



Article Autofluorescence-Based Investigation of Spatial Distribution of Phenolic Compounds in Soybeans Using Confocal Laser Microscopy and a High-Resolution Mass Spectrometric Approach

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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Abstract: In this research, we present a detailed comparative analysis of the bioactive substances of soybean varieties k-11538 (Russia), k-11559 (Russia), k-569 (China), k-5367 (China), k-5373 (China), k-5586 (Sweden), and Primorskaya-86 (Russia) using an LSM 800 confocal laser microscope and an amaZon ion trap SL mass spectrometer. Laser microscopy made it possible to clarify in detail the spatial arrangement of the polyphenolic content of soybeans. Our results revealed that the phenolics of soybean are spatially located mainly in the seed coat and the outer layer of the cotyledon. High-performance liquid chromatography (HPLC) was used in combination with an amaZon SL BRUKER DALTONIKS ion trap (tandem mass spectrometry) to identify target analytes in soybean extracts. The results of initial studies revealed the presence of 63 compounds, and 45 of the target analytes were identified as polyphenolic compounds.

Keywords: *Glycine* Willd; flavonols; laser microscopy; HPLC-MS/MS; tandem mass spectrometry; polyphenols

1. Introduction

Glycine Willd (soybean) is an economically important member of the Fabaceae family. The center of origin of the soybean is located in East Asia [1], where it has been used as food for more than 5000 years [2]. As a well-known source of cheap concentrated protein and vegetable oil, soybean occupies a place of world importance among crops. Accounting for a 53% global production share of all oilseed crops, soybean occupies a significant place in the agricultural production systems of most major countries, such as the USA, China, Brazil, Argentina, and India [3]. In recent years, soybean production in Russia has shown stable growth due to the expansion of crop acreage. In total, Russia produced more than 3 million tons of soybeans in 2016 [4].

There has been considerable interest among researchers and consumers in the potential role of soybean and soy foods in the prevention of diseases. Clinical and scientific evidence has revealed the medicinal benefits of the components of soybean against metabolic disorders and other chronic diseases (cardiovascular diseases, diabetes, obesity, cancer, osteoporosis, menopausal syndrome, anemia, etc.) [2]. As a step toward understanding the mechanisms of the influence of the food components on health, it is important to investigate chemical compositions to reveal the active components responsible for beneficial effects. It

was shown that the health benefits of soybean are due to its secondary metabolites, such as isoflavones, phytosterols, lecithins, saponins, etc. [2]. In particular, Omoni et al. (2005) pointed out that isoflavones appear to work in conjunction with proteins to protect against cancer, cardiovascular disease, and osteoporosis [5].

In addition, for various crops, a relationship between the presence of phenolic compounds and the degree of plant resistance to adverse environmental conditions has been reported. Phenolic acids are important secondary plant metabolites that function as cell wall structural components, biosynthesis intermediates, and signaling and defense molecules [6]. Flavonoids, including chalcones, flavanols, flavones, flavonols, and anthocyanins, usually accumulate in the epidermal layer of plants. They are associated defense responses to ultraviolet radiation and other abiotic and biotic stresses. Thus, flavonoid distribution in the epidermal layer is an important factor for plant survival in stressful environments and is indispensable to understand the mechanisms underlying stress response and tolerance in living plant tissues and cells [7].

Polyphenolic compounds, including phenolic acids and their derivatives, tannins, and flavonoids, represent the largest group of natural plant nutrients. They determine the color of fruits and seeds and play an important role in disease resistance [8]. In soybean, the concentrations of phenolic compounds such as flavonoids and anthocyanins correlate with seed coat color [9].

One of the most important classes of phenolics is anthocyanins, which are well known for their antioxidant activity [10]. In connection with the considerable potential of anthocyanins as components of functional nutrition, knowledge about their genetic control is in demand, as they are used in breeding programs aimed at creating new varieties of cultivated plants with an increased content of these compounds that are valuable for human health. Unfortunately, as crops are cultivated, a significant portion of their biodiversity is lost, which explains the increased research interest in the study of the biodiversity of wild forms of various crops.

New progressive research methods are becoming more widespread, such as laser microscopy, a method that exploits the ability of chemicals to fluoresce when excited by a laser and can be used to solve problems of visualization. Currently, microscopic images are successfully used to visualize the location of chemicals in organs and tissues of various plants [11,12]. However, previous autofluorescence-based microscopic studies of soybean were focused on visualization of anatomical features, such as the three-dimensional (3D) internal structure of a soybean seed [13] and the leaf anatomy of *Glycine max* (L.) Merr. [14].

Although the use of various microscopy methods is common in the study of soybeans, most of these approaches focus only on optical microscopy, specific staining of proteins or polysaccharides, and analysis of the signals of specific antibodies with a fluorescence label [15–17].

Therefore, we investigated the polyphenolic composition of soybean, in particular anthocyanins, and showed their localization in seeds based on the autofluorescence. Such a simple method as recording autofluorescence signals is significantly underestimated and can provide a sufficiently large amount of information without complex sample preparation. Despite the insufficiency of using this method without the support of deeper analysis data, such as RAMAN spectroscopy or MALDI spectrometry, in this study, we show that the method is applicable to deeper analysis of seeds in terms of classes of compounds present and that the obtained data correlate with more complex methods. Thus, the proposed method promising for obtaining preliminary data and analyzing a large number of varietal samples. The use of this approach is time- resource-, and reagentsaving and can help to increase the level of research in laboratories that do not have more complex equipment.

