between the different varieties studied. However, since the colorimetric method used does not allow us to the specific composition of the extracts, the phytochemical profile of the five varieties under study was analysed by LC-MS/MS. In addition to not allowing us to know the specific composition of the samples, Folin-Ciocalteu method results are influenced by the different reactivity of phenolic compounds and by the fact of many compounds that can act as interfering agents (such as thiols, amino acids, proteins, some vitamins, and inorganic ions) [55].

Table 2 - Comparison of recovery yield, free phenolic content, bound phenolic content, ratio and de sum of this contents (expressed in µg of GAE per (milli)gram of polyphenol-rich extract or dry weight matter) between coloured and non-coloured wheat flours. Data is presented as mean values ± standard deviation.

Wheat flour	Yield (%) (free phenolics extraction)	Free phenolic content (µg GAE[1]/g dry weight)	Bound phenolic content (µg GAE [2]/g dry weight)	Ratio bound:free phenolics	Sum (bound + free phenolics)
Bohemia	1.15 ± 0.57	609 ± 22	1868 ± 280	3.1	2477 ± 281
Bona Vita	0.67 ± 0.23	388 ± 13	1878 ± 124	4.8	2265 ± 125
AF Jumiko	0.73 ± 0.24	550 ± 14	2147 ± 49	3.9	2698 ± 51
AF Oxana	0.81 ± 0.21	294 ± 8	1941 ± 174	6.6	2235 ± 174
AF Zora	0.73 ± 0.02	593 ± 12	2077 ± 74	3.5	2670 ± 75

[1] Linear correlation: Abs @765 nm = 9.598 x [gallic acid] (0.00 - 0.25 mg/mL), R^2 = 0.9989, LOD = 0.01 mg/mL, LOQ = 0.04 mg/mL.

[2] Linear correlation: Abs @765 nm = 9.530 x [gallic acid] (0.00 - 0.25 mg/mL), R^2 = 0.9954, LOD = 0.03 mg/mL, LOQ = 0.09 mg/mL.

3.2. Anthocyanin profiling and quantification

The presence of 14 different cyanidin, delphinidin, peonidin, petunidin, malvidin and pelargonidin glucosyl derivatives was confirmed by SRM (*Figure 13*). As expected, we observed that purple (Jumiko), blue (Oxana) or black (Zora) varieties are richer in anthocyanins compared to red (Bohemia) or yellow (Bona Vita) varieties. The major differences were in the cyanidin-, delphinidin- and petunidin-3-O-glucoside content. Cyanidin-3-O-glucoside was found to be the dominant anthocyanin in purple wheat, while delphinidin-3-O-glucoside was the main in blue variety. In black wheat predominated the cyanidin-3-O-glucoside and delphinidin-3-O-glucoside (*Table 3*).

In plants, anthocyanins act as a defence mechanism against harsh conditions like extreme temperatures, drought, or high salinity by preventing lipid oxidation and shielding the plasma membrane from harm. Additionally, because pathogen infections often induce oxidative stress, anthocyanins may contribute to improving a plant's tolerance to such environmental stress factors [56].

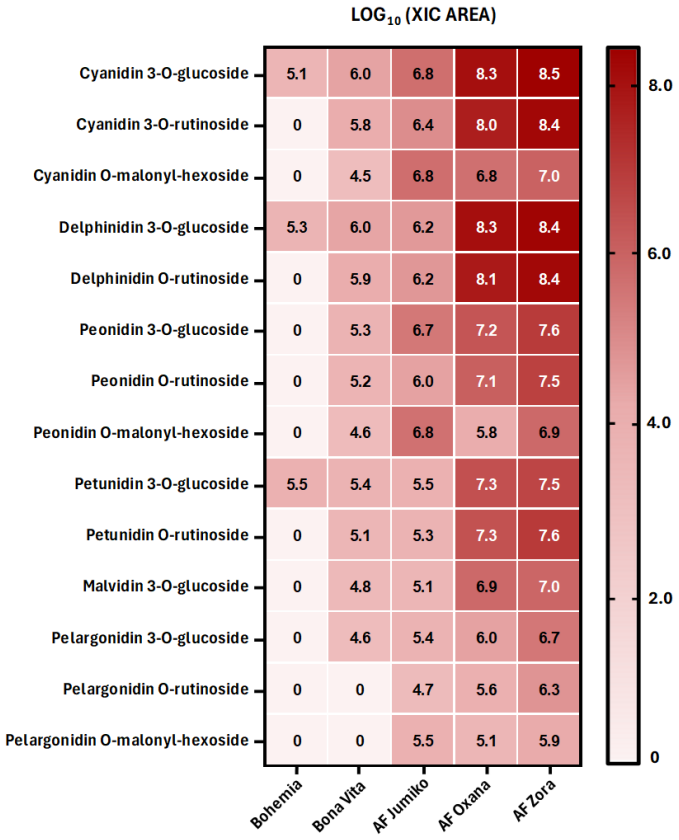


Figure 13 - Heatmap analysis of anthocyanin occurrence in pigmented vs non-pigmented wheat grains. XIC, extracted ion chromatogram. Logarithmic values do not account for differences in anthocyanin ionization and response in the mass spectrometer.

Table 3 - Quantification of cyanidin 3-O-glucoside, delphinidin 3-O-glucoside, and petunidin 3-O-glucoside content (expressed in µg of anthocyanin per kilogram of dry weight matter) in pigmented and non-pigmented wheat flours. Data is presented as mean values ± standard deviation.

Wheat variety	**Cyanidin 3-O-glucoside [1] (µg/Kg dry weight)**	**Delphinidin 3-O-glucoside [2] (µg/Kg dry weight)**	**Petunidin 3-O-glucoside [3] (µg/Kg dry weight)**
Bohemia	2.0 ± 1.0	2.9 ± 1.6	5.2 ± 4.2
Bona Vita	8.9 ± 0.0	9.1 ± 3.1	2.1 ± 0.6
AF Jumiko	60.5 ± 6.2	14.3 ± 0.7	3.1 ± 0.5
AF Oxana	1889 ± 32	2085 ± 173	207 ± 13
AF Zora	2740 ± 221	2718 ± 543	278 ± 52

[1] Linear correlation: XIC area = 1.62 x 10^8 x [cyanidin 3-O-glucoside] (0.001 - 10 µg/mL), R^2 = 0.9994, LOD = 0.42 µg/mL, LOQ = 1.26 µg/mL.

[2] Linear correlation: XIC area = 1.42 x 10^8 x [delphinidin 3-O-glucoside] (0.001 - 10 µg/mL), R^2 = 0.9995, LOD = 0.39 µg/mL, LOQ = 1.17 µg/mL.

[3] Linear correlation: XIC area = 1.40 x 10^8 x [petunidin 3-O-glucoside] (0.001 - 10 µg/mL), R^2 = 1, LOD = 0.09 µg/mL, LOQ = 0.28 µg/mL.

3.3. Metabolic profiling

In addition to the 14 anthocyanins, 105 other compounds were identified, namely 2 hydroquinones, 33 phenolic acids, 35 flavonoides, 19 organic acids, 1 nucleoside, 2 vitamins, 1 benzoxazinoides and 12 aminoacids/peptides (*Figure 14*). Phenolic compounds, in general, showed the highest relative abundance in the wheat varieties studied (*Figure 15*).

