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Supercritical CO_2-Based Extraction and Detection of Phenolic Compounds and Saponins from the Leaves of Three *Medicago varia* Mart. Varieties by Tandem Mass Spectrometry

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Abstract: A comparative metabolomic study of three varieties of alfalfa (*Medicago varia* Mart.) was performed via extraction with supercritical carbon dioxide modified with ethanol (EtOH) and the detection of bioactive compounds via tandem mass spectrometry. Several experimental conditions were investigated in the pressure range of 50–250 bar, with ethanol used as a co-solvent in an amount of 1% of the total volume in the liquid phase at a temperature in the range of 31–70 °C. The most effective extraction conditions were as follows: a pressure of 250 Bar and a temperature of 60 °C for *M. varia*. *M. varia* contains various phenolic compounds and sulfated polyphenols with valuable biological activity. Tandem mass spectrometry (HPLC-ESI–ion trap) was applied to detect the target analytes. A total of 103 bioactive compounds (59 polyphenols and 44 compounds belonging to other chemical groups) were tentatively identified in extracts from aerial parts of alfalfa. For the first time, twenty-one chemical constituents from the polyphenol group (flavones: Formononetin, Chrysoeriol, Cirsimaritin, Cirsiliol, Cirsilineol, tricin-*O*-hexoside, Apigenin *C*-glucose *C*-deoxyhexoside, Apigenin 7-*O*-diglucuronide, 2′-Hydroxygenistein 4′,7-*O*-diglucoside, etc.) and six from other chemical groups (saponins: Soyasaponin II, Soyasaponin *gamma* g, Soyasaponin I, Soyasaponin Bd, Soyaysaponin *beta* g, etc.) were identified in the aerial parts of *M. varia*.

Keywords: alfalfa changeable; *Medicago varia* Mart.; tandem mass spectrometry; SC-CO_2 extraction; polyphenols; metabolome

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1. Introduction

One of the most versatile and cost-effective crops is alfalfa (*Medicago varia* Mart., family Fabaceae Lindl.), which plays an important role not only in sustainable agriculture but also in expanding the raw material base of the food, cosmetic and pharmaceutical industries. For instance, studies have shown that Medicago varieties contain bioactive compounds, identified as phenolic compounds, in particular, flavonoids. The flavonoids found include flavones (luteolin and apigenin), isoflavones (genistein), flavanones (naringenin), flavanols (catechin and epicatechin) and anthocyanidins (cyanidin and delphinidin) [1,2]. The interest in flavonoid research increased because of the potential of these substances to prevent or treat factors related to metabolic disorders [3]. GC–MS analysis of *M. sativa* seeds revealed their enrichment in crude protein (33.79%), crude oil (8.11%), squalene,

hexadecanoic acid methyl ester, n-hexadecanoic acid, 9,12-octadecadienoic acid methyl ester, 9-octadecenamide and vitamin E. Moreover, Medicago sativa seed inclusion in the diet is recommended to normalize serum cholesterol levels in type II hyperlipoproteinemia patients [4–6]. Additionally, there are several interesting scientific studies showing the presence of simple phenolic compounds in *Medicago* [7,8]. These compounds have a range of biological activities, including anti-inflammatory, antioxidant, phytoestrogenic and anti-carcinogenic abilities, as well as their interactions with intracellular signaling pathways and regulation of cell survival. Extracts and balms of *M. sativa* have long been used as traditional herbal medicines in many countries, such as China, India and America [9,10]. Most varieties of alfalfa are autotetraploids (2n = 4x = 32), with the main number of chromosomes being eight [11,12]. Alfalfa is characterized by its exceptional ability to grow under a wide range of natural conditions, its stable global yield, and its longevity and reproduction of soil fertility through fixation of atmospheric nitrogen. This crop is used as fodder in its green form or for the preparation of fodder (hay, haylage and grass meal). Lucerne hay is a quality forage containing high levels of protein, phosphorus, calcium and essential amino acids [13]. Increased forage production is possible through the development of more productive and higher-quality lucerne varieties. Each soil–climatic zone requires a diverse set of complementary varieties adapted to different extreme growing conditions [14]. Therefore, the study of the genetic diversity of alfalfa source material is of great theoretical and practical importance. *M. varia* contains omega-3 fatty acids, which are necessary to improve milk quality and increase meat production in ruminants [15]. The plant complex of alfalfa, which contains substances necessary for humans (especially in unfavorable environmental conditions), is used in technology for the production of fermented milk products [16]. It is also worth noting the insecticidal and fungicidal potential of the use of saponins identified in *Medicago*. The combined deterrent and toxic effects on insects make *Medicago* saponins suitable for use against insect pests in agriculture and horticulture [17]. An effective way of using *Medicago* saponins against insect herbivores is to select varieties that accumulate high levels of saponins [17]. For example, the development, survival and reproduction of pea aphids fed on high-saponin alfalfa were reduced compared to those fed on low-saponin alfalfa. In addition, it has been shown that zanhic acid tridesmoside and medicagenic acid, which accumulate in a high-saponin cultivar, are the main compounds contributing to the resistance of alfalfa to pea aphids [18,19]. The nematocidal activity of saponins allows the use of *Medicago* biomass as a biological agent to control plant-parasitic nematodes, which are widespread in the soil. The antifungal activity of alfalfa saponins may also reduce the presence of phytopathogenic fungi in the soil. Total saponins and selected compounds from different *Medicago* species have been shown to prevent fusarium on tulip bulbs [20,21]. Saponins against phytopathogenic fungi and nematodes in plant material make *Medicago* biomass particularly useful as an agent against soil-borne plant pathogens and as a biological fertilizer. Supercritical fluid extraction with the use of pressured CO_2 (SC-CO_2) has been used over the last 50 years in analytical methodologies to investigate the composition of food products, for the removal of undesirable substances and for the isolation of valuable molecules. The goal of the present work was to identify and select bioactive compounds from *M. varia* via extraction with SC-CO_2. Also, a tandem mass spectrometry protocol was used for the detailed screening of phytochemicals present in three varieties of *M. varia*.

2. Materials and Methods

2.1. Materials

The subject of the study was the green mass of *M. varia* varieties (Demetra, Nakhodka and Sarga) (Figure 1A–D) collected and grown at the Sakhalin Agricultural Scientific Research Institute—Branch of N.I. Vavilov All-Russian Institute of Plant Genetic Resources. Standard agronomic practices were used for growing the accessions/varieties in their respective locations. The aerial parts of *M. varia* were harvested at the end of July 2023. All plant tissues used in this work conformed to the standard established by the State Pharmacopoeia of the Russian Federation [22].

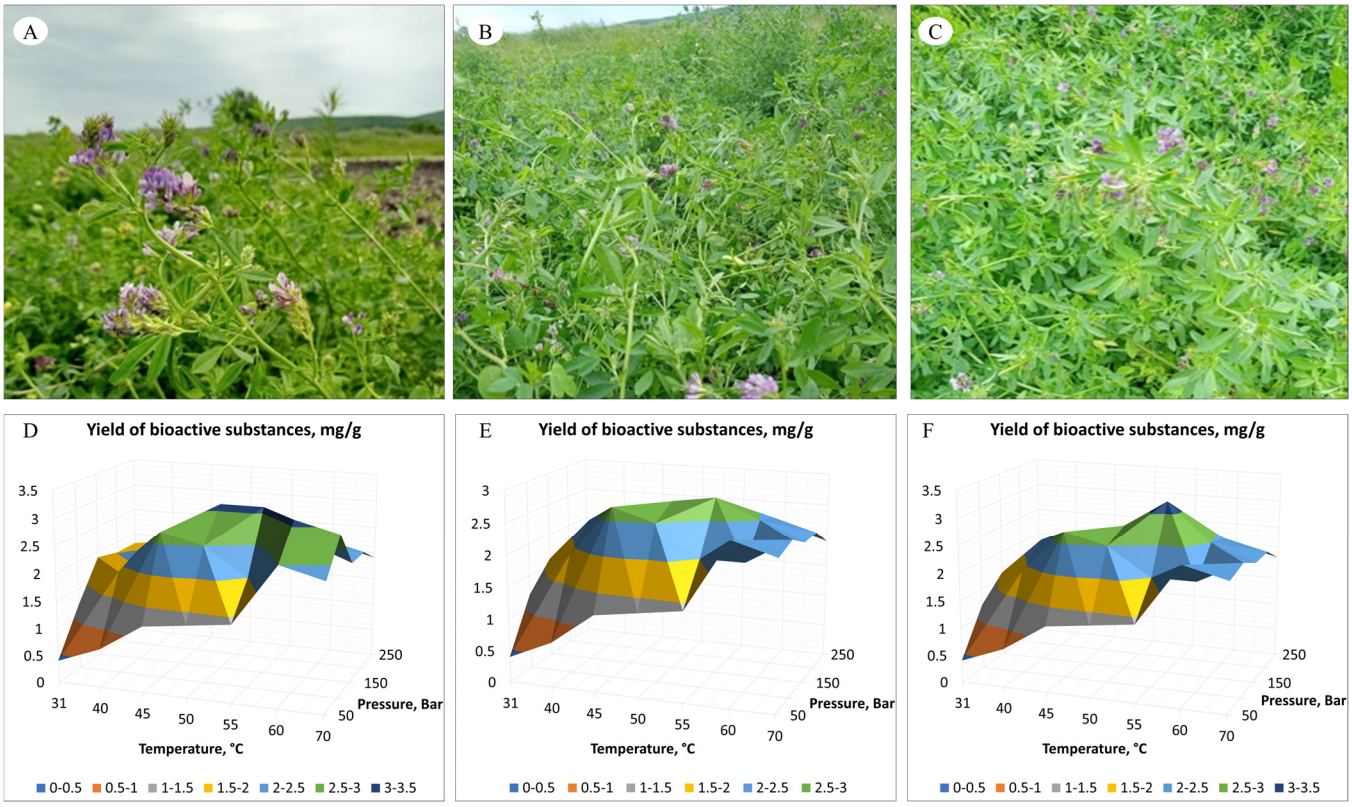

Figure 1. *M. varia* varieties: (**A**) Sarga; (**B**) Nakhodka; (**C**) Demetra. (Photos by E. Ivanova.) (**D**) Three-dimensional graph of the global yield of biologically active substances during SC-CO_2 extraction of the *M. varia* varieties Sarga, (**E**) Nakhodka and (**F**) Demetra.