2. Materials and Methods

2.1. Materials

As an object of research, we used the following soybean varieties cultivated at the N.I. Vavilov All-Russian Institute of Plant Genetic Resources: k-11538 (*G. soja*, cultivated form OLMIK-76, Russia), k-11559 (*G. soja*, wild, Russia), k-569 (*G. gracilis*, China), k-5367 (*G. gracilis*, E-Shen-Dow, China), k-5373 (*G. gracilis*, Harbin semiwild, China), k-5586 (*G. max*, 856-3-3, Sweden), and Primorskaya-86 (*G. max*, Russia).

Seeds from the VIR collection were selected, and the maximum diversity in appearance was taken into account. Seeds were obtained from the research fields of the N. I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) according to the developed VIR Guidelines. Because the purpose of this study was to investigate the diversity of polyphenolic compounds of soybean, the 5 most colored varieties and two control light-colored varieties were selected from the VIR collection (Figure 1).



Figure 1. Soybean varieties k-11538 (Russia), k-11559 (Russia), k-569 (China), k-5367 (China), k-5373 (China), k-5586 (Sweden), and Primorskaya-86 (Russia).

2.2. Chemicals and Reagents

HPLC-grade acetonitrile was purchased from Fisher Scientific (Southborough, UK), and MS-grade formic acid was obtained from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was prepared using a SIEMENS ULTRA clear (SIEMENS water technologies, Munich, Germany), and all other chemicals were of analytical grade. The results were obtained using the equipment of the Center for Collective Use of Scientific Equipment of the Tambov State University named after G.R. Derzhavin.

2.3. Fractional Maceration

A fractional maceration technique was applied to obtain highly concentrated extracts [18]. From 500 g of the sample, 1 g of soy seeds was randomly selected for maceration. The total amount of the extractant (reagent-grade methyl alcohol) was divided into three parts and consistently infused with the grains with the first, second, and third parts with a solid–solvent ratio of 1:20. The infusion of each part of the extractant lasted 7 days at room temperature.

After maceration, the samples were centrifuged to precipitate sediment at an acceleration of $5000 \times g$ and a temperature of 4 °C for 20 min; then, a 3 mL aliquot of the sample was filtered on syringe filters with a pore size of 0.45 µm, and the first 2 mL of filtrate was discarded for non-specific sorption on the membrane, and only the last milliliter was used for analysis. The filtered milliliter of the sample was diluted with 1 mL of deionized water.

2.4. Optical Microscopy

Dry, untreated soybean seeds were used for confocal laser scanning microscopy. The transverse dissection of seeds was performed with an MS-2 sled microtome (Tochmedpribor, Kharkiv, Ukraine). The autofluorescence parameters were determined using an inverted confocal laser scanning microscope in lambda mode (LSM 800, Carl Zeiss Microscopy GmbHAG, Jena, Germany). We carried out a lambda experiment with excitation lasers at 405, 488, 561, and 740 nm and registered emissions in the range of 400 to 700 nm with a step of 5 nm. The maxima of fluorescence were registered with the following parameters: excitation by a violet laser (405 nm) with emission in the range of 400–475 nm (blue); excitation by a blue laser (488 nm) with the emission in the range of 500–545 nm (green) and 620–700 nm (red). Images were obtained using 63× magnification and ZEN 2.1 software (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.5. Liquid Chromatography

HPLC was performed using an LC-20 Prominence HPLC (Shimadzu, Kyoto, Japan) equipped with a UV sensor and a C₁₈ silica reverse-phase column (4.6 × 150 mm, particle size: 2.7 µm) for separation of multicomponent mixtures. A gradient elution program with two mobile phases (A, deionized water; B, acetonitrile with formic acid 0.1% v/v) was performed as follows: 0–2 min, 0% B; 2–50 min, 0–100% B; control washing 50–60 min, 100% B. The entire HPLC analysis was performed with an SPD-20A UV-vis detector (Shimadzu, Japan) at wavelengths of 230 nm and 330 nm; the temperature was 50 °C, and the total flow rate was 0.25 mL min⁻¹. The injection volume was 10 µL. Additionally, liquid chromatography was combined with a mass spectrometric ion trap to identify compounds.

2.6. Mass Spectrometry

MS analysis was performed on an amaZon SL ion trap (BRUKER DALTONIKS, Bremen, Germany) equipped with an ESI source in negative and positive ion mode. The optimized parameters were obtained as follows: ionization source temperature, 70 °C; gas flow, 9/min; nebulizer gas (atomizer), 7.3 psi; capillary voltage, 4500 V; end-plate bend voltage, 1500 V; fragmentary voltage, 280 V; collision energy, 60 eV. An ion trap was used in the scan range of m/z 100–1.700 for MS and MS/MS. All experiments were repeated three times. A four-stage ion separation mode (MS/MS mode) was implemented.

3. Results and Discussion

3.1. Optical Microscopy of Soybean Components

The observation of autofluorescence makes it possible to draw conclusions about the presence and localization of fluorescent substances in plant tissues. An increased level of fluorescence signal in individual areas reflects the main accumulation sites of certain classes of compounds. Figures 2–8 show transverse sections of soybean seeds under a confocal laser microscope. Microscopic examination showed the presence of fluorescent substances in the soybean seeds.

We observed three main autofluorescence maxima: in the blue (400–475 nm), green (500–545 nm), and red (620–700 nm) regions of the spectrum. According to the literature data, the blue fluorescence in plants is mainly due to the presence of phenolic hydrox-ycinnamic acids [19]. The main fluorescent component is ferulic acid, but other hydrox-ycinnamic (e.g., p-coumaric and caffeic) acids can also contribute to fluorescence [20]. Moreover, lignin is a well-known source of blue fluorescence in plants. It has a wide emission range, owing to the presence of multiple fluorophore types within the molecule and can be observed when excited by UV and visible light [21]. Previous studies have shown that the lignin content of legume seed coat is low [22,23] and that the cotyledons are poorly lignified [24]. Therefore, we concluded that most of the blue fluorescence in soybean seeds comes from hydroxycinnamic acids.