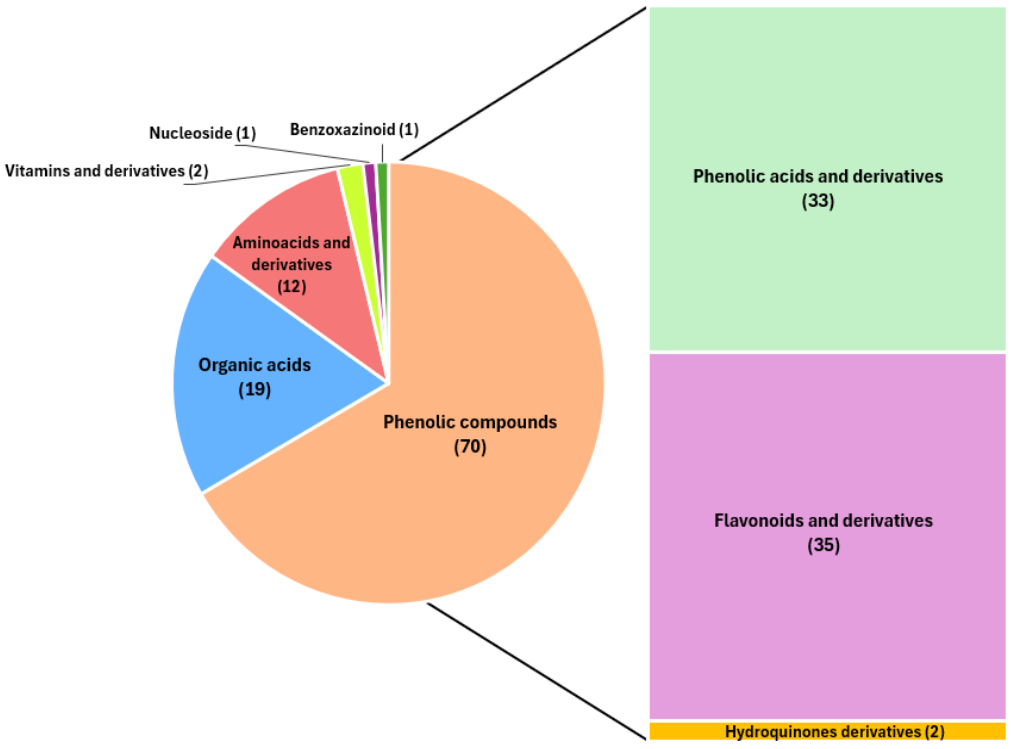


Figure 14 - Distribution of compound classes identified in the phytochemical profiling analysis.

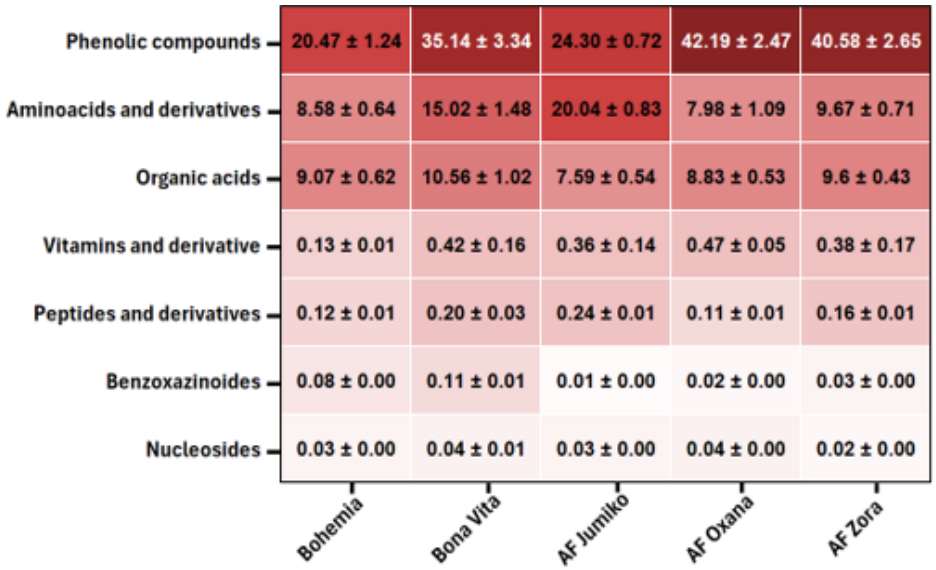


Figure 15 - Heatmap analysis of relative abundance of different classes of compound in wheat varieties. Data is presented as relative abundance values ± standard deviation.

3.3.1. Phenolic compounds

Within phenolic compounds, phenolic acids and flavonoids were the subclasses with major relative abundance, which is in line with was described by Wang et al., 2020 in metabolic analysis carried out with coloured wheat varieties originating in China [5] (*Figure 16*).

Starting with phenolic acids, the number of hydroxycinnamic acids (16) identified was higher than the number of hydroxybenzoic acids (10). Jumiko, Oxana and Zora were the varieties with the highest relative abundance of phenolic acids (*Figure 16*).

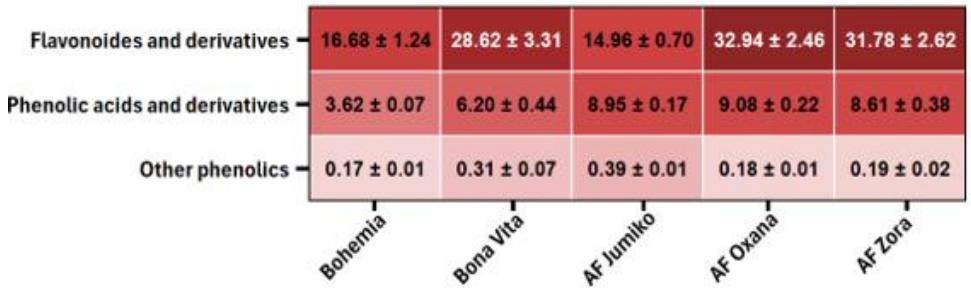


Figure 16 - Heatmap analysis of relative abundance of different subclasses of phenolic compound in wheat varieties. Data is presented as relative abundance values ± standard deviation.

3.3.1.1. Hydroxybenzoic acids

Dihydroxybenzoic acid was the phenolic acid with the higher relative abundance in Jumiko and Zora and the hydroxybenzoic acid with the major relative abundance in all the varieties (*Figure 17*). In this compound, there were statistically significant differences between varieties, as it was present higher values in Jumiko and Zora. There were important differences also in one of its derivatives, hydroxybenzoic acid hexoside, which was the hydroxybenzoic acid with the second highest relative abundance in Zora, Oxana and Bona Vita (*Figure 17*). Their values decreased in the following order: Bona Vita > Zora > Oxana > Jumiko > Bohemia.

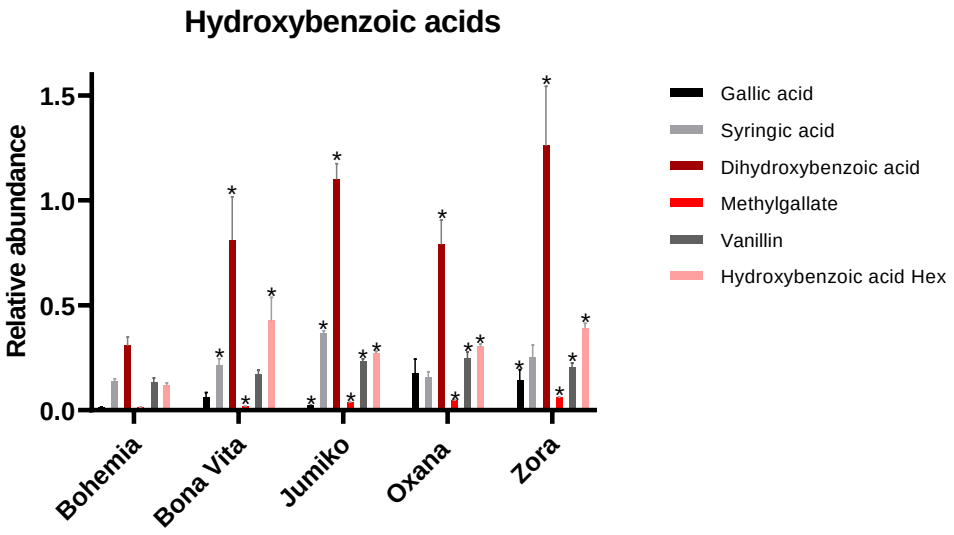


Figure 17 - Relative abundance of relevant hydroxybenzoic acids in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

Note that dihydroxybenzoic acid is one precursor in the biosynthesis of various hydroxybenzoic acids. Thus, the differences observed between the varieties in terms of their relative abundance was in line with the relative abundances of total hydroxybenzoic acids observed, which were also higher in Jumiko and Zora (*Figure 18*).