2.2. Chemicals and Reagents

All reagents used in the study were of analytical grade. HPLC-grade acetonitrile was purchased from Fisher Scientific (Kent, UK), and MS-grade formic acid and ethanol (EtOH) were purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was obtained from Siemens (SIEMENS water technologies, Munich, Germany).

2.3. Extraction

SC-CO_2 extraction was performed using the SFE-500 supercritical pressure extraction system (Thar SCF Waters, Milford, CT, USA). The system options include the following: a co-solvent pump (Thar Waters P-50 High Pressure Pump) for the extraction of polar samples; a CO_2-flow meter (Siemens, Munich, Germany) to measure the amount of CO_2 supplied to the system; and multiple extraction vessels to extract different sample sizes or to increase the throughput of the system. The flow rate was 10–25 mL/min for liquid CO_2 and 1.00 mL/min for EtOH. Extraction samples of 200 g *M. varia* were used. The extraction time was counted after reaching working pressure and equilibrium flow and was 60–90 min for each sample. This method of SC-CO_2 extraction of plant matrices was tested by the authors on numerous plant samples, including aboveground and underground parts of the plant [23–25].

2.4. Liquid Chromatography

High-performance liquid chromatography was carried out on a Shimadzu LC-20 Prominence HPLC (Shimadzu, Kyoto, Japan) instrument equipped with a UV sensor and a C18 silica reverse phase column (4.6 × 150 mm, particle size: 2.7 µm). Mobile-phase eluent A was deionized water containing 0.1% formic acid, and eluent B was acetonitrile containing 0.1% formic acid. The gradient elution was started at 0–2 min, 0% eluent B 2–50 min, 0–100% B; control washing: 50–60 min, 100% B. The mobile-phase flow rate

and column temperature were maintained at 0.3 mL/min and 30 °C, respectively. A UV–vis detector, the SPD-20A (Shimadzu, Kyoto, Japan), was used for detection and compound identification at a wavelength of 230 nm. The injection volume was 10 µL. Additionally, liquid chromatography was combined with a mass spectrometric ion trap to identify compounds.

2.5. Mass Spectrometry

MS analysis was performed on an ion trap, the amaZon SL (Bruker Daltoniks, Bremen, Germany), equipped with an ESI source in negative ion mode. MS analysis was carried out in electrospray ionization (ESI) mode using negative and positive polarity for all samples with data-independent MSE acquisition. The optimized parameters were obtained as reported earlier [23–25]. Similarly, the data collection and compound identification were carried out as per our previous reports [23–25].

2.6. Statistical Analysis

To more clearly present the similarities and differences of bioactive substances identified in different variants of *M. varia*, the team of authors used the Jaccard index. The Jaccard index, also known as the Jaccard similarity coefficient, is a statistical measure used to evaluate the similarity and diversity of sets of samples. Nine replicate samples were analyzed. Jaccard indices were calculated using a the "Compare Lists—Multiple List Comparator" hosted on molbiotools server (https://molbiotools.com/listcompare.php (accessed on 21 March 2024)).

3. Results

3.1. SC-CO_2 Extraction of Aerial Parts of M. varia

Three *M. varia* varieties, i.e., Sarga, Nakhodka and Demetra, were examined by SC-CO_2 extraction under different extraction conditions. The supercritical pressures applied ranged from 50 to 250 bar, and the extraction temperature ranged from 31 to 70 °C. The co-solvent, EtOH, was used in an amount of 1% of the total solvent amount. The Table 1 shows the global yield of bioactive compounds (variety Sarga) by SC-CO_2 extraction. Figure 1D shows a 3D plot of the global yield of bioactive compounds during SC-CO_2 extraction of the aerial parts (variety Sarga).

Table 1. The global yield of extract (mg/100 mg) after SC-CO_2 extraction of *M. varia*, variety Sarga.

Temperature (°C)	Pressure (Bar)				
	50 Bar	100 Bar	150 Bar	200 Bar	250 Bar
31 °C	0.4	1.2	1.9	1.8	1.8
40 °C	0.7	1.9	2.1	1.9	1.9
45 °C	1.2	2.7	2	2	2.1
50 °C	1.3	2.5	3.1	2.5	2.2
55 °C	1.4	2	3.1	2.6	2.1
60 °C	2.5	2.9	2.5	2.4	2.1
70 °C	2.3	2.9	2.2	2.2	1.9

The maximum global yield of bioactive substances from alfalfa aerial parts (variety Sarga) was observed under the following extraction conditions:

- Pressure: 150 Bar, extraction temperature: 50 °C, extraction time: 1 h; the global yield of biologically active substances was 3.1 mg/100 mg of plant sample; the share of the EtOH modifier was 2%;
- Pressure: 150 Bar, extraction temperature: 55 °C, extraction time: 1 h; the global yield of biologically active substances was 3.1 mg/100 mg of plant sample; the share of the EtOH modifier was 2%.

Table 2 showing the global yield of bioactive compounds (variety Nakhodka) by SC-CO_2 extraction is presented below.

Table 2. The global yield of bioactive compounds (variety Nakhodka) by SC-CO_2 extraction.

Pressure	50 Bar	100 Bar	150 Bar	200 Bar	250 Bar
31 °C	0.4	1.2	1.6	1.7	1.7
40 °C	0.7	1.9	2.3	1.9	1.9
45 °C	1.2	2.7	2	2	2.1
50 °C	1.3	2.5	2.7	2.2	2.2
55 °C	1.4	2	2.8	2.4	2.1
60 °C	2.5	2	2.5	2.4	2.1
70 °C	2.3	2.4	2.2	2.2	1.9

Figure 1E shows a 3D graph of the global yield of biologically active substances during SC-CO_2 extraction of alfalfa aerial parts (variety Nakhodka). The maximum global yield of bioactive substances from alfalfa aerial parts (variety Nakhodka) was observed under the following extraction conditions:

– Pressure: 150 Bar, extraction temperature: 50 °C, extraction time: 1 h; the global yield of biologically active substances was 2.7 mg/100 mg of plant sample; the share of the EtOH modifier was 2%;
– Pressure: 150 Bar, extraction temperature: 55 °C, extraction time: 1 h; the global yield of biologically active substances was 2.8 mg/100 mg of plant sample; the share of the EtOH modifier was 2%.

Table 3 shows the global yield of bioactive compounds (variety Demetra) by SC-CO_2 extraction.

Table 3. The global yield of bioactive compounds (variety Demetra) by SC-CO_2 extraction.

Pressure	50 Bar	100 Bar	150 Bar	200 Bar	250 Bar
31 °C	0.4	1.2	1.6	1.7	1.7
40 °C	0.7	1.9	2.3	1.9	1.9
45 °C	1.2	2.7	2	2	2.1
50 °C	1.3	2.5	2.7	2.2	2.2
55 °C	1.4	2	3.2	2.4	2.1
60 °C	2.5	2	2.5	2.4	2.1
70 °C	2.3	2.4	2.2	2.2	1.9

Figure 1F shows a 3D graph of the global yield of biologically active substances during SC-CO_2 extraction of alfalfa aerial parts (variety Demetra). The maximum global yield of bioactive substances from alfalfa aerial parts (variety Demetra) was observed under the following extraction conditions:

– Pressure: 150 Bar, extraction temperature: 50 °C, extraction time: 1 h; the global yield of biologically active substances was 3.2 mg/100 mg of plant sample; the share of the EtOH modifier was 2%.