Figure 2. A transverse section of a soybean seed (variety k-11538): (**a**) excitation at 405 nm with emission in the range of 400–475 nm (blue); (**b**) excitation at 488 nm with emission in the range of 500–545 nm (green); (**c**) excitation at 488 nm with emission in the range of 620–700 nm (red); (**d**) merged; cot, cotyledon; pl, palisade layer; sc, seed coat.

The blue-light-induced green autofluorescence in the range of 500–545 nm can be explained by the presence of flavins and flavonols (myricetin, quercetin, and kaempferol) and their derivatives [7,25,26]. The emission in the red spectrum mainly occurs due to the presence of anthocyanins and anthocyanidins [27,28].

We studied the seeds of three different soybean species (*G. soja* in cultivated and wild forms, as well as *G. gracilis* and *G. max*) and found that the spatial distribution of fluorescent substances has features that correlate with the color of the seeds.



Figure 3. A transverse section of a soybean seed (variety k-11559): (**a**) excitation at 405 nm with emission in the range of 400–475 nm (blue); (**b**) excitation at 488 nm with emission in the range of 500–545 nm (green); (**c**) excitation at 488 nm with emission in the range of 620–700 nm (red); (**d**) merged; cot, cotyledon; pl, palisade layer; sc, seed coat.

In general, our study showed the maximum of blue fluorescence, which reflects the content of hydroxycinnamic acids, in the outer cotyledon layer. A weaker signal was observed in the rest of the cotyledon parenchyma cells. In the seed coat of the dark-colored seeds, the signal was almost absent. On the contrary, the light-colored seeds (yellow) showed a solid blue signal (Figures 7a and 8a). Similar results were obtained in the other studies on the chemical composition of legume seeds. It was reported that coumaric and ferulic acids are dominant phenolic acids in the white seed coat of pea, as compared with colored seed coats [29].



Figure 4. A transverse section of a soybean seed (variety k-569): (**a**) excitation at 405 nm with emission in the range of 400–475 nm (blue); (**b**) excitation at 488 nm with emission in the range of 500–545 nm (green); (**c**) excitation at 488 nm with emission in the range of 620–700 nm (red); (**d**) merged; cot, cotyledon; pl, palisade layer; sc, seed coat.

Green fluorescence is most pronounced in the outer layer of the cotyledon. The signal is also present in the seed coat but it is usually weaker than that in the outer layer. The brightest green fluorescence of the palisade layer of the seed coat is observed in yellow seeds. This fluorescence is the most expressed among all investigated varieties and comparable to that of the outer cotyledon layer (Figures 7b and 8b).

The level of the red fluorescence signal correlates well with the color of the seeds. Microscopic examination showed that the palisade layer of black-seeded varieties has the brightest red fluorescence, whereas yellow-seeded varieties have the weakest red fluorescence. The brown-seeded variety demonstrated red fluorescence in the form of scattered inclusions (Figure 5c). It was previously reported that the black color of the seed coat in legumes is the result of a large amount of anthocyanins [30]. This confirms that bright red fluorescence is caused by such chemicals.



Figure 5. A transverse section of a soybean seed (variety k-5367): (**a**) excitation at 405 nm with emission in the range of 400–475 nm (blue); (**b**) excitation at 488 nm with emission in the range of 500–545 nm (green); (**c**) excitation at 488 nm with emission in the range of 620–700 nm (red); (**d**) merged; cot, cotyledon; pl, palisade layer; sc, seed coat.

Our result show that various phenolic substances are responsible for autofluorescence in soybean. The total fluorescence signal is maximal in the seed coat of all varieties. Our results are consistent with numerous publications indicating that the total concentration of phenolic compounds is always much higher in the seed coat than in the cotyledons of legumes [31,32]. The accumulation of phenolics mainly in the outer layers of the seed



may be associated with their protective function during seed development, as well as their protective function against detrimental agents in the environment [33].

Figure 6. A transverse section of a soybean seed (variety k-5373): (**a**) excitation at 405 nm with emission in the range of 400–475 nm (blue); (**b**) excitation at 488 nm with emission in the range of 500–545 nm (green); (**c**) excitation at 488 nm with emission in the range of 620–700 nm (red); (**d**) merged; cot, cotyledon; pl, palisade layer; sc, seed coat.

3.2. Tandem Mass Spectrometric Analysis

The most-consumed extracts of soybeans were analyzed by HPLC-MS/MS ion trap to better interpret the diversity of available phytochemicals. All of the examined extracts have a rich bioactive composition. Each compound was structurally identified on the basis of their accurate mass and MS/MS fragmentation by HPLC-ESI ion trap MS/MS. Sixty-three biologically active compound were successfully identified and characterized by comparing fragmentation patterns and retention times. Other compounds were identified by comparing their MS/MS data with available literature data. All identified compounds, along with molecular formulae, calculated and observed m/z, MS/MS data, and their comparative profile for soybeans (seven varieties), are summarized in Table 1.



Figure 7. A transverse section of a soybean seed (variety k-5586): (**a**) excitation at 405 nm with emission in the range of 400–475 nm (blue); (**b**) excitation at 488 nm with emission in the range of 500–545 nm (green); (**c**) excitation at 488 nm with emission in the range of 620–700 nm (red); (**d**) merged; cot, cotyledon; pl, palisade layer; sc, seed coat.

