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Whey and water-ethanol solution as extractants of biologically active substances: Comparative analysis

Anna A. Stepanova[1], Alice D. Mitina[1], Natalia S. Velichkovich[1,*], Oksana V. Kozlova[1], Timothy A. Larichev[1], Larisa A. Proskuryakova[1], Vladimir V. Martirosyan[2]

[1] Kemerovo State University, Kemerovo, Russia
[2] Scientific Research Institute for the Baking Industry, Moscow, Russia

* e-mail: velichkovich@yandex.ru

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Abstract:
Industrial whey wastes contain organic substances that cause serious harm to the environment, which makes whey recycling a relevant research objective. Whey can serve as an extractant of biologically active substances from plant raw materials. This research compared a 70% aqueous-ethanol solution and whey as extractants of bioactive components from a plant blend of *Heracleum sibiricum* L., *Syringa vulgaris* L., *Arctium tomentosum* Mill., *Achillea millefolium* L., *Thymus vulgaris* L., and *Pulmonaria officinalis* L. The method of high-performance liquid chromatography revealed catechin, chlorogenic acid, astragalin, ferulic acid, coumaric acid, and some trace amounts of other compounds. The samples subjected to water-ethanol extraction demonstrated a better quantitative and qualitative profile than those processed with whey: 637.92 vs. 380.45 mg/kg for chlorogenic acid, 93.05 vs. 2.34 mg/kg for coumaric acid, and 53.12 vs. 3.09 mg/kg for astragalin. Whey proved unable to extract catechin and ferulic acid. However, whey made it possible to obtain compounds that water-ethanol solution failed to extract, namely rutin and caffeic acid. By fractionating the extracts and isolating individual biologically active substances, we obtained chlorogenic acid (90.0%), ferulic acid (92.0%), and coumaric acid (88.0%) with water and ethanol, as well as caffeic acid (84.5%) and rutin (78.0%) with whey. Both ethanol and whey proved industrially relevant and demonstrated good prospects for commercial extraction of biologically active substances from various plant raw materials.

Keywords: Extraction, biologically active substances, whey, plant extract, extractant, fractionation, blending

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INTRODUCTION

The recent decades have seen a growing scientific interest in medicinal plants with therapeutic effects. New methods for the extraction, isolation, and analysis of phytochemical components make it possible to utilize their pharmacological potential in all their diversity [1–3]. Such advanced analytical techniques as high-performance liquid chromatography and gas chromatography-mass spectrometry have revolutionized the authentication and analysis of bioactive molecules [4–6]. In combination with genomic technologies, e. g., DNA barcoding, these techniques optimize the phytochemical synthesis in order to increase the yield and range of bioactive compounds, thus promoting novel plant-based therapies [7, 8]. The high therapeutic potential of plant materials is applied to treating diabetes mellitus and some cancers, as well as Alzheimer's and Parkinson's diseases, not to mention various microbial and fungal infections [9–11]. The holistic properties of plant extracts result from a complex mix of bioactive compounds, i. e., alkaloids, flavonoids, terpenes, phenols, etc. Each of these compounds provides therapeutic benefits, enhanced by their synergistic interactions. The synergetic effect and natural origin, combined with lower risks of side effects, make plant compounds promising alternatives to synthetic pharmaceuticals [12, 13].

In addition, the food industry is currently facing external problems caused by epidemics, military conflicts, etc. In this respect, medicinal plants offer a solution to the so-called hidden hunger issue, i. e., a deficiency of

phytonutrients in the national diet [14, 15]. A comprehensive use of plant components upgrades traditional products into functional foods. Their chemical composition contains non-traditional plant components responsible for bioactive properties with a long-term physiological antioxidant, antiseptic, immunomodulatory, radioprotective, or anticancer effects [16–19].

Countries that boast plant biodiversity have certain advantages in the fields of national economic growth and healthcare. By stimulating R&D in herbal medicines and plant food products, they turn their natural resources into innovative solutions in the food industry and healthcare [8, 20].

Phytoextraction is a complex process that depends on a number of factors. Extraction remains the main chemical method of producing bioactive compounds from plant raw materials [21]. An effective extraction technique produces a maximal yield of target components that render the resulting extracts with the highest biological activity possible. However, the yield of biologically active substances and their activity also depend on the solvent used. Traditional solvents applied in phytoextraction include methanol, ethanol, acetone, and water. Bioactive compounds differ in solubility, which means that the process of solvent selection should take into account the specifics of plant materials and target compounds. Therefore, choosing an optimal extraction solvent is a difficult matter that depends on each individual plant material [22].