3.2. Global Metabolome Profile of M. varia

The structural identification of each compound was carried out on the basis of its accurate mass and MS/MS fragmentation by HPLC-ESI–ion trap–MS/MS. A total of 103 chemical compounds were identified from the extracts of the three *M. varia* varieties (Table 4). Fifty-nine and forty-four chemical compounds were classified as polyphenols and others, respectively (see the chemical structure of some of these compounds in Figure 2). The polyphenols detected in our study were categorized as flavones, flavonols, flavan-3-ols, anthocyanidins, phenolic acids, lignans, coumarins, stilbenes, etc. In total, the metabolites detected in our study belonged to 19 compound classes. The highest number of metabolites

was recorded for flavones (24), followed by flavonols (20), anthocyanins (6) and flavan-3-ols (3). These numbers of compounds in respective groups indicate that *M. varia* extracts are rich in flavonoids. The highest numbers of chemical compounds from other groups were recorded for polysaccharides (8) and saponins (11).

Table 4. Jaccard indices for three varieties of *M. varia*.

	Variety Demetra (71)	Variety Nakhodka (63)	Variety Sarga (38)
Variety Demetra (71)	--	41 0.4409	25 0.2976
Variety Nakhodka (63)	41 0.4409	--	22 0.2785
Variety Sarga (38)	25 0.2976	22 0.2785	--

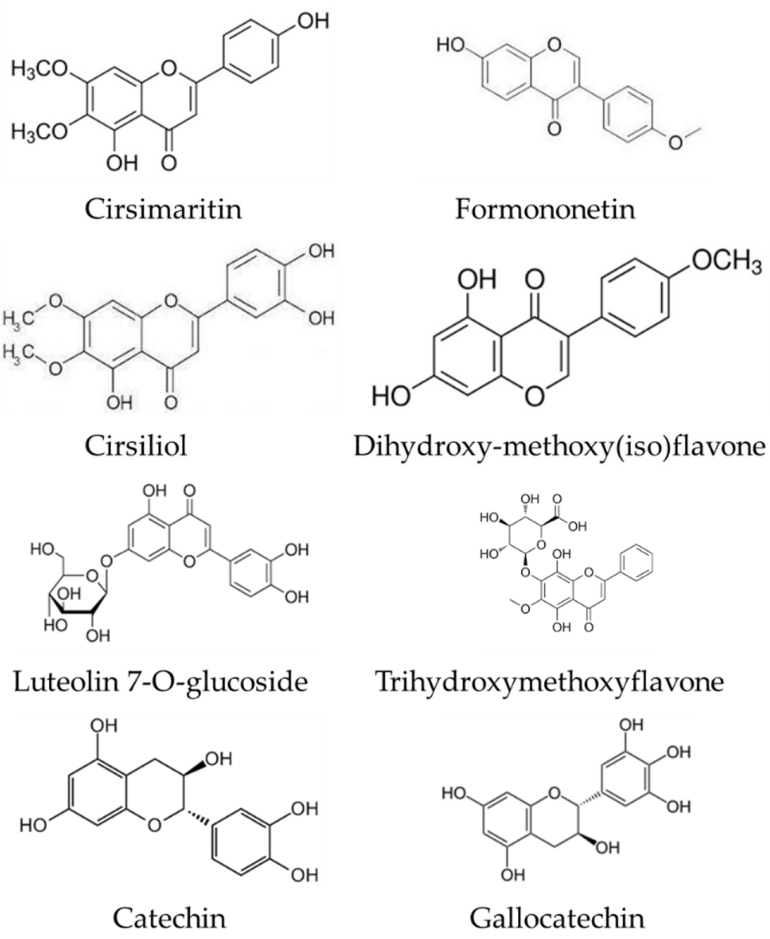

Figure 2. Chemical structures of some phenolic compounds identified in *M. varia*.

Figure 3 shows the numbers of common and specific compounds. Nineteen compounds were commonly detected from the three *M. varia* varieties. These nineteen compounds belong to compound classes such as flavones, flavonols, anthocyanins and saponins, suggesting that flavonoids and saponins are major active compounds in *M. varia* leaves. The applied methods were able to detect 71 (Demetra), 63 (Nakhodka) and 38 (Sarga) compounds.

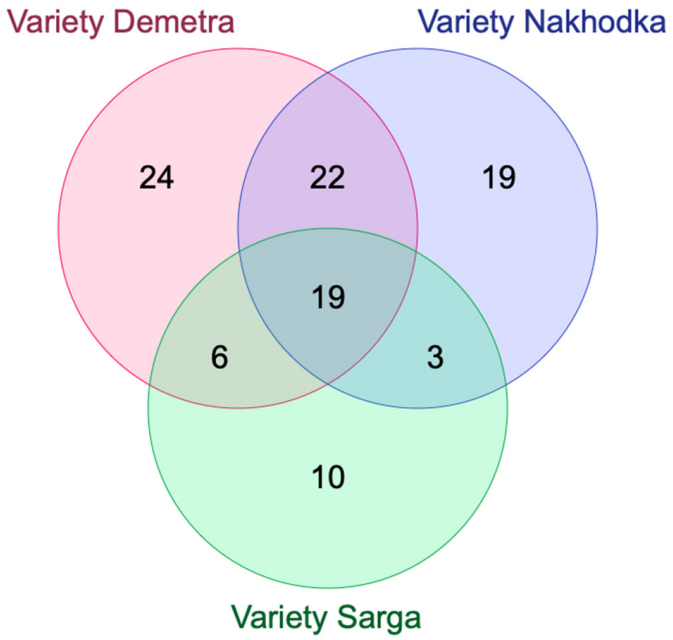

Figure 3. Venn diagram showing numbers of common and specific compounds in *M. varia* varieties.

Moreover, to present the similarities and differences in bioactive substances in different variations of *M. varia*, we used the Jaccard index (Table 4). The Jaccard index, also known as the Jaccard similarity coefficient, is a statistic used to evaluate the similarity and diversity of sets of samples [26–28]. It showed that the highest degree of similarity existed between the varieties Demetra and Nakhodka—0.4409.

3.2.1. Flavones

Hydroxy(iso)flavones

7-hydroxyisoflavone formononetin (compound **1**) and monohydroxy flavone apigenin-7,4′-dimethyl ether (compound **6**) have already been reported in *Astragali Radix* [29], Huolisu oral liquid [30], *Dracocephalum jacutense* [31], *Maackia amurense* [32], the Chinese herbal formula for the Jian-Pi-Yi-Shen pill [33], *Ocimum* [34] and propolis [35]. Thus, our results indicate that both the flavones formononetin and apigenin-7,4′-dimethyl ether were tentatively identified components in the extracts from *M. varia* (varieties Demetra and Nakhodka) (Table 2). The CID (collision-induced spectrum) in positive ion mode of flavone formononetin from variety Demetra is shown in Figure 4A. $[M + H]^+$ ions produced two fragment ions (FIs) with m/z 254.12 and m/z 213.20 (Figure 4A). The FI with m/z 254.12 produced one characteristic daughter ion with m/z 237.13.

Dihydroxyflavones

The flavones acacetin (compound **3**), dihydroxy-methoxy(iso)flavone (compound **4**), cirsimaritin (compound **9**), dihydroxy-dimethoxy(iso)flavone (compound **10**), nevadensin (compound **12**), cirsilineol (compound **13**) and 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone (compound **15**) (Table 2) have already been characterized as components of *Mentha* [36], *Ocimum* [34], Mexican lupine species [37], *Wissadula periplocifolia* [38], *Artemisia annua* [39], *Rosmarinus officinalis* [40], *Astragali radix* [29] and propolis [35]. We also tentatively identified these flavones in extracts from the three varieties of *M. varia* (Nakhodka, Sarga and Demetra). The CID in positive ion mode of cirsimaritin from extracts from *M. varia* (variety Nakhodka) is shown in Figure 4B. $[M + H]^+$ ions produced four FIs with m/z 287.18, m/z 259.20, m/z 216.08 and m/z 167.17 (Figure 4B). The FI with m/z 259.20 produced two characteristic daughter ions with m/z 227.15 and m/z 171.18. The FI with m/z 227.15 generated an ion with m/z 198.18.