In the present study, 45 polyphenolic compounds were identified and characterized, including 17 flavones, 10 flavonols, 3 flavan-3-ols, 1 flavanone, 3 anthocyanidins, 3 condensed tannins, 5 phenolic acids, 1 lignan, 1 stilbene, and 1 hydroxycoumarin. Additionally, 18 compounds of other classes were identified in soybeans, with some identified for the

first time, for example, steroidal alkaloids Alpha-chaconine and solanidadiene solatriose. Table 2 lists the identified polyphenolic compounds in seven varieties of soybeans. In our research, the richest polyphenolic content was observed in the Chinese variety k-5373 (Harbin semiwild). In this variety, 30 polyphenolic compounds were identified during primary studies. The Russian variety k-11538 (OLMIK-76) is in second place in terms of the richness of compounds, with 23 compounds identified.



Figure 8. A transverse section of a soybean seed (variety Primorskaya-86): (**a**) excitation at 405 nm with emission in the range of 400–475 nm (blue); (**b**) excitation at 488 nm with emission in the range of 500–545 nm (green); (**c**) excitation at 488 nm with emission in the range of 620–700 nm (red); (**d**) merged; cot, cotyledon; pl, palisade layer; sc, seed coat.

№	Class of Compound	Identified Compound	Formula	Mass	Molecular Ion [M – H] [–]	Molecular Ion [M + H] ⁺	2 Fragmenta- tion MS/MS	3 Fragmenta- tion MS/MS	4 Fragmenta- tion MS/MS	References
1	Amino acid	L-Leucine [(S)-2-Amino- Methylpentanoic acid]	C ₆ H ₁₃ NO ₂	131.1729		132	114			Potato leaves [34]; Vigna unguiculata [35]; Lonicera japonica [36]; Camellia kucha [37]
2	Benzaldehyde	Vanillin	C ₈ H ₈ O ₃	152.15		153	151	136		Potato [38,39]; Triticum [40]; millet grains [41]
3	Trans-cinnamic acid	Ferulic acid	C ₁₀ H ₁₀ O ₄	194.184		195	177; 141	126		Lonicera japonica [36]; Potato [38,39]; Zostera marina [42]; Andean blueberry [43]; Tomato [44]; Codonopsis Radix [45]; Bougainvillea [46]
4	Amino acid	L-Tryptophan [Tryptophan; (S)-Tryptophan]	$C_{11}H_{12}N_2O_2$	204.2252		205	188	144	118	Vigna unguiculata [35]; Camellia kucha [37]; Perilla frutescens [47]; Passiflora incarnata [48]; Vigna inguiculata [49];
5	Stilbene	Resveratrol [trans-Resveratrol; 3,4',5- Trihydroxystilbene; Stilbentriol]	C ₁₄ H ₁₂ O ₃	228.2433		229	210	141; 169	123	Embelia [50]; Red wines [51]; vinery products [52]; A. cordifolia; F. glaucescens; F. herrerae [53]; Radix polygoni multiflori [54]
6	Isoflavone	Daidzein [4',7 -Dihydroxyisoflavone; Daidzeol]	$C_{15}H_{10}O_4$	254.2375		255	227; 199; 137	181		Hedyotis diffusa [55]; Isoflavones [56]
7	Ribonucleoside composite of adenine (purine)	Adenosine	$C_{10}H_{13}N_5O_4$	267.2413		268	136			<i>Lonicera japonica</i> [36]; Huolisu Oral Liquid [57]
8	7- hydroxyisoflavone	Formononetin [Biochanin B; Formononetol]	$C_{16}H_{12}O_4$	268.2641		269	254; 159; 118	237; 181; 118	237; 181	Astragali Radix [45]; Isoflavones [56]; Huolisu Oral Liquid [57];

Table 1. Compounds identified from the extracts of seven soybean varieties in positive and negative ionization modes by HPLC ion trap MS/MS: k-11538 (Russia),k-11559 (Russia), k-569 (China), k-5367 (China), k-5373 (China), k-5586 (Sweden), and Primorskaya-86 (Russia).

№	Class of Compound	Identified Compound	Formula	Mass	Molecular Ion [M – H] [–]	Molecular Ion [M + H] ⁺	2 Fragmenta- tion MS/MS	3 Fragmenta- tion MS/MS	4 Fragmenta- tion MS/MS	References
9	Flavone	Apigenin [5,7-Dixydroxy-2- (40Hydroxyphenyl)- 4H-Chromen-4-One]	$C_{15}H_{10}O_5$	270.2369		271	153; 215	111		Lonicera japonica [36]; millet grains [41]; Andean blueberry [43]; Hedyotis diffusa [55]; Mexican lupine species [58]; Wissadula periplocifolia [59]
10	Anthocyanin	Pelargonidin [Pelargonidol chloride]	C ₁₅ H ₁₁ O ₅₊	271.2493		271	215; 197; 153	197; 169; 141	169	acerola [60]
11	Flavan-3-ol	Epiafzelechin [(epi)Afzelechin]	$C_{15}H_{14}O_5$	274.2687		275	247; 193; 147	193; 175		A. cordifolia; F. glaucescens; F. herrerae [53]; Cassia granidis [61]; Cassia abbreviata [62]
12	Omega-3 fatty acid	Stearidonic acid [6,9,12,15- Octadecatetraenoic acid; Moroctic acid]	$C_{18}H_{28}O_2$	276.4137		277	217	190		G. linguiforme [53]; Salviae Miltiorrhizae [63]; Rhus coriaria [64]
13	Sceletium alkaloid	4'-O-desmethyl mesembranol	C ₁₆ H ₂₃ NO ₃	277.3587	276		234	218	218	A. cordifolia [53]
14	Omega-3 fatty acid	Linolenic acid (Alpha-Linolenic acid; Linolenate)	$C_{18}H_{30}O_2$	278.4296						Salviae [63]; rice [65]; Pinus sylvestris [66]
15	Octadec-9-enoic acid	Oleic acid (Cis-9-Octadecenoic acid; Cis-Oleic acid)	$C_{18}H_{34}O_2$	282.4614		283	209; 153			Zostera marina [42]; Sanguisorba officinalis [67]; Pinus sylvestris [66]
16	Flavone	Acacetin [Linarigenin; Buddleoflavonol]	C ₁₆ H ₁₂ O ₅	284.2635		285	270; 224	241		Mexican lupine species [58]; Wissadula periplocifolia [59]; Mentha [68,69]; Dracocephalum palmatum [70]
17	Flavone	6,7-Dihydroxy-4'- methoxyisoflavone	C ₁₆ H ₁₂ O ₅	284.2635		285	270; 229; 145	242; 152		Mentha [68]