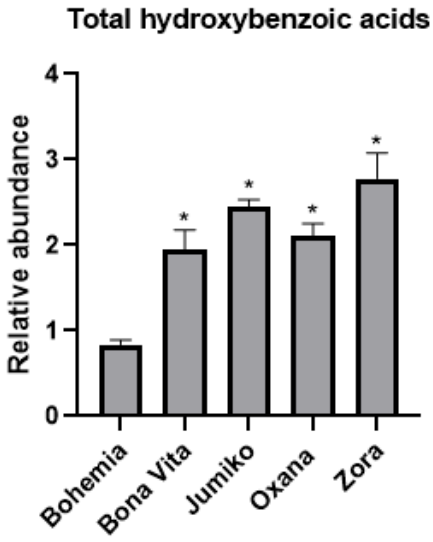


Figure 18 - Relative abundance of hydroxybenzoic acids in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured wheat varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

Others hydroxybenzoic acids identified are syringic acid, vanillin and gallic acid. Syringic acid was the second most important hydroxybenzoic acid in Bohemia and Jumiko and was present in major relative abundance in Jumiko (*Figure 17*). This hydroxybenzoic acid is an antioxidant and has antibacterial and hepatoprotective effects [57].

Syringic acid can be synthesized from vanillic acid, which in turn is an oxidized form of vanillin, one of the hydroxybenzoic acids identified. In vanillin, major differences were recorded in Jumiko, Oxana and Zora, which had higher relative abundance values than Bohemia and Bona Vita (*Figure 17*). Finally, also in gallic acid, there were differences between the varieties, and was present in higher relative abundance in Oxana and Zora (*Figure 17*). This has been shown to offer various health benefits, such as antioxidant, anti-inflammatory, and antineoplastic effects. It has demonstrated therapeutic potential

in treating gastrointestinal, neuropsychological, metabolic, and cardiovascular disorders [58].

According to existing studies, gallic acid can be converted into methyl gallate [59], a compound present in lower relative abundance in the varieties studied. In this compound, the values decreased in the following order: Zora > Oxana > Jumiko > Bona Vita > Bohemia (*Figure 17*).

3.3.1.2. Hydroxycinnamic acids

As for hydroxycinnamic acids, ferulic acid was the most relevant hydroxycinnamic acid in Bohemia, Jumiko, Oxana and Zora and present the second higher relative abundance in Bona Vita (*Figure 19*). For this compound, the main statistically significant differences were found in Jumiko and Oxana, which had higher values compared to the other varieties. Note that ferulic acid has antioxidant, anticancer, UV-absorbing, and anti-inflammatory properties [60].

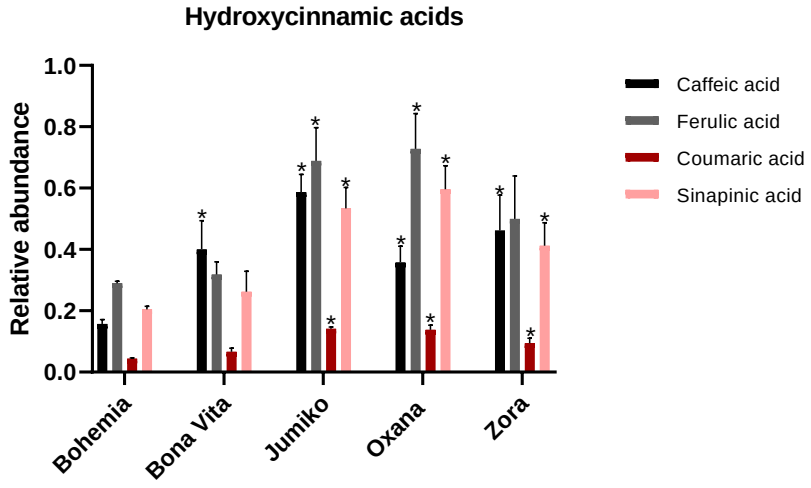


Figure 19 - Relative abundance of relevant hydroxycinnamic acids in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

Other important hydroxycinnamic acids identified were coumaric, caffeic and sinapinic acids. Coumaric acid was present in major relative abundance in Jumiko, Oxana and Zora, while the differences between Bohemia and Bona Vita were not statistically significant (*Figure 19*). Note that this compound is one of the first precursors in biosynthesis of hydroxycinnamic acids. Thus, the differences observed in the relative abundance of this compound was consistent with the fact that Jumiko, Oxana and Zora were those with the highest relative abundance of the total hydroxycinnamic acids (*Figure 20*).

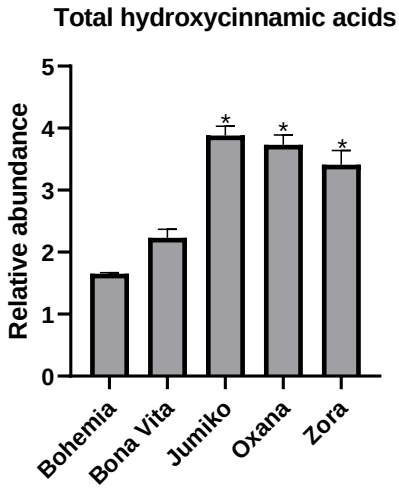


Figure 20 - Relative abundance of hydroxycinnamic acids in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

In terms of health benefits, coumaric acid and its derivatives have been shown to have antioxidant, antibacterial, anticancer, anti-inflammatory, antiplatelet aggregation, and other health properties [60].

Caffeic acid can be synthesized from coumaric acid and was the most important hydroxycinnamic acid in Bona Vita (*Figure 19*). Its relative abundance decreased in the following order: Jumiko > Zora > Bona Vita > Oxana > Bohemia. This compound has anti-inflammatory, antibacterial, anticancer, and antidiabetic properties [60].

Finally, sinapinic acid derives from caffeic acid and was found in major relative abundance in Jumiko, Oxana and Zora (*Figure 19*). For this compound, differences

between Bohemia and Bona Vita were not statistically important. Recently, this acid has aroused interest for its chemopreventive properties [60].

In plants, in addition to other important effects, the phenolic acids identified have shown confer resistance against Fusarium head blight (FHB), one of the most devastating diseases of small grain cereals, which is caused by fungi of the gender *Fusarium*. The effectiveness of this compounds seems to be related to their antioxidant activity [56]. Caffeic, syringic, *p*-coumaric, *p*-hydroxybenzoic and ferulic acids are examples of compounds that have already been shown to be toxic to *Fusarium graminearum* with effect increase in the following order: *p*-hydroxybenzoic acid < caffeic acid < syringic acid < *p*-coumaric acid < ferulic acid [56]. Hydroxycinnamic acids, such as caffeic, ferulic and *p*-coumaric acids, thus, are most recognized as contributors to FHB resistance. In particular, ferulic acid has a significant effect on the growth of all *Fusarium* species. Therefore, the coloured wheat varieties studied, especially Oxana, Jumiko and Zora, being richer in derivatives of cinnamic acid, and particularly ferulic acid, compared to common wheat varieties, are likely resistant to infections by fungi of the *Fusarium* gender, one of the main problems that affects small grain cereals, as already mentioned. However, second Trávníčková et al., 2024, the higher FHB resistance is only observed in the purple and black wheat varieties, since there are more factors that modulate tolerance to this type of disease (such as morphological factors) [61]. On the other hand, it should be highlight that, during grain development, changes occur in the phenolic acid profile [62]. The resistance of the coloured wheat varieties studied to certain diseases may therefore also differ depending on the stage of the phenological cycle to the grain.