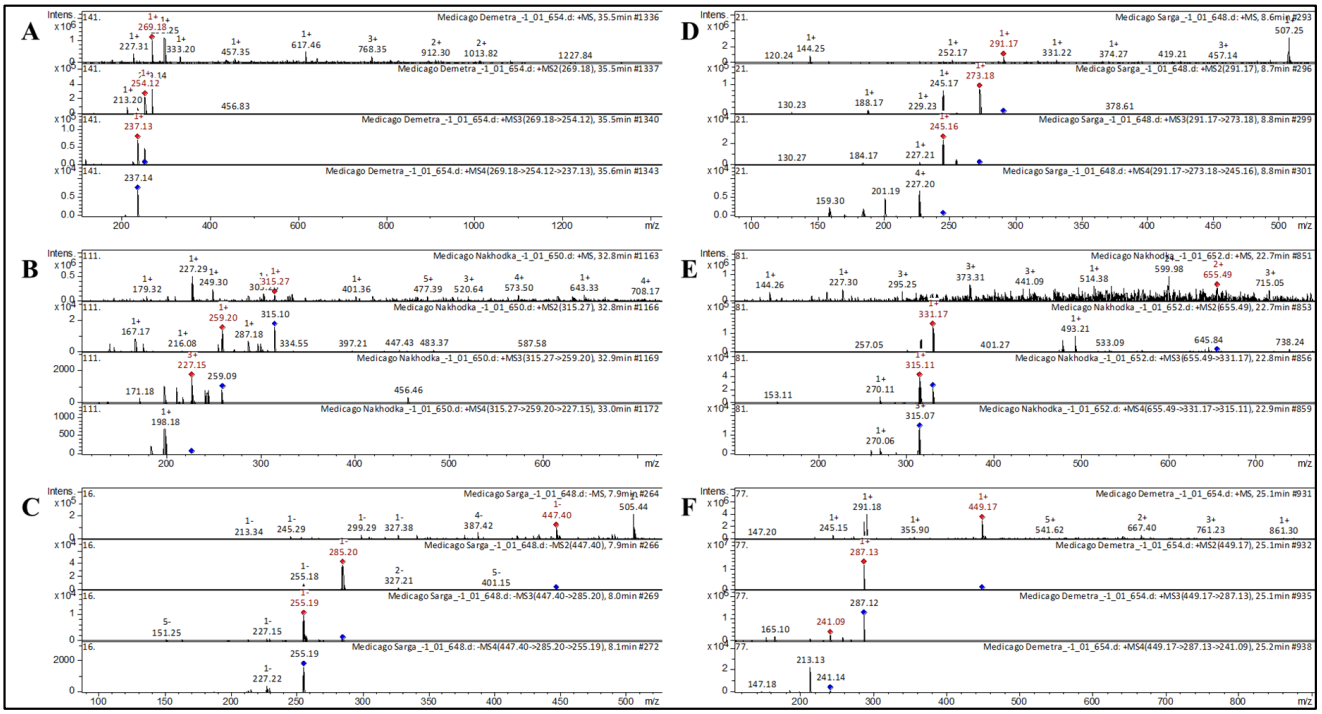

Figure 4. (**A**) Collision-induced spectrum of Formononetin from *M. varia* (variety Demetra), *m*/*z* 269.18. (**B**) CID of Cirsimaritin from *M. varia* (variety Nakhodka), *m*/*z* 315.27. (**C**) CID of Astragalin from *M. varia* (variety Sarga), *m*/*z* 447.40. (**D**) CID of Catechin from *M. varia* (variety Sarga), *m*/*z* 291.17. (**E**) CID of Malvidin 3-*O*-(6-*O*-*p*-caffeoyl) glucoside from berries of *M. varia* (variety Nakhodka), *m*/*z* 655.49. (**F**) CID of Cyanidin-3-*O*-glucoside from berries of *M. varia* (variety Demetra), *m*/*z* 449.17.

Trihydroxyflavones

The flavones apigenin (compound **2**), trihydroxymethoxyflavone (compound **7**), chrysoeriol [chrysoeriol] (compound **8**), cirsiliol (compound **11**), luteolin 7-*O*-glucoside [cynaroside] (compound **16**), kaempferide [4′-*O*-methylkaempferol] (compound **26**), rhamnocitrin (compound **27**), kaempferol-3-*O*-*α*-L-rhamnoside (compound **33**), astragalin [kaempferol 3-*O*-glucoside] (compound **34**) and isorhamnetin 3-*O*-glucoside (compound **36**) (Table 2) have been already characterized as a components of *Inula gaveolens* [41], *Phlomis (Lamiaceae*) [42], *Lonicera henryi* [43], *Ribes meyeri* [44], *Lonicera japonica* [45], *Stevia rebaudiana* [46], propolis [35] and *Jatropha* [47]. There trihydroxyflavones were tentatively identified in extracts from *M. varia* (varieties Nakhodka, Sarga and Demetra). The CID in negative ion mode of astragalin from extracts of *M. varia* is shown in Figure 4C. $[M - H]^-$ ions produced four FIs with *m*/*z* 285.20, *m*/*z* 401.15, *m*/*z* 327.21 and *m*/*z* 255.18 (Figure 4C). The FI with *m*/*z* 285.20 produced one characteristic daughter ion with *m*/*z* 255.19, *m*/*z* 227.15 and *m*/*z* 151.250. The FI with *m*/*z* 255.19 produced one characteristic daughter ion with *m*/*z* 227.22. Astragalin has been reported in extracts from *Juglans mandshurica* [48], bee pollen [49], *Lonicera japonica* [45], *Ribes meyeri* [44] and potato [50].

3.2.2. Flavan-3-ols

Catechin (compound **45**), (epi)-catechin (compound **46**) and gallocatechin (compound **47**) (Table 2) have already been characterized as components of *Carpinus betulus* [51], *Solanaceae* [52], *Glottiphyllum linguiforme* [53], *Embelia* [54], *Inula viscosa* [55], *Juglans mandshurica* [48], *Ribes meyeri* [44], *Radix polygoni multiflori* [56], *Glycine* soja [57,58] and *Jatropha* [47]. These flavan-3-ols were tentatively identified in extracts from *M. varia* (varieties Nakhodka, Sarga and Demetra). The CID in positive ion mode of Catechin from extracts from *M. varia* (variety Sarga) is shown in Figure 4D. $[M - H]^-$ ions produced five FIs with *m*/*z* 273.18, *m*/*z* 245.17, *m*/*z* 229.23, *m*/*z* 188.17 and *m*/*z* 130.23 (Figure 4D). The FI

with *m*/*z* 273.18 produced four characteristic daughter ions with *m*/*z* 245.16, *m*/*z* 227.21, *m*/*z* 184.17 and *m*/*z* 130.27. The FI with *m*/*z* 245.16 produced three characteristic daughter ions with *m*/*z* 227.20, *m*/*z* 201.19 and *m*/*z* 159.30.

3.2.3. Anthocyanins

Cyanidin-3-*O*-glucoside (compound **48**), malvidin 3-*O*-glucoside (compound **49**), cyanidin 3-(6″-malonylglucoside) (compound **50**), cyanidin-3-*O*-dioxayl-glucoside (compound **51**), peonidin 3-*O*-(6-*O*-*p*-coumaroyl) glucoside (compound **52**) and malvidin 3-*O*-(6-*O*-*p*-caffeoyl) glucoside (compound **53**) (Table 2) have been already characterized as components of *Gaultheria mucronata*, *Gaultheria antarctica* [59], *Berberis microphylla* [60], *Bougainvillea* [61], *Grape* [62], vines [63] and many other plant species whose organs (mainly fruits) accumulate pigments and exhibit a range of colors. These anthocyanins were tentatively identified in extracts from *M. varia* (varieties Nakhodka and Demetra). The CID in positive ion mode of malvidin 3-*O*-(6-*O*-*p*-caffeoyl) glucoside from extracts from *M. varia* (variety Nakhodka) is shown in Figure 4E. $[M + H]^+$ ions produced two FIs with *m*/*z* 331.17 and *m*/*z* 257.05 (Figure 4E). The FI with *m*/*z* 331.11 produced three characteristic daughter ions with *m*/*z* 315.11, *m*/*z* 270.11 and *m*/*z* 153.11. The anthocyanin malvidin 3-*O*-(6-*O*-*p*-caffeoyl) glucoside was tentatively identified in results for extracts from *Grape* [62] and *Bougainvillea* [61]. The CID in positive ion mode of cyanidin-3-*O*-glucoside from extracts from *M. varia* (variety Demetra) is shown in Figure 4F. $[M + H]^+$ ions produced one FI with *m*/*z* 287.13 (Figure 4F). The FI with *m*/*z* 287.13 produced two characteristic daughter ions with *m*/*z* 241.09 and *m*/*z* 165.10. The anthocyanin cyanidin-3-*O*-glucoside was tentatively identified in the results for extracts from several plant species, some of which were mentioned at the beginning of this paragraph, as well as others, such as *Ribes magellanicum* [64] and *Rubus ulmifolius* [65].

3.3. Newly Detected Chemical Compounds in M. varia

Of the detected metabolites in the three *M. varia* varieties, twenty-one compounds from the polyphenol group and six compounds from other chemical groups were identified for the first time. The newly identified polyphenols include flavones (formononetin, chrysoeriol, cirsimaritin, cirsiliol, cirsilineol, tricin-*O*-hexoside, apigenin *C*-glucose *C*-deoxyhexoside, apigenin 7-*O*-diglucuronide and 2′-hydroxygenistein 4′,7-*O*-diglucoside malonylated), flavonols (ampelopsin, astragalin, kaempferol 3-(6″-malonylglucoside), rhamnosylhexosyl-methyl-quercetin, quercetin 3,4′-di-*O*-*β*-glucopyranoside, isorhamnetin-di-*O*-hexoside, quercetin-7-*O*-(acetyl-hexoside)-3-*O*-rhamnoside and isorhamnetin-3-*O*-6-*O*-acetyl-*β*-*D*-glucopyranosyl), the flavan-3-ol gallocatechin, vimalin (phenylpropanoid), syringaresinol (lignan), fraxetin (coumarin), etc. Interestingly, the other compound classes that we also detected in *M. varia* were saponins (soyasaponin II, soyasaponin *gamma* g, soyasaponin I, soyasaponin Bd and soyasaponin *beta* g), steroidal alkaloids (alpha-chaconine), etc. (Table 5).