N⁰	Class of Compound	Identified Compound	Formula	Mass	Molecular Ion [M – H] [–]	Molecular Ion [M + H] ⁺	2 Fragmenta- tion MS/MS	3 Fragmenta- tion MS/MS	4 Fragmenta- tion MS/MS	References
18	Flavonol	Kaempferol [3,5,7- Trihydroxy-2-(4-hydro- xyphenyl)-4H- chromen-4-one]	C ₁₅ H ₁₀ O ₆	286.2363	285		257; 184; 117	117		Potato leaves [34]; <i>Lonicera</i> <i>japonica</i> [36]; Potato [38]; Andean blueberry [43]; <i>Rhus coriaria</i> [64]; Rapeseed petals [71]
19	Flavan-3-ol	Catechin	C ₁₅ H ₁₄ O ₆	290.2681		291	243; 189	215; 197		Potato [39]; <i>Triticum</i> [40]; millet grains [41]; Eucalyptus [72]; <i>Vaccinium macrocarpon</i> [73]
20	Flavan-3-ol	(epi)catechin	C ₁₅ H ₁₄ O ₆	290.2681		291	273; 117	255; 145		millet grains [41]; C. edulis [53]; Radix polygoni multiflori [54]; Camellia kucha [37]
21	Flavone	Chrysoeriol [Chryseriol]	C ₁₆ H ₁₂ O ₆	300.2629		301	299; 253; 152	226		Dracocephalum palmatum [70]; Rhus coriaria [64]; Rice [65]; Mentha [68]; Mexican lupine species [58]
22	Hydroxybenzoic acid	Ellagic acid [Benzoaric acid; Elagostasine; Lagistase; Eleagic acid]	C ₁₄ H ₆ O ₈	302.1926		303	275; 202	157	139	Rhus coriaria [64]; strawberry [74]; Rubus occidentalis [75]; vinery products [52]; Chamaecrista nictitans [76]; Punica granatum [77]
23	Flavonol	Quercetin	C ₁₅ H ₁₀ O ₇	302.2357		303	244; 202; 184	175; 156	129	Potato leaves [34]; <i>Triticum</i> [40]; Tomato [44]; millet grains [41]; Red wines [51]; vinery products [52]; <i>Rhus coriaria</i> [64]; Eucalyptus [72]; <i>Vaccinium</i> <i>macrocarpon</i> [73]
24	Flavanone	Hesperitin [Hesperetin]	C ₁₆ H ₁₄ O ₆	302.2788		303	202; 257; 185	156		Andean blueberry [43]; [78]; Red wines [51]; Mentha [79]

Molecular Molecular 2 Fragmenta-4 Fragmenta-3 Fragmenta-Class of № **Identified Compound** Ion Ion tion tion tion References Formula Mass Compound $[M - H]^{-}$ $[M + H]^+$ MS/MS MS/MS MS/MS Tanshinone IIB [(S)-6-(Hydroxymethyl)-1,6-Dimethyl-6,7,8,9-C19H18O4 *Salviae miltiorrhiza* [63] 25 Diterpenoid 310.3438 311 292; 189; 135 217; 135 Tetrahydrophenanthro [1,2-B]Furan-10, 11-Dione] 26 Flavone 5,7-Dimethoxyluteolin C₁₇H₁₄O₆ 314.2895 313 212; 185; 113 113 *Syzygium aromaticum* [80] Omega-hydroxy-19-Hydroxynonalong-chain C₁₉H₃₈O₃ 314.5032 315 169; 124 A. cordifolia [53] 27 287; 241; 187 241; 187 decanoic acid fatty acid Rhamnetin I Rhus coriaria L. (Sumac) [64]; [beta-Rhamnocitrin; 299; 243; C₁₆H₁₂O₇ 317 147; 123 28 Flavonol 316.2623 Ouercetin 7-Methyl 189;165; 123 Mangifera indica [81] ether] Isorhamnetin Andean blueberry [43]; [Isorhamnetol: Eucalyptus [72]; Astragali 29 Flavonol Quercetin 3'-Methyl C₁₆H₁₂O₇ 316.2623 317 288; 243; 189 260; 242; 187 Radix [45]; Embelia [50]; ether; *Rapeseed petals* [71]; 3-Methylquercetin] Syzygium aromaticum [80] millet grains [41]; Red wines [51]; Andean blueberry [43]; Sanguisorba 30 Flavonol Myricetin C₁₅H₁₀O₈ 318.2351 319 271; 217 243; 189; 171 171 officinalis [67]; F. glaucescens [53]; *Clidemia rubra* [82] Umbelliferone 306; 289; 225; C₁₅H₁₆O₈ G. linguiforme [53] 31 Hydroxycoumarin 324.2827 325 145 hexoside 163 Long-Chain Docosahexaenoic acid 327; 281; 181; Polyunsaturated $C_{22}H_{32}O_2$ 328.4883 329 199 Marine extracts [83] 32 [Doconexent; 115 **Fatty Acid** Cervonic acid]