Environmental protection and sustainability are another issue to be considered in relation to extraction and processing. Whey is a non-conventional raw material that can act as an extractant for bioactive substances of plant origin. It may reduce environmental burden by replacing inorganic extractants. As whey is rich in organic substances, its industrial wastes are bad for the environment. Its recycling as an industrial extractant is a relevant solution to all the above-mentioned problems. However, whey requires a detailed comparative analysis with other extractants to confirm its effectiveness.

Surprisingly, the whey extractant issue has gained a rather narrow scientific coverage. Velichkovich *et al.* [23] tested plant extracts obtained with whey for flavonoid compounds, antioxidant activity, and effective extraction parameters.

Melnikova *et al.* [24] obtained dairy-plant extracts from yacon tubers (*Polymnia sonchifolia*) using a curd whey ultrafiltrate. The resulting optimal parameters included 50–60 min extraction time, 40–60°C, and pH 4–5. The optimal ratio of yacon pulp and whey ultrafiltrate ranged from 1:4 to 1:6. The method improved the efficiency of nutrient extraction, thus increasing the nutritional and biological value of the finished product.

Kaledina *et al.* [25] extracted vitamin C and rutin from green tea, thyme, and rose hips using unsalted cheese and curd whey. The optimal parameters included 30 min extraction time and 40–60°C. The optimal amount of raw material was 1–10% of extractant mass.

Khalanskaya *et al.* [26] extracted bioactive substances from aloe, hawthorn, and licorice using whey and skim milk permeate. The optimal parameters included 30 min extraction time and 40–60°C. The most effective ratio of raw material to solvent was 1:2.

Lodygin *et al.* [27] used whey to isolate bioactive components from milk thistle, peppermint leaves, sage leaves, and purple echinacea. The optimal processing parameters included 2 h extraction time, 55–60°C, and 70 rpm. The optimal whey-to-plant ratios were 1:8 for milk thistle and peppermint and 1:10 for sage and purple echinacea. The experimental whey extracts demonstrated a higher antioxidant activity than the aqueous ones. The yield of phenolic compounds was higher at the ratio of 1:8. The sage extracts yielded much more phenolic compounds than the extracts of purple echinacea.

Sargin *et al.* [28] obtained a water-fat extract from plant raw materials. The procedure involved calendula, St. John's wort, and chamomile as herbs or inflorescences and a water-fat emulsion as an extractant, i. e., whole natural milk, milk or curd whey, or their mixes. The plant raw material was soaked in deionized water until swollen. The water-fat emulsion had a 3–15% fat content. The extraction relied on the percolation method. The ratios of plant raw materials to the extractant ranged from 1:25 to 1:10; the extraction time was 2–24 h at 10–80°C. The 1:1 ratio of calendula flowers and a milk-and-whey mix served as an example. The optimal processing parameters included 2 h, 80°C, and 1:10 calendula-extractant ratio. Using the HPLC method, they detected 8% terpenoids and 4.5% tannins in the extracts. The extraction of St. John's wort herbs and flowers with a 1:1 mix of milk and curd whey had the following optimal parameters: 24 h, 10°C, 1:10 of raw material and extractant. The resulting extract contained a total 12% of terpenoids and tannins, 10% carotene, and 3.2% vitamin C. The optimal parameters for extracting bioactive substances from chamomile with whey included 3 h, 40°C, and a chamomile-to-whey ratio of 1:20. The liquid chromatography revealed 13.5% tannins and terpenoids, 14.1% carotenes, 2.7% polyphenols, and 2.1% vitamin C.

Daudova & Ramazanova used milk whey to extract bioactive compounds from barberry [29] and cranberry [30]. The berries were ground, extracted with milk whey, and filtered. The optimal ratios of raw materials to extractant were 1:10–1:20 at 40 or 60°C for 60–90 min. The purified extract could be used as a food additive in bakery products.