Table 5. Chemical compounds identified from the SC-CO_2-extracts of *M. varia* in positive and negative ionization modes by HPLC–ion trap–MS/MS.

	Class of Compound	Identification	Formula	Calculated Mass	Observed Mass $[M - H]^-$	Observed Mass $[M + H]^+$	MS/MS Stage 1 Fragmentation	MS/MS Stage 2 Fragmentation	MS/MS Stage 3 Fragmentation	References
		Phenolic compounds								
1	**7-Hydroxyisoflavone**	**Formononetin** [Biochanin B; Formononetol] *	$C_{16}H_{12}O_4$	**268.2641**		**269**	254; 213	237	237	*Astragali Radix* [29]; Huolisu oral liquid [30]; *Dracocephalum jacutense* [31]; *Maackia amurense* [32]; Chinese herbal formula, Jian-Pi-Yi-Shen pill [33]
2	**Flavone**	**Apigenin** [5,7-Dixydroxy-2-(40Hydroxyphenyl)-4H-Chromen-4-One]	$C_{15}H_{10}O_5$	**270.2369**		**271**	271; 153			Propolis [35]; *Inula gaveolens* [41]; *Phlomis (Lamiaceae)* [42]; *Lonicera henryi* [43]; *Ribes meyeri* [44]; *Lonicera japonica* [45]; *Stevia rebaudiana* [46]; *Jatropha* [47]
3	**Flavone**	**Acacetin** [Linarigenin; Buddleoflavonol]	$C_{16}H_{12}O_5$	**284.2635**		**285**	270	269; 242	213; 185	Propolis [35]; *Mentha* [36]; Mexican lupine species [37]; *Wissadula periplocifolia* [38]
4	**Flavone**	**Dihydroxy-methoxy(iso)flavone**	$C_{16}H_{12}O_5$	**284.2635**		**285**	270	269	227	Propolis [35]
5	**Flavone**	**Luteolin**	$C_{15}H_{10}O_6$	**286.2363**		**287**	213; 165	157		Propolis [35]; *Artemisia absinthium* [39]; *Inula gaveolens* [41]; *Lonicera henryl* [43]; *Ribes meyeri* [44]; *Lonicera japonica* [45]; *Jatropha* [47]; potato [50]
6	**Flavone**	**Apigenin-7, 4′-dimethyl ether**	$C_{17}H_{14}O_5$	**298.2901**		**299**	284	256; 169	255; 132	*Ocimum* [34]; propolis [35]
7	**Flavone**	**Trihydroxymethoxyflavone**	$C_{16}H_{12}O_6$	**300.2629**		**301**	286	258		*Artemisia absinthium* [39]
8	**Flavone**	**Chrysoeriol** [Chryseriol] *	$C_{16}H_{12}O_6$	**300.2629**		**301**	286	258		Propolis [35]; *Mentha* [36]; Mexican lupine species [37]; *Rhus coriaria* [66]
9	**Flavone**	**Cirsimaritin** [Scrophulein; 4′,5-Dihydroxy-6,7-Dimethoxyflavone] *	$C_{17}H_{14}O_6$	**314.2895**		**315**	287; 259; 216	227; 171	198	*Ocimum* [34]; *Artemisia annua* [39]; *Rosmarinus officinalis* [40]
10	**Flavone**	**Dihydroxy-dimethoxy(iso)flavone**	$C_{17}H_{14}O_6$	**314.2895**		**315**	287; 259; 216	227; 171	198	*Astragali radix* [29]; propolis [35]; *Rosmarinus officinalis* [50]
11	**Flavone**	**Cirsiliol** *	$C_{17}H_{14}O_7$	**330.2889**		**332**	315; 271			*Ocimum* [34]; *Juglans mandshurica* [48]; *Inula viscosa* [55]
12	**Flavone**	**Nevadensin**	$C_{18}H_{16}O_7$	**344.3154**		**345**	312; 222; 181	284; 256	283; 269; 255	*Ocimum* [34]; *Mentha* [36]
13	**Flavone**	**Cirsilineol** [Eupatrin; Fastigenin] *	$C_{18}H_{16}O_7$	**344.3154**		**345**	312; 222; 181	284; 256	283; 269; 255	*Ocimum* [34]

Table 5. *Cont.*

	Class of Compound	Identification	Formula	Calculated Mass	Observed Mass $[M - H]^-$	Observed Mass $[M + H]^+$	MS/MS Stage 1 Fragmentation	MS/MS Stage 2 Fragmentation	MS/MS Stage 3 Fragmentation	References
14	**Flavone**	**Tetrahydroxy-dimethoxyflavone**	**$C_{17}H_{14}O_8$**	**346.2883**	**345**		330	315	287	*Artemisia absinthium* [39]
15	**Flavone**	**5,6-Dihydroxy-7,8,3′,4′-tetramethoxyflavone**	**$C_{19}H_{18}O_8$**	**374.3414**		**375**	368; 348; 325; 304	358; 301; 226		Mentha [36]
16	**Flavone**	**Luteolin 7-*O*-glucoside** [Cynaroside; Luteoloside]	**$C_{21}H_{20}O_{11}$**	**448.3769**		**449**	287	241; 165	213; 147	Mexican lupine species [37]; *Lonicera henryi* [43]; *Lonicera japonica* [45]
17	**Flavone**	**Luteolin 8-*C*-Glucoside** [Orientin; Orientin (Flavone); Lutexin]	**$C_{21}H_{20}O_{11}$**	**448.3769**		**449**	430; 373; 328; 285; 215			*Lemon*, *passion fruit* [67]; *P. aculeata* [68]; *Phyllostachys nigra* [69]; *Aspalathus linearis* [70]; *bamboo* [71]
18	**Flavone**	**Tricin *O*-hexoside** *	**$C_{23}H_{24}O_{12}$**	**492.4295**		**493**	331	315		*Triticum aestivum* L. [72]; *bamboo* [71]
19	**Flavone**	**Dihydroxy-trimethoxyflavone-*O*-hexoside**	**$C_{23}H_{22}O_{14}$**	**506.414**		**507**	331	315; 270	270	Citrus species [73]
20	**Flavone**	**Apigenin *O*-pentosyl hexoside**	**$C_{26}H_{28}O_{14}$**	**564.4921**		**565**	433; 288	415; 334; 271; 163	127	*F. glaucescens* [53]
21	**Flavone**	**Apigenin *C*-glucose *C*-deoxyhexoside** *	**$C_{27}H_{30}O_{14}$**	**578.5187**		**579**	547; 488; 403; 365			*Passiflora incarnata* [74]
22	**Flavone**	**Apigenin 7-*O*-diglucuronide** *	**$C_{27}H_{26}O_{17}$**	**622.4851**		**623**	447	271	153	*Perilla frutescens* [75]
23	**Flavone**	**Tricin di-*O*,*O*-hexoside**	**$C_{29}H_{34}O_{17}$**	**654.5701**		**655**	331; 493	315; 270; 153	270	*Triticum aestivum* L. [72,76]
24	**Flavone**	**2′-Hydroxygenistein 4′, 7-*O*-diglucoside malonylated** *	**$C_{30}H_{32}O_{19}$**	**696.5637**		**697**	287	241; 165; 121	185	Mexican lupine species [37]
25	**Flavonol**	**Kaempferol**	**$C_{15}H_{10}O_6$**	**286.2363**		**287**	241	165		*Ribes meyeri* [44]; *Lonicera japonicum* [45]; *Juglans mandshurica* [48]; *Rhus coriaria* [66]
26	**Flavonol**	**Kaempferide** [4′-*O*-Methylkaempferol]	**$C_{16}H_{12}O_6$**	**300.2629**		**301**	286	258		*Ribes meyeri* [44]; *Alpinia officinarum* [77]; Brazilian propolis [78]
27	**Flavonol**	**Rhamnocitrin**	**$C_{16}H_{12}O_6$**	**300.2629**		**301**	273; 163	243	227	*Astragali radix* [29]; *Lonicera caerulea* [79]
28	**Flavonol**	**Quercetin**	**$C_{15}H_{10}O_7$**	**302.2357**		**303**	285; 167	257; 197	257	*Inula gaveolens* [41]; *Juglans mandshurica* [48]; *Inula viscosa* [55]; *Black soja* [57]
29	**Flavonol**	**Dihydroquercetin** (Taxifolin; Taxifoliol)	**$C_{15}H_{12}O_7$**	**304.2516**		**305**	285; 211; 175	268; 185; 124	168	*Juglans mandshurica* [48]; *Glycine soja* [58]; *Camellia kucha* [80]