№	Class of Compound	Identified Compound	Formula	Mass	Molecular Ion [M – H] [–]	Molecular Ion [M + H] ⁺	2 Fragmenta- tion MS/MS	3 Fragmenta- tion MS/MS	4 Fragmenta- tion MS/MS	References
33	Trihydroxyflavone	Jaceosidin [5,7,4'-trihydroxy-6',5'- dimetoxyflavone]	C ₁₇ H ₁₄ O ₇	330.2889		331	329; 285; 231; 191; 163	328; 286; 216		Mentha [68,84]
34	Trihydroxyflavone	5,7-Dimethoxy-3,3',4'- trihydroxyflavone	$C_{17}H_{14}O_7$	330.2889		331	303; 185	157		Oxalis corniculata [85]
35	Flavonol	Myricetin 5-Methyl ether [5-O-Methylmyricetin]	$C_{16}H_{12}O_8$	332.2617		333	287; 241; 205; 177	177; 149	149; 123	Vitis amurensis [86]; Rhodiola rosea [87]
36	Alpha, omega- dicarboxylic acid	Eicosatetraenedioic acid	$C_{20}H_{30}O_4$	334.4498		335	307; 289; 233	277; 246; 207		G. linguiforme [53]
37	Flavone	Syringetin	C ₁₇ H ₁₄ O ₈	346.2883		347	317; 290; 219; 169	289; 272; 219	261; 173	C. edulis [53]
38	Lignan	Matairesinol [(–)-Matairesinol; Artigenin Congener]	$C_{20}H_{22}O_{6}$	358.3851		359	325; 289; 258; 198	143	127	Punica granatum [88]; Lignans [89]
39	Flavone	5,6-Dihydroxy-7,8,3',4'- tetramethoxyflavone	C ₁₉ H ₁₈ O ₈	374.3414		375	346; 219; 173	319; 273; 219; 173	273; 219; 173	Mentha [68]
40	Hydroxycinnamic acid	Caffeic acid derivative	C ₁₆ H ₁₈ O ₉ Na	377.2985	377		341; 215	179		Bougainvillea [46]; Embelia [50]
41	Sterol	Campesterol [Dihydrobrassicasterol]	C ₂₈ H ₄₈ O	400.6801		401	381; 304; 225; 171	363; 345; 279; 225; 169	345; 261; 202	A. cordifolia; C. edulis [53]
42	Sterol	Stigmasterol [Stigmasterin; Beta-Stigmasterol]	C ₂₉ H ₄₈ O	412.6908		413	301; 279; 189	171		Hedyotis diffusa [55]; A. cordifolia; F. pottsii [53]; Olive leaves [90]; Salvia [91]
43	Sterol	Beta-Sitostenone [Stigmast-4-En-3-One; Sitostenone]	C ₂₉ H ₄₈ O	412.6908		413	395; 345; 301; 171	189; 171		F. herrerae [53]; Cryptomeria japonica bark [92]; Terminalia laxiflora [93]

Tal	ole	1.	Cont.

№	Class of Compound	Identified Compound	Formula	Mass	Molecular Ion [M – H] [–]	Molecular Ion [M + H] ⁺	2 Fragmenta- tion MS/MS	3 Fragmenta- tion MS/MS	4 Fragmenta- tion MS/MS	References
44	Hydroxybenzoic acid	Salvianolic acid D	$C_{20}H_{18}O_{10}$	418.3509		419	373; 293; 212; 127	329; 271; 192; 127		Mentha [69,94]; Salvia multiorrizae [95]
45	Iridoid monoterpenoid	Dihydroisovaltrate	$C_{22}H_{32}O_8$	424.4847		425	365; 327; 281; 207	309; 253	235	Rhus coriaria [64]
46	Flavone	Apigenin-7 -O- glucoside [Apigetrin; Cosmosiin]	C ₂₁ H ₂₀ O ₁₀	432.3775		433	271	153; 214		Tomato [44]; Grataegi fructus [45]; Mexican lupine species [58]; Dracocephalum palmatum [70]; Mentha [84]; Malva sylvestris [96]
47	Hydroxybenzoic acid	Ellagic acid pentoside [Ellagic acid 4-O-xylopyranoside]	C ₁₉ H ₁₄ O ₁₂	434.3073	433		257	227; 157	199; 127	Strawberry [74]; Chamaecrista nictitans [76]; Punica granatum [77]; Rubus ulmifolius [97]
48	Flavonol	Dihydrokaempferol-3- O-rhamnoside	$C_{21}H_{22}O_{10}$	434.3934	433		259	258; 229	199	Vitis vinifera [98]
49	Dihydroflavonol	Aromadendrin 7-O-rhamnoside	$C_{21}H_{22}O_{10}$	434.3934		435	261; 243	243; 165	215; 161	Eucalyptus [72]
59	Flavone	Calycosin-7-O-beta-D- glucoside	$C_{22}H_{22}O_{10}$	446.4041		447	285	270; 225; 145	242; 152	Astragali radix [99]; [100]; Huolisu Oral Liquid [57];
51	Flavone	Acacetin O -glucoside	$C_{22}H_{22}O_{10}$	446.4041		447	285	269; 227; 145	241	Mexican lupine species [58]
52	Flavonol	Kaempferol-3-O- hexoside	C ₂₁ H ₂₀ O ₁₁	448.3769		449	329; 203	303; 257; 203; 185; 157		Andean blueberry [43]; vinery products [52]; F. glaucescens [53]; Rhus coriaria [64]; Punica granatum [77]; Cytisus multiflorus; Malva sylvestris [96]
53	Anthocyanin	Cyanidin-3- O- glucoside [Cyanidin 3-O-beta-D-Glucoside; Kuromarin]	C ₂₁ H ₂₁ O ₁₁ +	449.3848		449	287	213; 175	213; 185; 141	Triticum [40,101]; acerola [60]; Rice [65]; Clidemia rubra [82]; Rapeseed petals [71]; Vigna sinensis [102]; Vitis labrusca [103]