3.3.1.3. Other phenolic acids

In addition to hydroxycinnamic and hydroxybenzoic acids, other types of phenolic acids were identified, such as phenylacetic acids, which can be synthesized from phenylalanine. 4-Hydroxyphenyllactic acid was the most significant phenylacetic acid in all the varieties studied and its relative abundance decreased in the following order: Jumiko > Bona Vita > Zora > Oxana > Bohemia (*Figure 21*).

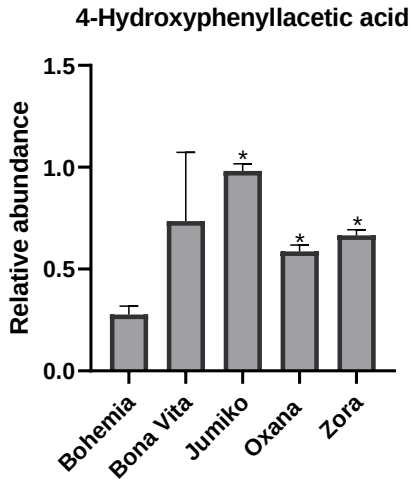


Figure 21 - Relative abundance of 4-Hydroxyphenyllacetic acid in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

Finally, 3,4,5-trihydroxy-6-{[4-hydroxy-5-(3-hydroxyphenyl)pentanoyl]oxy}oxane-2-carboxylic acid (*Figure 22)*, not belonging to any of the already mentioned subclasses of phenolic acids, was the most important phenolic acid in Bohemia, Bona Vita and Oxana. This compound was also present in higher relative abundance in Oxana, Jumiko and Zora, compared to Bohemia and Bona Vita (*Figure 23*).

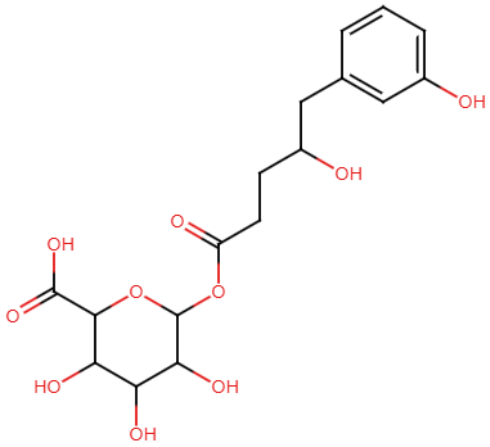


Figure 22 - Structure of 3,4,5-trihydroxy-6-{[4-hydroxy-5-(3-hydroxyphenyl)pentanoyl]oxy}oxane-2-carboxylic acid.

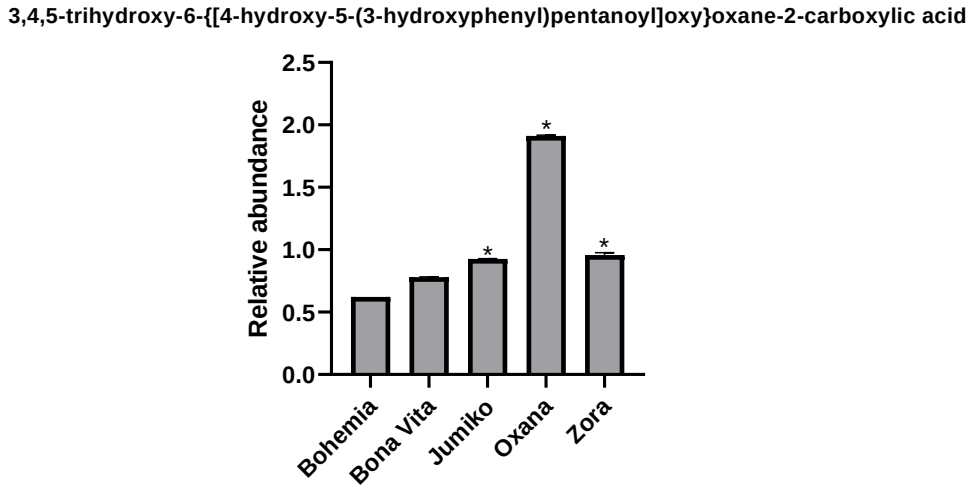


Figure 23 - Relative abundance of 3,4,5-trihydroxy-6-{[4-hydroxy-5-(3-hydroxyphenyl)pentanoyl]oxy}oxane-2-carboxylic acid in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

3.3.1.4. Flavonoids

On to another class of compounds, most of the phenolic compounds identified in the different varieties of wheat belonged to the flavonoid class and, within this class, the majority were flavones (26). Oxana and Zora were the varieties where flavonoids and, in particular, flavones present higher relative abundance values (*Figure 16* and *Figure 24*).

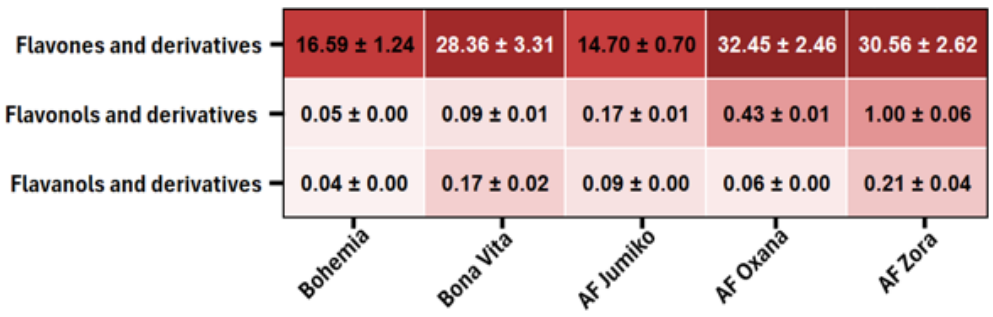
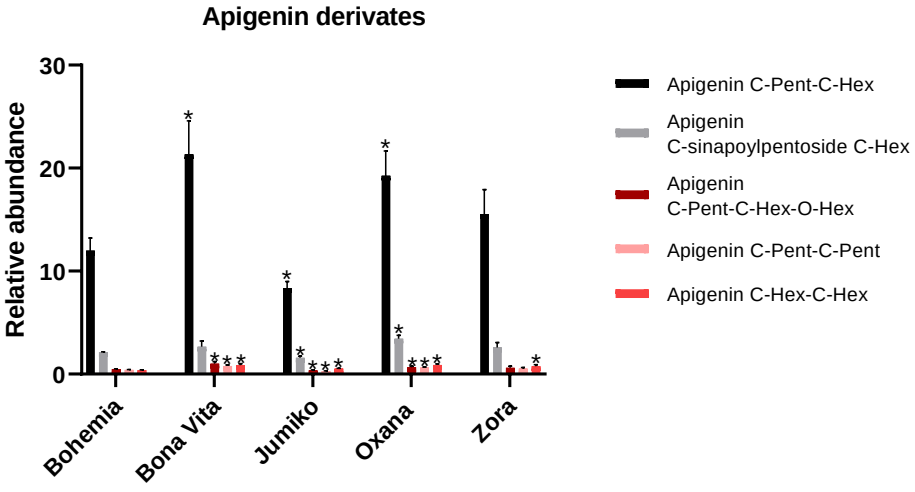


Figure 24 - Heatmap analysis of relative abundance of different subclasses of flavonoids in wheat varieties. Data is presented as relative abundance values ± standard deviation.