Table 5. *Cont.*

	Class of Compound	Identification	Formula	Calculated Mass	Observed Mass $[M - H]^-$	Observed Mass $[M + H]^+$	MS/MS Stage 1 Fragmentation	MS/MS Stage 2 Fragmentation	MS/MS Stage 3 Fragmentation	References
30	**Flavonol**	**Isorhamnetin** [Isorhamnetol; Quercetin 3′-Methyl ether; 3-Methylquercetin]	$C_{16}H_{12}O_7$	**316.2623**		**317**	302	274; 153	229; 153	Propolis [35]; *Rosmarinus officinalis* [40]; *Stevia rebaudiana* [46]; *Inula viscosa* [55]; *Lonicera caerulea* [79]; *Phoenix dactylifera* [81]
31	**Flavonol**	**Myricetin**	$C_{15}H_{10}O_8$	**318.2351**	**317**		273; 295	260	238	*Juglans mandshurica* [48]; *F. glaucescens* [53]; *Taraxacum officinale* [82]
32	**Flavonol**	**Ampelopsin** [Dihydromyricetin; Ampeloptin] *	$C_{15}H_{12}O_8$	**320.251**		**321**	301; 201	267; 201		*Juglans mandshurica* [48]; *Rnus coriaria* [66]; *Impatients glandulifera* Royle [83]
33	**Flavonol**	**Kaempferol-3-*O*-α-*L*-rhamnoside**	$C_{21}H_{20}O_{10}$	**432.3775**		**433**	427; 340; 287			*Carpinus betulus* [51]; *C. edulis*; *F. glaucescens* [53]; *Taraxacum officinale* [82]; *Cassia abbreviata* [84]
34	**Flavonol**	**Astragalin** [Kaempferol 3-*O*-glucoside] *	$C_{21}H_{20}O_{11}$	**448.3769**	**447**		285; 255	255; 227; 151	227	*Juglans mandshurica* [48]; bee pollen [49]; *Lonicera japonica* [45]; *Ribes meyeri* [44]; *potato* [50]
35	**Flavonol**	**Quercetin 3-*O*-glucoside** [Isoquercetin; Isoquercitrin; Hirsutrin; Quercetin-3-*O*-Glucopyranoside]	$C_{21}H_{20}O_{12}$	**464.3763**		**465**	303; 258; 164	243; 179		*Juglans mandshurica* [48]; Black soja [57]; *Ribes meyeri* [44]; *Lonicera henryi* [43]; *Lonicera japonica* [45]; *Vaccinium myrtillus* [85]; *Solanaceae* [52]; *Rhus coriaria* [66]; *Embelia* [54]
36	**Flavonol**	**Isorhamnetin 3-*O*-glucoside**	$C_{22}H_{22}O_{12}$	**478.4029**		**479**	317	301; 257; 177	274; 218	*Artemisia annua* [86]; *Eucalyptus* [87]; *Capsicum annuum* [88]; *Senecio clivicolus* [89]
37	**Flavonol**	**Isorhamnetin 3-*O*-glucoronide**	$C_{22}H_{20}O_{13}$	**492.3864**		**493**	317	302	274	*Anethum graveolens* [90]; Strawberry [91];
38	**Flavonol**	**Kaempferol 3-(6″-malonylglucoside)** *	$C_{24}H_{22}O_{14}$	**534.4231**		**535**	287	241; 165	213	*A. cordifolia* [53]; Strawberry [91]
39	**Flavonol**	**Rhamnosylhexosyl-methyl-quercetin** *	$C_{26}H_{28}O_{17}$	**612.4903**		**613**	595; 540; 489	521; 337; 241	503; 349; 239	*Phoenix dactylifera* [81]
40	**Flavonol**	**Quercetin 3,4′-di-*O*-*beta*-glucopyranoside** [Quercetin diglucoside] *	$C_{27}H_{30}O_{17}$	**626.5169**		**627**	465; 393; 303	303	257; 165	Potato leaves [92]; potato [50]; rapeseed petals [93]
41	**Flavonol**	**Quercetin-*O*-dihexoside**	$C_{27}H_{30}O_{17}$	**626.5169**		**627**	465; 393; 303	303	257; 165	*Inula viscosa* [55]; *Artemisia absinthium* [39]; Chilean currants [64]; *Phoenix dactylifera* [81]; *Taraxacum formosanum* [94]

Table 5. *Cont.*

	Class of Compound	Identification	Formula	Calculated Mass	Observed Mass $[M - H]^-$	Observed Mass $[M + H]^+$	MS/MS Stage 1 Fragmentation	MS/MS Stage 2 Fragmentation	MS/MS Stage 3 Fragmentation	References
42	**Flavonol**	**Isorhamnetin-di-*O*-hexoside** [Methyl quercetin-*O*-dihexoside] *	$C_{28}H_{32}O_{17}$	**640.5435**		**641**	317	302	285; 228; 169	*Artemisia absinthium* [39]; passion fruit [67]; *Phoenix dactylifera* [81]
43	**Flavonol**	**Quercetin-7-*O*-(acetyl-hexoside)-3-*O*-rhamnoside** *	$C_{29}H_{32}O_{17}$	**652.5542**		**653**	301	286; 153	258	*Capsicum annuum* [88]
44	**Flavonol**	**Isorhamnetin-3-*O*-6-*O*-acetyl-*beta*-*D*-glucopyranosyl** *	$C_{30}H_{34}O_{18}$	**682.5802**		**683**	331	315; 270		*Rosa rugosa* [95]
45	**Flavan-3-ol**	**Catechin**	$C_{15}H_{14}O_6$	**290.2681**		**291**	273; 188	245	227; 201	*Inula viscosa* [55]; *Juglans mandshurica* [48]; *Black soja* [57]; *Glycine soja* [58]
46	**Flavan-3-ol**	**(epi)-Catechin**	$C_{15}H_{14}O_6$	**290.2681**		**291**	261; 173	173		Black soja [57]; *Glycine soja* [58]; *Jatropha* [47]
47	**Flavan-3-ol**	**Gallocatechin** [+(-)Gallocatechin] *	$C_{15}H_{14}O_7$	**306.2675**		**307**	289	260; 175	244; 171	*Carpinus betulus* [51]; *Solanaceae* [52]; *G. linguiforme* [53]; *Ribes meyeri* [44]; *Embelia* [54]
48	**Anthocyanin**	**Cyanidin-3-*O*-glucoside** [Cyanidin 3-*O*-*beta*-*D*-Glucoside; Kuromarin]	$C_{21}H_{21}O_{11}+$	**449.3848**		**449**	287	241; 165	213; 147	Black soybean [57]; *Glycine soja* [58]; *Ribes magellanicum* [64]; *Rubus ulmifolius* [65]; *B. ilicifolia*; *B. empetrifolia*; *R. maellanicum*; *R. cucullatum*; *M. nummalaria*; *G. mucronata*; *G. antarctica*; *Fuchsia magellanica* [59]; *B. microphylla* [60]
49	**Anthocyanin**	**Malvidin 3-*O*-glucoside [Oenin]**	$C_{23}H_{25}O_{12}$	**493.4374**		**493**	331	315; 270	315; 270	*G. mucronata*; *G. antarctica* [59]; *Berberis microphylla* [60]
50	**Anthocyanin**	**Cyanidin 3-(6″-malonylglucoside)**	$C_{24}H_{23}O_{14}$	**535.4310**		**535**	287	241; 165	213	Strawberry [91]; *Zostera marina* [25]
51	**Anthocyanin**	**Cyanidin-3-*O*-dioxayl-glucoside**	$C_{31}H_{28}O_{12}$	**592.5468**		**592**	287	241	227; 209; 144	*Rubus ulmifolius* [65]
52	**Anthocyanin**	**Peonidin 3-*O*-(6-*O*-*p*-coumaroyl) glucoside**	$C_{31}H_{29}O_{13}$	**609.554**		**609**	303	257; 153	229	Grape [62]; vines [63]
53	**Anthocyanin**	**Malvidin 3-*O*-(6-*O*-*p*-caffeoyl) glucoside**	$C_{32}H_{31}O_{15}$	**655.5795**		**655**	331; 493	315; 270; 153	270	Grape [62]; Bougainvillea [61]
54	**Phenolic acid**	**Coniferyl aldehyde** [4-Hydroxy-3-methoxycinnamaldehyde; Coniferaldehyde; Ferulaldehyde]	$C_{10}H_{10}O_3$	**178.1846**		**179**	161; 133; 119	119		*Juglans mandshurica* [48]; potato [50]; *A. cordifolia* [53]