№	Class of Compound	Identified Compound	Formula	Mass	Molecular Ion [M – H] [_]	Molecular Ion [M + H] ⁺	2 Fragmenta- tion MS/MS	3 Fragmenta- tion MS/MS	4 Fragmenta- tion MS/MS	References
54	Anabolic steroid	Vebonol	C ₃₀ H ₄₄ O ₃	452.6686		453	444; 389; 340; 276	435; 395; 336; 259	417; 331; 268	Rhus coriaria [64]; Hylocereus polyrhizus [104]
55	Anthocyanin	Pelargonidin 3-O-(6-O-malonyl- beta-D-glucoside)	$C_{24}H_{23}O_{13}$	519.4388		519	271	215; 153	197	<i>Gentiana lutea</i> [105]; Wheat [101]; Strawberry [106]
56	Indole sesquiterpene alkaloid	Sespendole	C ₃₃ H ₄₅ NO ₄	519.7147		520	184; 502	166		Rhus coriaria [64]; Hylocereus polyrhizus [104]
57	Flavonol	Kaempferol diacetyl hexoside	$C_{25}H_{24}O_{13}$	532.4503		533	285	270; 229; 145	242; 224; 152	A. cordifolia [53]
58	Flavone	AcacetinO-glucoside malonylated	$C_{25}H_{24}O_{13}$	532.4503		533	285	269; 228; 145	196; 152	Mexican lupine species [58]
59	Condensed tannin	Procyanidin A-type dimer	$C_{30}H_{24}O_{12}$	576.501		577	547; 493; 425; 245; 181	217	189; 161	<i>Vaccinium macrocarpon</i> [73]; grape juice [107]; pear [108]
60	Condensed tannin	Proanthocyanidin B1 [Procyanidin B1; Procyanidin Dimer B1; (-)-epicatechin-(4beta- >8)-(+)-catechin; Epicatechin-(4beta->8)- ent-epicatechin]	C ₃₀ H ₂₆ O ₁₂	578.5202		579	409; 343; 291; 247; 205	287; 259; 203; 163	245	Camellia kucha [37]; millet grains [41]; Vigna inguiculata [49]; vinery products [52]; Andean blueberry [43]; Vaccinium macrocarpon [73]; strawberry [74]; grape juice [107]; pear [108]; Senna singueana [109]
61	Condensed tannin	Procyanidin B2 [Epicatechin-(4beta->8)- epicatechin]	$C_{30}H_{26}O_{12}$	578.5202		579	427; 291; 247; 211	408; 327; 227; 139	379; 287; 257; 163	millet grains [41]; <i>F. esculentum</i> [110]; Red wines [51]; blackberry [111]
62	Steroidal alkaloid	Alpha-chaconine	C ₄₅ H ₇₃ NO ₁₄	852.0594		852	706	560	398	Potato [39,112–114]
63	Steroidal alkaloid	Solanidadiene solatriose	C ₄₅ H ₇₃ NO ₁₅	868.9588		868	706; 661; 560; 477	560; 398	382; 327	Potato [113]

№	Class of Compound	Identified Compound	Formula	k-569 (China)	k-5586 (Sweden)	k-5367 (China)	k-5373 (China)	k-11538 (Russia)	k-11559 (Russia)	Primorskaya-86 (Russia)
1	Isoflavone	Daidzein [4′,7 -Dihydroxyisoflavone; Daidzeol]	$C_{15}H_{10}O_4$							
2	7-hydroxyisoflavone	Formononetin [Biochanin B; Formononetol]	$C_{16}H_{12}O_4$							
3	Flavone	Apigenin	C ₁₅ H ₁₀ O ₅							
4	7-hydroxyisoflavone	Formononetin [Biochanin B; Formononetol]	$C_{16}H_{12}O_4$		-					
5	Flavone	Apigenin	C ₁₅ H ₁₀ O ₅							
6	Flavone	Acacetin [Linarigenin; Buddleoflavonol]	C ₁₆ H ₁₂ O ₅							
7	Flavone	6,7-Dihydroxy-4'- methoxyisoflavone	$C_{16}H_{12}O_5$							
8	Flavone	Chrysoeriol [Chryseriol]	C ₁₆ H ₁₂ O ₆							
9	Flavone	5,7-Dimethoxyluteolin	C ₁₇ H ₁₄ O ₆							
10	Trihydroxyflayone	Iaceosidin	C17H14O7							
11	Trihydroxyflavone	5,7-Dimethoxy-3,3',4'- trihydroxyflavone	C ₁₇ H ₁₄ O ₇							
12	Flavone	Syringetin	C ₁₇ H ₁₄ O ₈							
13	Flavone	5,6-Dihydroxy-7,8,3',4'- tetramethoxyflavone	C ₁₉ H ₁₈ O ₈							
14	Flavone	Apigenin-7-O-glucoside	C ₂₁ H ₂₀ O ₁₀							
15	Flavone	Calycosin-7-O-beta-D- glucoside	$C_{22}H_{22}O_{10}$							
16	Flavone	Acacetin O-glucoside	C ₂₂ H ₂₂ O ₁₀							
17	Flavone	Acacetin O-glucoside malonylated	$C_{25}H_{24}O_{13}$							
18	Flavonol	Kaempferol	C ₁₅ H ₁₀ O ₆							
19	Flavonol	Quercetin	C ₁₅ H ₁₀ O ₇							
20	Flavonol	Rhamnetin I	C ₁₆ H ₁₂ O ₇							
21	Flavonol	Isorhamnetin	C ₁₆ H ₁₂ O ₇							
22	Flavonol	Myricetin	C ₁₅ H ₁₀ O ₈							
23	Flavonol	Myricetin 5-Methyl ether [5-O-Methylmyricetin]	C ₁₆ H ₁₂ O ₈							

 Table 2. Polyphenolic compounds identified in seven varieties of soybean.