Various derivatives of apigenin, luteolin, chrysoeriol and tricin were identified, with statistically significant differences observed. The tricin derivatives were detected in low relative abundance, which makes them less relevant for comparison purposes. In general, Zora showed the highest relative abundance of chrysoeriol derivatives and Oxana showed the highest relative abundance of luteolin derivatives. As for the apigenin derivatives detected in this analysis, the general trend was Oxana with the highest relative abundance and Jumiko with the lowest (*Figure 25*).

Derivates of tricin had important antioxidant properties and can be used as agent for glycaemic control [63]. In addition, chrysoeriol, apigenin and luteolin having anti-inflammatory effects, relaxing smooth muscle, and reducing apoptosis and cell death [64].



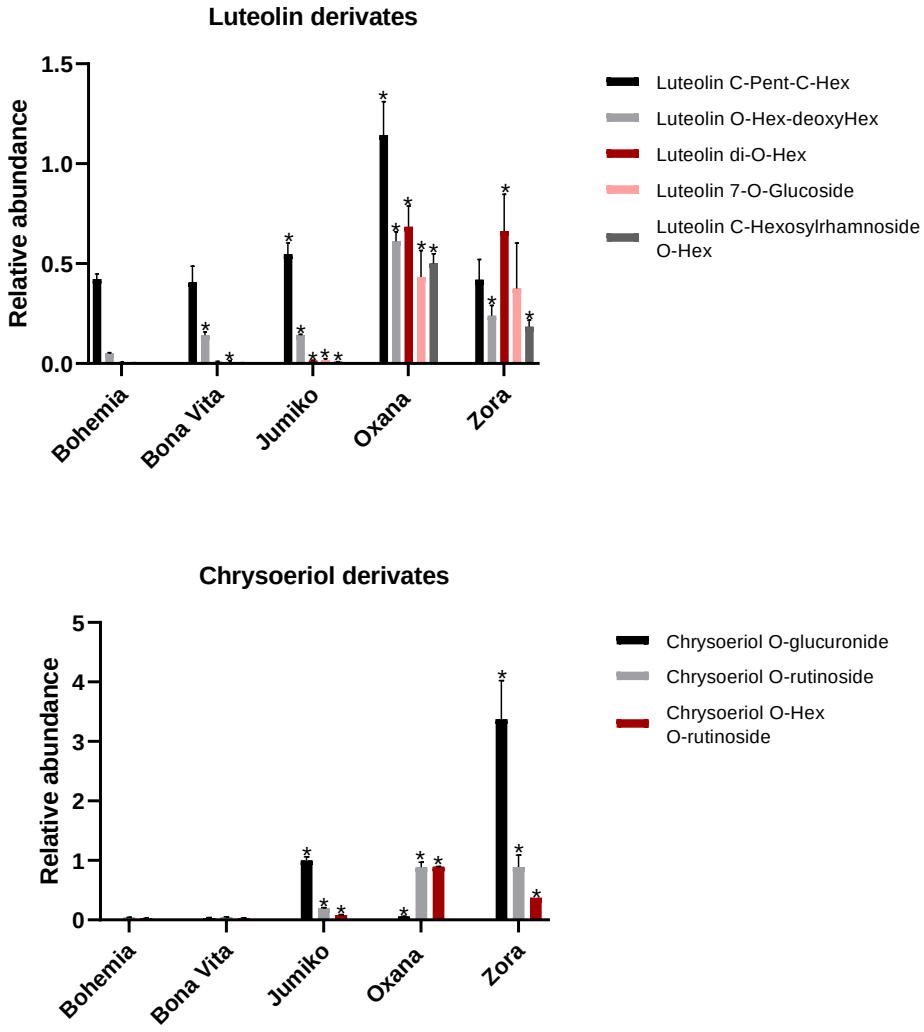


Figure 25 - Relative abundance of apigenin, luteolin and chrysoeriol derivates in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

Apigenin C-pentoside-C-hexoside was the most important phenolic compound in all the wheat varieties studied. The statistically significant differences were recorded in Bona Vita, Jumiko and Oxana. Bona Vita and Oxana had higher relative abundance values compared to Bohemia, while Jumiko had lower values.

In addition, 9 flavonols and 2 flavanols (dihydroflavonols) were identified within the flavonoid class. Within the flavonols subclass, the compounds found were derivates of kaempferol, quercetin, isorhamnetin and syringetin. To highlight quercetin-3-O-rutinoside, which was the flavonol with the highest relative abundance in Zora and Oxana.

Its values decreased in the following order: Zora > Oxana > Jumiko > Bona Vita > Bohemia (*Figure 26*).

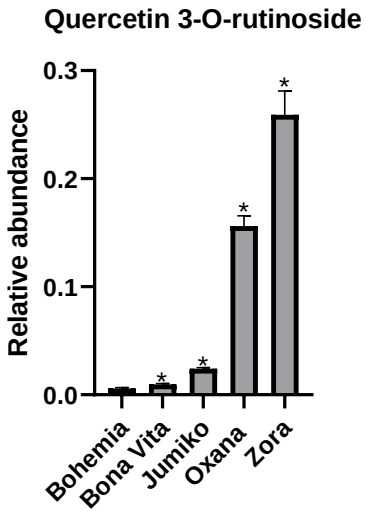


Figure 26 - Relative abundance of quercetin 3-O-rutinoside in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

Note that all the flavonoids mentioned share precursors in the flavonoid biosynthesis pathway and are related to each other. In more detail, phenylalanine, through a series of condensation, decarboxylation and isomerization reactions, gives rise to naringenin, a compound that has not been identified. All flavonoids are then formed from this compound. Naringenin gives rise to apigenin and kaempferol which, in turn, can be converted into luteolin and quercetin, respectively. Luteolin can still give rise to chrysoeriol. Finally, tricin is a tricetin derivative obtained from luteolin.

In the plant, flavonoids also seem to contribute to the resistance to fungi of gender *Fusarium*. Levels of apigenin, kaempferol, luteolin, quercetin and rutin (quercetin 3-O-rutinoside), for example, were significantly increased after inoculation with *Fusarium culmorum* in previous studies. The genes of the phenylpropanoid pathway responsible for the biosynthesis of flavonoids have even been considered capable of reducing the growth of pathogens, due to antifungal and antioxidant properties of the flavonoids [65].

3.3.2. Other compounds

In addition to phenols, other types of compounds extracted were identified (organic acids, nucleosides, peptides, vitamins, benzoxazinoides and aminoacids), as has already been explained. Citric acid was the most relevant organic acid in the varieties studied and is associated with the Krebs cycle. This metabolic pathway produces precursors necessary for the production of phenolic compounds [66]. Another compound associated with the Krebs cycle identified was *cis*-aconitate. This compound was the citric acid derivative with the major relative abundance in the varieties studied (apart from citric acid itself). In these 2 compounds, the most significant differences were found at the level of Jumiko (*Figure 27*).