Table 5. *Cont.*

	Class of Compound	Identification	Formula	Calculated Mass	Observed Mass [M − H]⁻	Observed Mass [M + H]⁺	MS/MS Stage 1 Fragmentation	MS/MS Stage 2 Fragmentation	MS/MS Stage 3 Fragmentation	References
55	**Phenolic acid**	**Caffeic acid derivative**	**$C_{16}H_{18}O_9Na$**	**377.2985**	**377**		341	215		*Bougainvillea* [61]; *Embelia* [54]
56	**Hydroxybenzoic acid (Phenolic acid)**	**Ellagic acid** [Benzoaric acid; Elagostasine; Lagistase; Eleagic acid]	**$C_{14}H_6O_8$**	**302.1926**		**303**	257; 229; 165	229; 201	201	*Juglans mandshurica* [48]; *Rhus coriaria* [66]; *Eucalyptus* [87]
57	**Phenylpropanoid (cinnamic alcohol glycoside)**	**Vimalin ***	**$C_{16}H_{22}O_7$**	**326.3417**		**327**	309; 195	241; 195		*Rhodiola rosea* [96]
58	**Coumarin**	**Fraxetin ***	**$C_{10}H_8O_5$**	**208.1675**		**209**	167			*Embelia* [54]; *Jatropha* [47]; *Artemisia martjanovii* [97]
59	**Lignan**	**Syringaresinol ***	**$C_{22}H_{26}O_8$**	**418.4436**		**419**	326; 253; 184	298; 254; 174	252; 226; 182	Wheat [98]; *Annona montana* [99]; *Lonicera caerulea* [79]
		Others								
60		**2,3-Dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one [DDMP]**	**$C_6H_8O_4$**	**144.1253**		**145**	127			*Radix polygoni multiflori* [56]
61	**Aliphatic amino acid**	**L-Glutamic acid [L-Glutamate]**	**$C_5H_7NO_4$**	**145.1134**		**146**	144; 118			*Medicago truncatula* [100]; soybean leaves [101]; *Lonicera japonica* [45]
62	**Amino acid**	**L-Histidine**	**$C_6H_9N_3O_2$**	**155.1546**		**156**	110			*Medicago truncatula* [100]; *Lonicera japonica* [45]; *Camellia kucha* [80]; *Actinidia deliciosa* [102]; *Lonicera caerulea* [79]
63	**Amino acid**	**Phenylalanine** [L-Phenylalanine]	**$C_9H_{11}NO_2$**	**165.1891**		**166**	120			*Medicago truncatula* [100]; *Juglans mandshurica* [48]; soybean [58]; soybean leaves [101]; *Lonicera japonica* [45]; potato leaves [92]; *Camellia kucha* [80]
64	**Cyclohexenecarboxylic acid**	**Shikimic acid** [L-Schikimic acid]	**$C_7H_{10}O_5$**	**174.1513**		**175**	157			*Medicago truncatula* [100]; soybean [58]; *Camellia kucha* [80]; *Ribes meyeri* [44]
65	**Tricarboxylic acid**	***cis*-Aconitic acid**	**$C_6H_6O_6$**	**174.1082**		**175**	157			*Medicago truncatula* [100]
66	**Tricarboxylic acid**	***Trans*-Aconitic acid** [trans-Aconitate]	**$C_6H_6O_6$**	**174.1082**		**175**	157			*Medicago truncatula* [100]; *Inula graveolens* [41]
67	**Amino acid**	**L-theanine** [Theanine; Theanin; N-Ethyl-L-glutamine]	**$C_7H_{14}N_2O_3$**	**174.1977**		**175**	157			*Camellia kucha* [80]
68	**Aromatic amino acid**	**Tyrosine** [(2S)-2-Amino-3-(4-Hydroxyphnyl)Propanoic acid]	**$C_9H_{11}NO_3$**	**181.1885**		**182**	177; 165	123		*Medicago truncatula* [100]; soybean leaves [101]; *Hylocereus polyrhizus* [103]; *Polygala sibirica* [104]
69	**Essential amino acid**	**L-Tryptophan** [Tryptophan; (S)-Tryptophan]	**$C_{11}H_{12}N_2O_2$**	**204.2252**		**205**	187	121		*Camellia kucha* [80]; *Rosa acicularis* [105]

Table 5. *Cont.*

	Class of Compound	Identification	Formula	Calculated Mass	Observed Mass $[M-H]^-$	Observed Mass $[M+H]^+$	MS/MS Stage 1 Fragmentation	MS/MS Stage 2 Fragmentation	MS/MS Stage 3 Fragmentation	References
70	**Carboxylic acid**	**Myristoleic acid** [Cis-9-Tetradecanoic acid]	$C_{14}H_{26}O_2$	**226.3550**		**227**	209; 165	121		*F. glaucescens* [53]; *Maackia amurensis* [32];
71	**Ribonucleoside composite of adenine (purine)**	**Adenosine**	$C_{10}H_{13}N_5O_4$	**267.2413**		**268**	136	121		*Lonicera japonica* [45]; Huolisu oral liquid [30]; *L. palustre* [106]; *Rosa acicularis* [105]
72	**Ribonucleoside composite of adenine (purine)**	**Inosine**	$C_{10}H_{12}N_4O_5$	**268.2261**		**269**	136			*Lonicera japonica* [45]
73	**Monosaccharides**	**6-Phosphogluconic acid**	$C_6H_{13}O_{10}P$	**276.1352**		**277**	259; 205; 188; 130			*Medicago truncatula* [100]
74	**Omega-3 fatty acid**	**Linolenic acid** (Alpha-Linolenic acid; Linolenate)	$C_{18}H_{30}O_2$	**278.4296**		**279**	219; 154	159		Soybean [58]; soybean leaves [101]; *Maackia amurense* [32]; *Polygala sibirica* [104]
75	**Polyunsaturated long-chain fatty acid**	**Hydroxy eicosatetraenoic acid**	$C_{20}H_{32}O_3$	**320.4663**		**321**	312; 256; 228; 193	224; 176; 143		*F. glaucescens*; *F herrerae* [53]
76	**Polysaccharides**	**Adenosine 5′-monophosphate [5′-adenylic acid]**	$C_{10}H_{14}N_5O_7P$	**347.2212**		**348**	341; 273; 233	205		*Medicago truncatula* [100]
77	**Phytosterol**	**Ergosterol** [Provitamin D2; Ergosterin]	$C_{28}H_{44}O$	**396.6484**		**397**	392; 311; 183; 129	361; 311; 226; 183; 130		*F. glaucescens* [53]
78	**Pentacyclic triterpenoid**	**Sophoradiol**	$C_{30}H_{50}O_2$	**442.7168**		**443**	425; 175	175	115	*Medicago truncatula* [107]
79	**Polysaccharides**	**Guanosine 5′-diphosphate**	$C_{10}H_{15}N_5O_{11}P_2$	**443.2005**		**444**	359; 323; 297; 274	189; 154	172	*Medicago truncatula* [100]
80	**Anabolic steroid**	**Vebonol**	$C_{30}H_{44}O_3$	**452.6686**		**453**	435; 336; 209	336; 226	209	*Rhus coriaria* [66]; *Hylosereus polyrhizus* [103]
81	**Triterpenic acid**	**Ursolic acid**	$C_{30}H_{48}O_3$	**456.7003**		**457**	411; 203	393; 283; 201	201	*Juglans mandshurica* [48]; *Ocimum* [34]; *Mentha* [36]
82	**Triterpenic acid**	**Oleanolic acid**	$C_{30}H_{48}O_3$	**456.7004**		**457**	411; 263; 203	393; 309; 177	375; 203; 145	*Medicago truncatula* [107]; *C. edulis* [53]; *Folium Eriobotryae* [108]
83	**Saponin**	**Soyasapogenol B** [24-Hydroxysophoradiol; Soyasapogenin B]	$C_{30}H_{50}O_3$	**458.7162**		**459**	452; 317; 279; 212	445; 298; 216		*Medicago truncatula* [107]
84	**Saponin**	**Soyasapogenol A**	$C_{30}H_{50}O_4$	**474.5434**		**475**	437; 343; 249	168; 127		*Medicago truncatula* [107]
85	**Polysaccharides**	**UTP [Uridine 5′-triphosphate]**	$C_9H_{15}N_2O_{15}P_3$	**484.1411**		**485**	478; 365; 321; 261			*Medicago truncatula* [100]
86	**Polysaccharides**	**Guanosine-5′-triphosphate [GTP]**	$C_{10}H_{16}N_5O_{14}P_3$	**523.1804**		**524**	494; 386; 303; 165			*Medicago truncatula* [100]
87	**Polysaccharides**	**UDP-arabinose [Uridine 5′-diphosphate arabinose]**	$C_{14}H_{20}N_2O_{16}P_2$	**534.2599**		**535**	275; 217; 159	220		*Medicago truncatula* [100]