N⁰	Class of Compound	Identified Compound	Formula	k-569 (China)	k-5586 (Sweden)	k-5367 (China)	k-5373 (China)	k-11538 (Russia)	k-11559 (Russia)	Primorskaya-86 (Russia)
24	Flavonol	Dihydrokaempferol-3-O- rhamnoside	$C_{21}H_{22}O_{10}$							
25	Dihydroflavonol	Aromadendrin 7-O-rhamnoside	$C_{21}H_{22}O_{10}$							
26	Flavonol	Kaempferol-3-O-hexoside	$C_{21}H_{20}O_{11}$							
27	Flavonol	Kaempferol diacetyl hexoside	$C_{25}H_{24}O_{13}$							
28	Flavan-3-ol	Epiafzelechin [(epi)Afzelechin]	$C_{15}H_{14}O_5$							
29	Flavan-3-ol	Catechin	$C_{15}H_{14}O_{6}$							
30	Flavan-3-ol	(epi)catechin	C ₁₅ H ₁₄ O ₆							
31	Flavanone	Hesperitin [Hesperetin]	$C_{16}H_{14}O_{6}$							
32	Anthocyanin	Pelargonidin [Pelargonidol chloride]	C ₁₅ H ₁₁ O ₅₊							
33	Anthocyanin	Cyanidin-3-O-glucoside	C ₂₁ H ₂₁ O ₁₁ +							
34	Anthocyanin	Pelargonidin 3-O-(6-O- malonyl-beta-D-glucoside)	$C_{24}H_{23}O_{13}$							
35	Condensed tannin	Procyanidin A-type dimer	$C_{30}H_{24}O_{12}$							
36	Condensed tannin	Proanthocyanidin B1	$C_{30}H_{26}O_{12}$							
37	Condensed tannin	Proanthocyanidin B2	$C_{30}H_{26}O_{12}$							
38	Phenolic acid	Ferulic acid	$C_{10}H_{10}O_4$					_		
39	Phenolic acid	Ellagic acid	$C_{14}H_6O_8$							
40	Phenolic acid	Caffeic acid derivative	C ₁₆ H ₁₈ O ₉ Na							
41	Phenolic acid	Salvianolic acid D	$C_{20}H_{18}O_{10}$							
42	Phenolic acid	Ellagic acid pentoside	$C_{19}H_{14}O_{12}$							
43	Stilbene	Resveratrol	C ₁₄ H ₁₂ O ₃							
44	Hydroxycoumarin	Umbelliferone hexoside	C ₁₅ H ₁₆ O ₈							
45	Lignan	Matairesinol	C ₂₀ H ₂₂ O ₆							

Figures 9 and 10 show examples of the decoding spectra (collision-induced dissociation (CID) spectrum) of the ion chromatogram obtained using tandem mass spectrometry. The mass spectrum in positive ion mode of Cyanidin 3-O-glucoside from extracts of soyabean k-5373 (China, Harbin semi-wild) is shown in Figure 9. The $[M + H]^+$ ion produced one fragment ion at m/z 287. The fragment ion with m/z 287 yielded two daughter ions at m/z 213 and m/z 137. This compound was identified in the bibliography as cyanidin 3-O-glucoside in extracts from *Clidemia rubra* [82], *Triticum* [40,101], *acerola* [60], rice [65], Disterigma [43], *Vigna sinensis* [102], *Vitis labrusca* [103], and rapeseed petals [71].



Figure 9. Mass spectrum of cyanidin 3-*O*-glucoside from extracts of soyabean k-5373 (China, Harbin semi-wild), *m*/*z* 448.88.



Figure 10. Mass spectrum of proanthocyanidin B1 from extracts of soyabean k-5373 (China, Harbin semi-wild), *m*/*z* 578.77.

The mass spectrum in positive ion mode of proanthocyanidin B1 from extracts from extracts of soyabean k-5373 (China, Harbin semi-wild) is shown in Figure 10. The $[M + H]^+$ ion produced five fragment ions at m/z 409, m/z 343, m/z 291, m/z 247, and m/z 205. The fragment ion with m/z 409 yielded four daughter ions at m/z 287, m/z 259, m/z 203, and m/z 163. The fragment ion with m/z 287 yielded two daughter ions at m/z 245 and m/z 203. To the best of our knowledge, proanthocyanidin B1 has been reported in millet grains [41], pear [108], *Vaccinium macrocarpon* [73], Andean blueberry [43], strawberry [74], *Vigna inguiculata* [49], *Senna singueana* [109], *Camellia kucha* [37], grape juice [107], vinery products [52], etc.

4. Conclusions

The results of a preliminary study showed the presence of 63 compounds corresponding to the Glycine Willd genus (soybean), some of which were identified for the first time in Glycine. The extracts of soybean k-5373 (China, Harbin semi-wild) contain the most polyphenolic complexes, which are biologically active compounds. Laser microscopy made it possible to clarify in detail the spatial arrangement of the polyphenolic content of soybeans. Results showed that phenolics of soybean are spatially located mainly in the seed coat and the outer layer of the cotyledon. Anthocyanins are especially abundant in the palisade layer of dark-colored varieties. The seed coat of yellow-seeded varieties contains more phenolic acids and flavonols than the seed coat of dark-seeded varieties. This information can be useful for rapid evaluation of varieties for selection and breeding with respect to those compounds.

Author Contributions: M.P.R., S.E. and K.S.G. conceived the idea. Y.N.Z. analyzed the data and wrote the manuscript. M.P.R., D.K.K., V.A.K., A.M.Z., S.E. and K.S.G. participated in the literature search and data analysis and provided technical guidance. M.P.R. and K.S.G. supervised the work and edited the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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