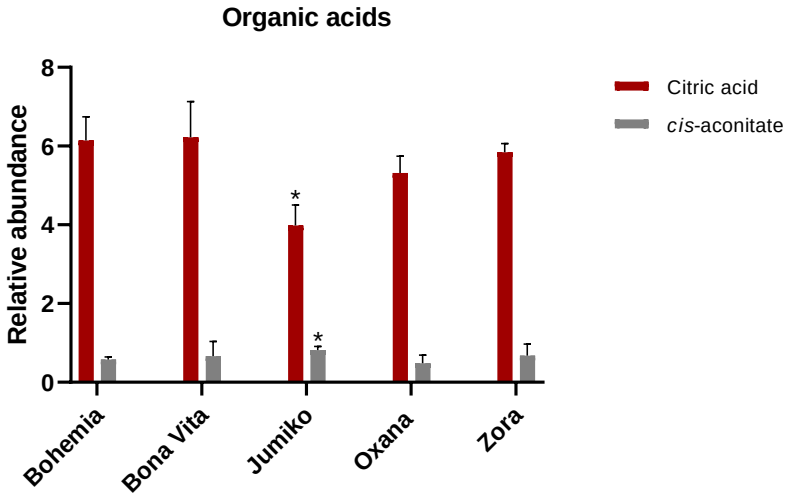


Figure 27 - Relative abundance of citric acid and *cis*-aconitate in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

As for the amino acids, most of those identified were essential aromatic amino acids (phenylalanine, tryptophan, tyrosine and their derivatives), which come from the shikimic acid pathway and are precursors of the phenolic compound biosynthesis pathways. Tryptophan was the most important aminoacid in Bohemia, Bona Vita, Jumiko and Zora and present the second highest relative abundance in Oxana. Their values decreased in

the following order: Jumiko > Bona Vita > Bohemia > Zora > Oxana (*Figure 28*). One tryptophan derivate, 5-hydroxy-*L*-tryptophan, was the second most relevant aminoacid in Bohemia, Bona Vita, Jumiko and Zora and the most important in Oxana. It was present major relative abundance in Bona Vita, Jumiko and Zora, which the differences observed between Bohemia and Oxana were not considered statistically significant.

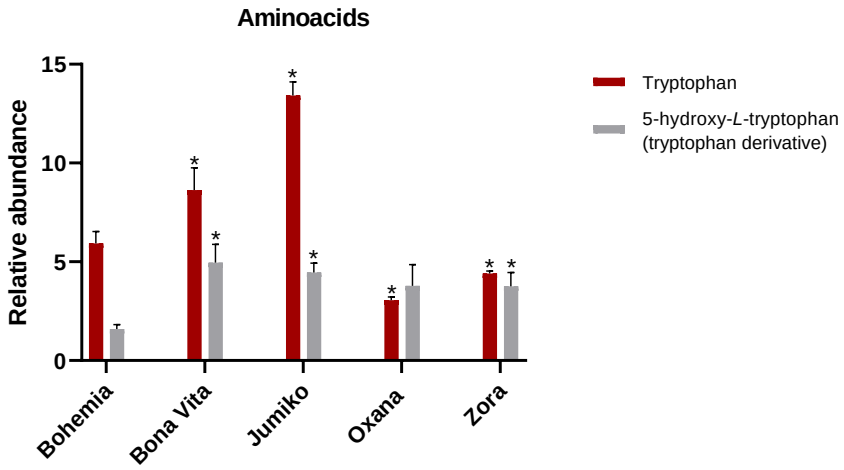


Figure 28 - Relative abundance of tryptophan and 5-hydroxy-*L*-tryptophan in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

Note that tryptophan is a precursor in metabolic pathways that give rise to indole-derived compounds, a type of compound also identified. In more detail, 3 indole acids have been identified, with (2-oxo-2,3-dihydro-1H-indol-3-yl) acetic acid showing the greatest differences between the varieties. Their relative abundance values decreased in the following order: Jumiko > Bona Vita > Zora > Oxana > Bohemia (*Figure 29*).

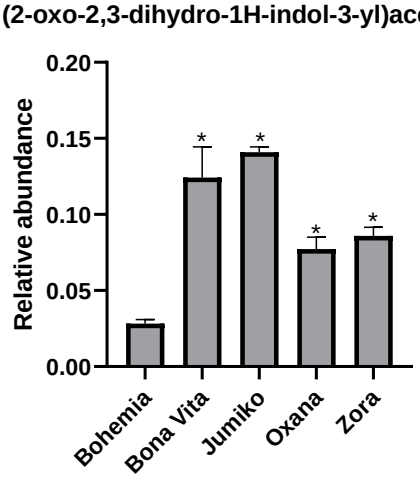


Figure 29 - Relative abundance of (2-oxo-2,3-dihydro-1H-indol-3-yl) acetic acid in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

This led to the conclusion that certain metabolic pathways that have tryptophan as a precursor are also active in the different wheat varieties studied.

Finally, phenylalanine and tyrosine, precursors in the synthesis of phenolic compounds, were also identified (*Figure 30*). By performing the already mentioned *t*-test, the differences between the phenylalanine values determined for Bona Vita, Jumiko, Oxana and Zora, and those determined for Bohemia, were not considered statistically significant. For tyrosine, which can also be synthetized from phenylalanine, the most significant differences were found in Jumiko, which had a lower relative abundance compared to Bohemia. Note that phenylalanine and tyrosine, in addition to being precursors in the biosynthesis of phenolic compounds, are also associated with many other metabolic pathways, so their content can not only relate to the activation of phenolic compound biosynthesis pathways. In addition, according to previous studies, metabolites such as phenylalanine and tyrosine become more essential to the plant in the early stages of wheat grain development [67]. Therefore, as the analyses were carried out on mature grain, it is natural that the relative abundance determined for these aminoacids was low and there were no important differences between the varieties studied.

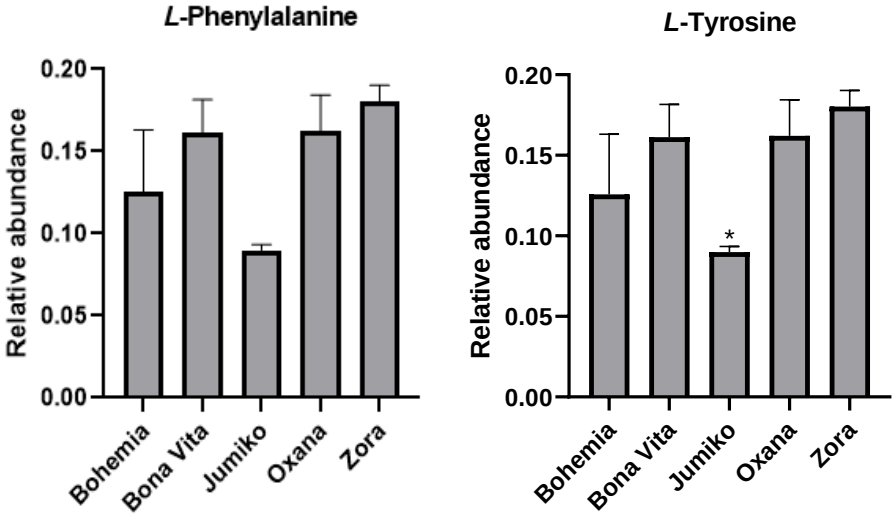


Figure 30 - Relative abundance of *L*-Phenylalanine and *L*-Tyrosine in wheat varieties. Data is presented as relative abundance values ± standard deviation. Asterisks indicate statistically significant differences between coloured varieties and Bohemia, using the *t*-test (*p*-value < 0.05).

4. Final remarks and future perspectives

In summary, the metabolic profiles of five winter wheat cultivars with different grain colours were effectively discriminated. Anthocyanin quantification by SRM revealed that wheat varieties with purple, blue, and dark grains contain higher levels of anthocyanins compared to red and yellow varieties, with significant differences in concentrations of cyanidin-, delphinidin-, and petunidin-3-O-glucoside (*Table 4*). Thus, it is suggested that the anthocyanin biosynthesis pathway is differentially active in the various varieties of coloured wheat. On the other hand, in the full scan + ddMS^2 analysis most of the compounds identified were phenolic compounds and, more precisely, flavonoids and phenolic acids, with differences between the varieties studied. In general, it was found that the coloured varieties had a higher (relative) abundance of these compounds compared to the common amber wheat variety. In addition to phenolic compounds, other types of compounds have been identified, such as organic acids associated with the Kebrs cycle (a metabolic pathway produces precursors necessary for the production of phenolic compounds) and aminoacids like tryptophan or phenylalanine and tyrosine. Thus, metabolic pathways that have tryptophan as a precursor may also be active in the

varieties under study. As for phenylalanine and tyrosine, their presence is essential for the synthesis of phenolic compounds.