Table 5. *Cont.*

	Class of Compound	Identification	Formula	Calculated Mass	Observed Mass $[M-H]^-$	Observed Mass $[M+H]^+$	MS/MS Stage 1 Fragmentation	MS/MS Stage 2 Fragmentation	MS/MS Stage 3 Fragmentation	References
88	**Polysaccharides**	**Uridine diphosphate-xylose [UDP-xylose]**	**$C_{14}H_{22}N_2O_{16}P_2$**	**536.2758**		**537**	277; 187	249; 136		*Medicago truncatula* [100]
89	**Polysaccharides**	**UDP-glucose** [Uridine diphosphate glucose]	**$C_{15}H_{24}N_2O_{17}P_2$**	**566.3018**		**567**	423; 385; 324; 283	405; 367; 297; 241		*Medicago truncatula* [100]
90	**Product of chlorophyll degradation**	**Chlorophyllide a**	**$C_{35}H_{34}MgN_4O_5$**	**614.9733**		**615**	583	565; 458; 269	520; 441	[109,110]
91		**Medicagenic acid -3-*O*-*beta*-*D*-glucopyranoside**	**$C_{36}H_{56}O_{11}$**	**664.8232**		**665**	635; 510; 452; 401	337	319	Pubchem
92	**Saponin**	**Azukisaponin II**	**$C_{42}H_{68}O_{14}$**	**796.9809**		**797**	519	429; 357; 243		*Leguminosae* [111]; *Glycine max* [112]
93	**Saponin**	**Soyasaponin Bb′ [Soyasaponin III]** *	**$C_{42}H_{68}O_{14}$**	**796.9809**		**797**	519	429; 357; 243		*Black soja* [57]
94	**Product of chlorophyll degradation**	**Pyropheophytin a**	**$C_{53}H_{72}N_4O_3$**	**813.1638**		**813**	535	435; 329		[110]
95	**Steroidal alkaloid**	**Alpha-chaconine** *	**$C_{45}H_{73}NO_{14}$**	**852.0594**		**852**	706; 560; 398	560	398	Potato [113,114]
96	**Product of chlorophyll degradation**	**Pheophytin A**	**$C_{55}H_{74}N_4O_5$**	**871.1999**		**871**	533	461		*Physalis peruviana* [115]; *Capsicum* [116]; [109, 110]
97	**Saponin**	**Soyasaponin II** [Soyasaponin II (SH); Soyasaponin Bc] *	**$C_{47}H_{76}O_{17}$**	**913.0961**	**912**		501	483; 425	425	Black soja [57]; *Leguminosae* [111]; soya [117]
98	**Saponin**	**Soyasaponin *gamma* g** *	**$C_{48}H_{74}O_{17}$**	**923.0910**		**924**	581; 423; 321	213		Black soja [57]; *Leguminosae* [111]; soya [117]
99	**Saponin**	**3-Rhamnose-galactose-glucuronic acid-soyasapogenol B**	**$C_{48}H_{78}O_{18}$**	**943.1221**		**944**	598; 423; 295	581; 419; 215	572	*Medicago truncatula* [100]; *Rhus coriaria* [66]
100	**Saponin**	**Soyasaponin I** [Soyasaponin Bb] *	**$C_{48}H_{78}O_{18}$**	**943.1221**		**944**	598; 423; 365; 281	205		*Leguminosae* [111]; soya [117]; Black soja [57];
101	**Saponin**	**Soyasaponin Bd** *	**$C_{48}H_{76}O_{19}$**	**957.1056**		**958**	456; 595; 718; 812	409; 247	271	Black soja [57]; *Leguminosae* [111]; soya [117]
102	**Saponin**	**6-deoxyhexose-hexoside-uronic acid-aglycone D**	**$C_{48}H_{78}O_{20}$**	**975.1209**		**975**	799; 715; 529; 477	301	286; 259; 201	*Medicago truncatula* [118]
103	**Saponin**	**Soyasaponin *beta* g** *	**$C_{54}H_{84}O_{21}$**	**1069.2322**	**1068**		967; 879; 741; 584	659; 483		Black soja [57]; *Leguminosae* [111]; soya [117]

* Compounds identified for the first time in *M. varia*.

4. Discussion

The biologically active compounds of aerial parts of plants are effectively extracted using organic solvents such as methanol and ethanol. But extraction products in the final phase require additional purification from trace amounts of used solvents. SC-CO_2 extraction can be used as an alternative to traditional extraction methods: maceration or Soxhlet extraction [119,120]. SC-CO_2 extraction has been used in the evaluation of food products, the isolation of bioactive substances, and the determination of lipid levels in foods and levels of toxic substances. With SC-CO_2 extraction, the products do not contain organic solvent residues that occur with conventional extraction methods, and the solvents can be toxic, as in the case of methanol and n-hexane, for example. Easy solvent removal from the final product, high selectivity and moderate extraction temperatures are the main attractions of SC-CO_2 technology, leading to a significant increase in research for applications in the food and pharmaceutical industries. When comparing possible supercritical solvents, carbon dioxide has the most attractive advantages, being a non-toxic, non-flammable, environmentally friendly and renewable resource [121]. Popova et al. investigated the influence of SC-CO_2 extraction parameters and the quality of *Ledum palustre* feedstock on the global yields of chlorophylls and carotenoids. The data obtained were significant for the pharmaceutical, food, and perfume and cosmetic industries, which require natural dyes and antioxidants [122]. Baananou et al. reported the anti-inflammatory activity of two extracts from the aerial parts of Rhododendron [123]. Aliev et al., in their research, have shown that SC-CO_2 extraction is an effective method for extracting a wide range of lipophilic fractions from plant materials in one experimental procedure, which provides additional opportunities for research [124].

Thus, the use of SC-CO_2 extraction is an effective approach for the extraction of bioactive compounds. Our results are consistent with these reports that SC-CO_2 extraction is a useful approach to extract and study bioactive compounds.

The extracts obtained showed both a high content of polyphenolic compounds and a high content of saponin group compounds. Earlier studies revealed the presence and detection of polyphenols in legume species. Chiriac et al. [125] used UHPLC-Q exactive hybrid quadrupole orbitrap high-resolution mass spectrometry and identified 29 compounds from the sprouts of *Medicago sativa* and *Trifolium pratense*, based on their mass, FIs, retention time and data in the literature. However, using SC-CO_2 extraction, the number of polyphenol compounds was higher in our results. This difference could be due to the extraction method or the different tissues under study. Other than *Medicago*, polyphenols are also abundant in other legume species, such as *Phaseolus vulgaris* [126], soybean [127], chickpea, grass pea, lentils [128], peanut [129], etc. These polyphenols act as dietary antioxidants in humans and impart protective effects against certain diseases [130].

Apart from polyphenols, the presence of saponins in *Medicago* varieties is a useful observation. These observations are consistent with earlier studies which reported the presence of saponins in *Medicago truncatula* [100,107,118]. Saponins are active compounds present in edible legumes and impart health benefits [131,132]. Bioinformatic analyses in legumes have revealed the presence of the triterpene biosynthesis pathway and conserved genes [133,134]. Thus, the detection of eleven compounds classified as saponins and two triterpenoid acids (ursolic acid and oleanolic acid) in the leaves of *M. varia* is consistent with the above-cited studies. Though several studies on soybean and other legumes have highlighted that seeds and roots [135] are the major sinks for saponins, their presence in leaves and stems has also been reported in, e.g., *Jatropha curcas* [136], *Acanthopanax sieboldianus* [137], *Quillaja lancifolia* [138], etc. The detection of saponins in the leaves of *M. varia* together with a range of polyphenols suggests this plant as a potential raw material for use in traditional medicine as well as modern pharmacology.

It is well recognized that using some fungicides can be harmful to both the environment and human health. As a result, during the past twenty years, an increasing amount of research has been carried out on the potential use of plant-based substances that would be less harmful than chemicals produced in factories. The increasing prospect of

employing saponins as natural fungicides is highlighted in a large number of international articles [139].

Research on 29 *Medicago* species has shown reliable results, including the identification of several species as having a high concentration of fungicidal saponins [140]. The saponins of some species, including *M. sativa* [141,142], *M. arabica* [143], *M. arborea* [144] and *M. hybrida* [145], have fungicidal actions.

Fourteen triterpene saponins from the roots of *Medicago hybrida* have been identified and their structures have been established [145]. Six fungi were tested in vitro to determine the antifungal activity of the roots' saponins, and eight main saponin glycosides were tested against *Botrytis tulipae*, one of the most sensitive fungi [20].

It should be noted that different concentrations of saponins equally inhibited the mycelial growth of *Botrytis cinerea* and *B. tulipae.* However, the higher concentrations inhibited the mycelial growth of *B. cinerea* somewhat less. It is assumed that lower concentrations of saponins are sufficient to block all active sites in the mycelial hyphae. It has also recently been shown that *M. hybrida* saponins have insecticidal activities as high as those of *M. arabica* and *Medicago murex* [146]. On the other hand, it is known that *M. arabica* and *M. murex* saponins are also rich in highly fungicidal saponins [147]. In conclusion, *Medicago* saponins have significant antifungal activity, and the roots of this plant can be a rich source of natural fungicides. Therefore, their detection in the aerial parts of *M. varia* has added to the existing list of beneficial compounds.

5. Conclusions

M. varia species contain many polyphenolic components and components of other chemical groups that have valuable biological activities. SC-CO_2 extraction of *M. varia* (three varieties) was successfully carried out by the team of authors. Certain extraction conditions were selected, and the extracts obtained showed both a high content of polyphenolic compounds and a high content of saponin group compounds. Tandem mass spectrometry (HPLC-ESI–ion trap) was used to detect target analytes. Mass spectrometric data were recorded on an ion trap equipped with an ESI source in negative and positive ion modes. A four-stage ion separation mode was implemented. One hundred and three different biologically active compounds were found in *M. varia* extracts. Twenty-seven phenolic compounds were tentatively identified for the first time. Also, for the first time, the following saponins were identified with reliable accuracy in *M. varia*: soyasaponin II, soyasaponin *gamma* g, soyasaponin I, soyasaponin Bd, soyasaponin *beta* g, steroidal alkaloids (alpha-chaconine), etc. Although our study aimed only to study the aerial parts of *Medicago varia*, which belongs to the legume family (Fabaceae), the same approach can be applied in the future to study factors influencing the metabolite profiles of legume seeds, including seasonal variations, cultivation and storage conditions. In addition, our study may provide new interesting details for future taxonomic studies, especially if they target larger genotypes.

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