Table 4 - Overview of the types of compounds identified, their properties and the differences observed between the wheat varieties studied.

Compounds	Properties/Relevance	Profiles observed
Anthocyanins	-**Human Health:** Antioxidant properties -**Agriculture:** defence mechanism against abiotic stress factors (like extreme temperatures, drought, or high salinity) and against pathogen infections	-Purple (Jumiko), blue (Oxana) or black (Zora) varieties are richer in anthocyanins compared to red (Bohemia) or yellow (Bona Vita) varieties, with the major differences observed in cyanidin- ,delphinidin- and petunidin-3-O-glucoside -Cyanidin-3-O-glucoside was found to be the dominant anthocyanin in purple wheat, while delphinidin-3-O-glucoside was the main in blue variety. In black wheat predominated the cyanidin-3-O-glucoside and delphinidin-3-O-glucoside
Flavonoids	-**Human Health:** Anti-inflammatory effects, smooth muscle relaxion; reduction of apoptosis and cell death (like derivates of apigenin, luteolin, chrysoeriol and tricin) **-Agriculture:** antifungal and antioxidant properties (resistance do Fusarium)	-The flavonoid subclass with the highest number of compounds identified was flavones, with many derivatives of apigenin, luteolin, chrysoeriol and tricin identified -Tricin was detected in low abundance, which makes it less relevant for comparison purposes. Zora showed the highest relative abundance of chrysoeriol derivatives, Oxana showed the highest relative abundance of luteolin derivatives and, for the apigenin derivatives, the general trend was Oxana with the highest relative abundance and Jumiko with the lowest

Phenolic acids	**-Human Health:** Antioxidant, anti-carcinogenic and anti-inflammatory properties (like coumaric and ferulic acids) **-Agriculture:** resistance do Fusarium	-The number of hydroxycinnamic acids identified was higher than the number of hydroxybenzoic acids -Caffeic acid, ferulic acid, coumaric acid and sinapinic acid are examples of hydroxycinnamic acids identified and gallic acid, syringic acid, dihydroxybenzoic acid, methylgallate, vanillin and hydroxybenzoic acid hexoside of hydroxybenzoic acids -In general, the coloured varieties showed higher relative abundance values than the non-coloured variety (Bohemia)
Organic acids	For example, citric acid and *cis*-aconitate have been identified, which are associated with the citric acid cycle (which produces precursors for the synthesis of phenolic compounds)	In citric acid and *cis*-aconitate the most significant differences were found at Jumiko, where relative abundance values were lowest
Aminoacids	Essential aromatic amino acids such as tryptophan, phenylalanine and tyrosine have been identified. Phenylalanine and tyrosine are precursors of phenolic compounds	-Tryptophan was the predominant aminoacid in Bohemia, Bona Vita, Jumiko and Zora and the second most abundant in Oxana. Their relative abundance values decreased in the following order: Jumiko > Bona Vita > Bohemia > Zora > Oxana -In phenylalanine, for example, the differences observed between the varieties were not considered statistically significant. For tyrosine, the most significant differences were found in Jumiko, where relative abundance values were lowest

It should be noted also that this work has resulted in a metabolic database for a more efficient and correct metabolic analyses of wheat grains and related cereals. In existing databases, plant metabolites are under-represented, which can lead to them being misidentified as non-plant compounds. We therefore build a mass list of the compounds present in wheat and related species, which was then validated using polyphenol-rich extracts prepared *in-house* from coloured wheat varieties.

A basis has thus been established for understanding the process of wheat grain colouring and for clarifying the advantages that the wheat varieties studied may have for health and at an industrial level. Note that biofortified wheat varieties may be more resistant to certain microorganisms or unfavourable environmental conditions, which could have positive impact on the industry. However, the major challenge will be to produce these varieties on a large scale, since environmental conditions and cultivation techniques have a significant influence on their phytochemical profile. Thus, were studied varieties originating in the Czech Republic with the objective of, in future, implementing its growing in Portugal.

4.1. Studies with Near Isogenic lines (NIL)

To be able to effectively understand which metabolic pathways are relevant in the process of colouring wheat grains, it will be necessary also analyse the activity of key enzymes involved in the flavonoid biosynthesis pathway, for instance through Near Isogenic Lines. Near Isogenic Lines are strains which genetic makeups are similar except for few specific locations or genetic loci. Thus, in this case, Near Isogenic Lines of the same wheat variety could be used that differ only in key genes involved in the phenolic compounds biosynthesis pathways (such as phenylalanine ammonia lyase (*TaPAL*) or chalcone isomerase (*TaCHI*)) in order to understand the consequences that altering certain metabolic pathways could have on the plant and on its potential health benefits.

4.2. *In vitro* digestion simulation protocols

Once the phytochemical/metabolic profile of the different varieties of coloured wheat has been studied, it will be also important to analyse the bioaccessibility and bioavailability of phenolic compounds. Although phenolic compounds in are relatively common in the human diet and are present in the wheat varieties under study, they may not be the most active in the body, either due to low intrinsic activity, poor absorption in the gastrointestinal tract, extensive metabolism, or rapid elimination [65]. Thus, the bioaccessibility of phenolic compounds present in different varieties of coloured wheat is already being studied through *in vitro* digestion simulation protocols. It should be note, therefore, that bioaccessibility corresponds to the amount of a compound available for absorption after the digestion process and help to predict its bioavailability.

According to some studies, during digestion, the majority of polyphenols appear to be released in the gastric phase. These compounds and their degradation products and metabolites with remaining phenolic structure are, thus, potentially available for absorption after digestion process [65].

4.3. Bioactivity and bioavailability assays

In addition to the above, bioactivity and bioavailability assays are also being carried out.

According to the literature, the anthocyanins identified, despite their important antioxidant properties, have very low bioavailability. During digestion, the pH increases in the small intestine which in turn promotes the degradation of these compounds and contributes to a low overall uptake into serum (typically below 1%) [68]. The bioavailability of the other phenolic compounds also appears to be low. According to several studies, in general, the bioavailability of bioaccessible polyphenols in the small intestine reporting values between 5% and 10% [64].

Low weight phenolics, like dihydroxybenzoic, syringic, gallic acids and phenolic acids in general are effectively absorbed by passive diffusion mechanisms in the enterocytes of the small intestine (where it is considered that most phenolic compounds are

absorbed). There are, however, specific situations that have already been studied, such as the case of caffeic and ferulic acids, which are absorbed through facilitated transport by monocarboxylic acid transporters (MCTs) [68]. As for excretion, another parameter able to affect the bioactivity of the compounds in question, it is thought that polyphenols are mostly eliminated via the kidneys, at least in the case of the more polar polyphenols (such as phenolic acids). The number of polyphenols and conjugates recovered in the urine varies greatly, the highest being gallic acid (over 60%). For other phenolic acids, like caffeic and ferulic acids the percentage of excretion via the kidney is 5-27% [68].

On the other hand, flavonoids derivates, for example, need to be cleaved from their sugar moieties by brush border enzymes (*i.e*, lactase-phloridzin hydrolase (LPH)) prior to cellular uptake. The lower lipophilicity of this compounds is another factor that dampen epithelial uptake that are taken up actively via sodium-glucose transport proteins, especially sodium-glucose-linked transporter 1 (SGLT1), as some studies suggest [68]. It should also be noted that this type of compounds can undergo many metabolic reactions (*i.e*, conjugating and hydrolyzing reactions) in the small intestine and liver to facilitate their excretion via urine and bile or in colon to can be more readily absorbed [68]. Studies have shown that quercetin, for example, undergoes methylation reactions during systemic absorption (up to 20-30% of the dose consumed) [68]. This compound is associated to antioxidative and radical scavenging capacity. However, the bioavailability of the aglycone quercetin is very low. Interesting, total quercetin concentrations in plasma increase considerably when quercetin glycosides, like quercetin 3-O-rutinoside, were consumed instead of the aglycone [69]. As for excretion, the percentage of flavonols recovered in urine, for example, are 0.3–1.4% [68].

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Appendix

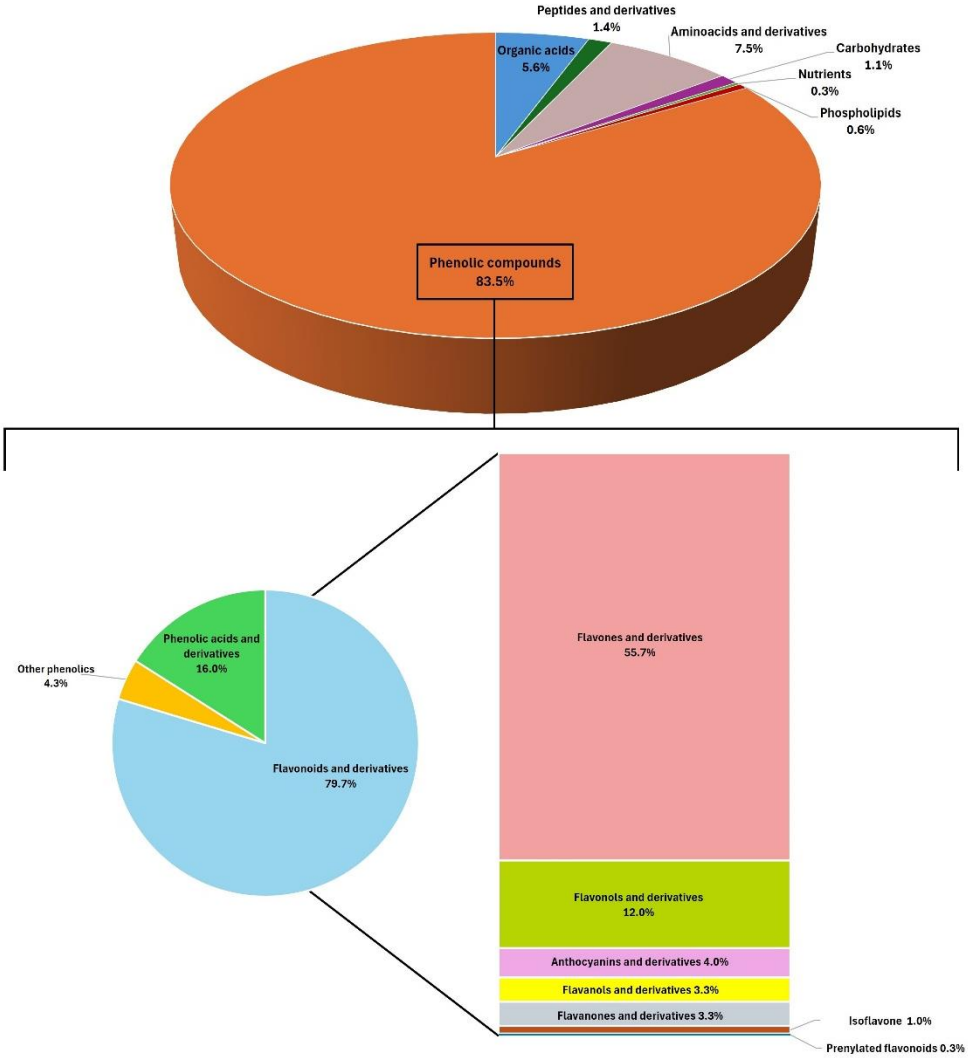


Figure S1 – Distribution of classes and subclasses of compounds in the mass list.

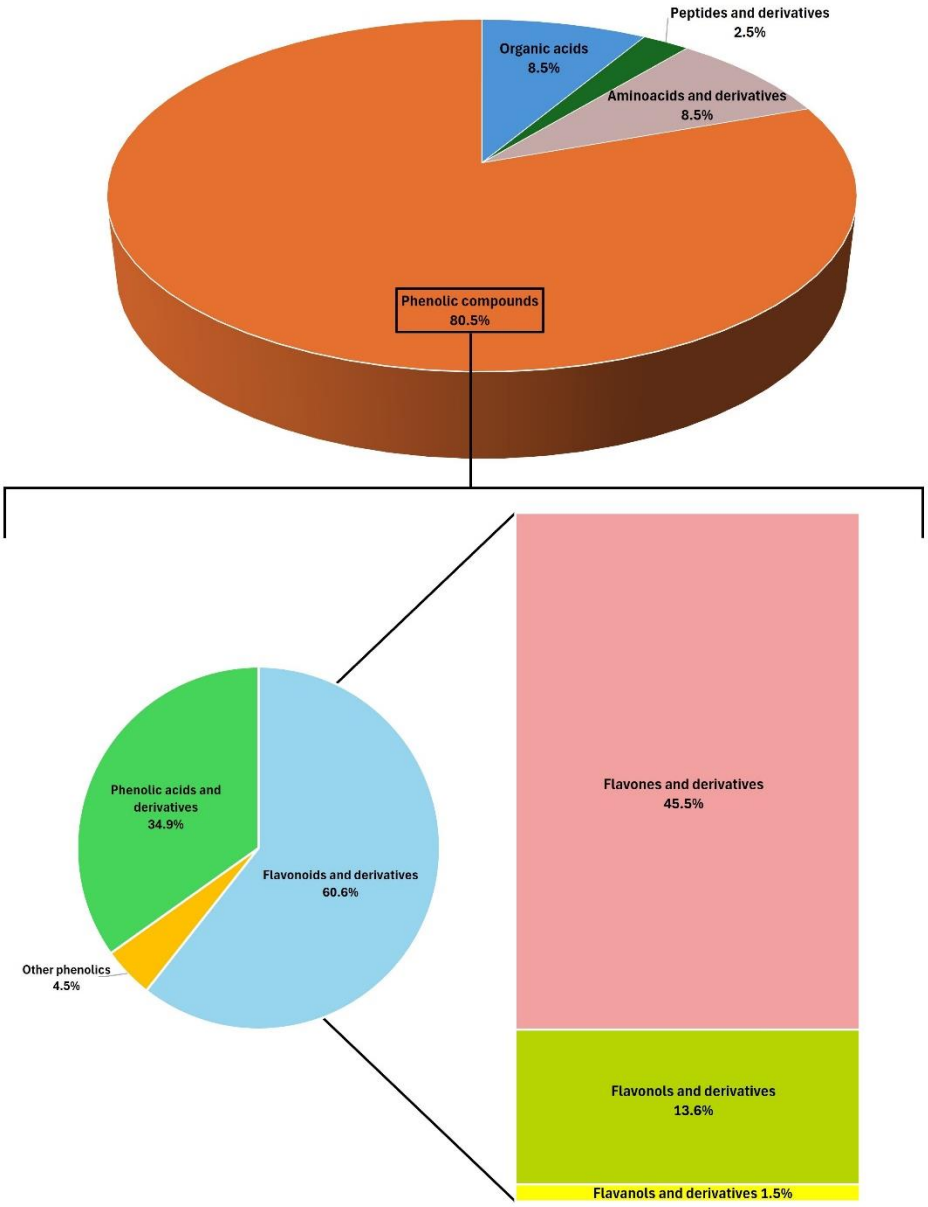


Figure S2 – Distribution of classes and subclasses of compounds in the spectral library (mzVault).