Doronin *et al.* [31] produced pectin powder from beet roots, using curd and cheese whey as extractant. They ground the beet and treated it with acidified citric acid to a pH of 3.5–3.8 at 95–100°C for 60–90 min. The extraction with curd or cheese whey lasted for 60–80 min at 90–95°C and 0.2–0.3 MPa; the ratio of pulp, water, acidified citric acid, and curd/cheese whey was 1:2:5–1:2:6. Another extract was obtained by extracting crushed beet leaves with curd or cheese whey at 1:7–1:12, 65–70°C, and 0.2–0.3 MPa for 60–80 min. Both extracts were mixed at 80–90–20–10, evaporated to 12–15% solids, and dried to 3–5% moisture. The resulting content of soluble pectin was 1.1–1.57%.

This study focused on a comparative analysis of ethanol and whey extracts of a blend of lilac, lungwort, yarrow, burdock, hogweed, and thyme. The research objective was to assess the effect of the extractant on the yield of bioactive substances.

STUDY OBJECTS AND METHODS

The research involved 70% water-ethanol solution and whey as extractants. The list of plant materials included hogweed (*Heracleum sibiricum* L.), lilac (*Syringa vulgaris* L.), burdock (*Arctium tomentosum* Mill.), lungwort (*Pulmonaria officinalis* L.), thyme (*Thymus vulgaris* L.), and yarrow (*Achillea millefolium* L.) [32, 33]. The extracts were obtained from the plant blend using whey and water-ethanol solution.

To compare the extractants used to extract bioactive compounds from the plant blend, we isolated the bioactive substances individually in several stages:

– composing a plant blend based on the metabolomic analysis of the extracts;
– obtaining extracts from the blend using whey and a 70% water-ethanol solution;
– phytochemical analysis;
– fractionation;
– isolating individual bioactive substances from one major peak per fraction; and
– drying the bioactive substances separately.

Metabolomic analysis of the extracts to compose the blend. We used the method of high-efficiency liquid chromatography (HPLC) to define the metabolome of the plant materials. The process involved a LC-20AB Shimadzu Prominence chromatograph (Shimadzu, Japan) with a binary pump, an SPD-M20A diode array detector, and a Zorbax 300SB-C18 4.6 Column (250 mm, 5 µm; Agilent Technologies, USA). The separation temperature was 40°C in the gradient elution mode. The data for the mobile phase were as follows: 0.1% eluent A, trifluoroacetic acid (TFA) in bi-distilled water, acetonitrile B, 1 mL/min flow rate, 254, 280, and 325 nm analytical wavelengths. The components were identified by their retention times and the spectra of standard substances. Their concentration was calculated using calibration equations. The metabolome analysis made it possible to compile a plant blend for further research.

Obtaining extracts from the blend with whey and 70% water-ethanol solutions. To prepare cheese whey, we heated it to 45°C and centrifuged it to separate the milk fat. After that, the solution was brought to 95°C and held for 15 min to coagulate the remaining whey protein and provide additional pasteurization. The heated whey was centrifuged to separate it into liquid (extract) and thick (protein) fractions. The resulting extract was passed through a cotton-gauze filter for additional purification [23]. This cheese whey was used as extractant. The extraction parameters were as follows: 3 h, 90 ± 1°C, 2.5 g raw material to 450 mL whey or 70% water-ethanol solution. Upon extraction, we filtered the extracts through a sterile cotton gauze and an ash-free filter. They were stored in the dark at +2 to +6°C in 500 mL conical flasks with rubber stoppers.

Phytochemical composition. The phytochemical analysis of the blends relied on the analytical HPLC method described above.

Fractionation. The blends were evaporated in a vacuum rotary evaporator at 40°C with silica gel (5 g silica gel per 80 mL extract) until the solvent was completely removed (Fig. 1).

The resulting mix was dried and loaded into the column of a Büchi Pur-C150 preparative chromatograph (Büchi, Switzerland). The processing parameters were as follows: flash mode, FP ECOFLEX Si 40 g, ELSD and UV detectors (254, 270, 280, and 320 nm). Vials from each peak were collected separately and evaporated.

We conducted the elution in a gradient mode in the hexane-methylene chloride and methylene chloride-methanol systems, increasing the content of the more polar solvent by 20% for each subsequent column volume (Table 1).

We defined the total fraction yield gravimetrically, analyzed their composition by analytical HPLC, and expressed in g/kg and mg/kg of plant material, respectively.

Isolating individual bioactive substances from one major fraction peak. To isolate individual bioactive substances from fractions of Blends 1 and 2, we used the preparative liquid chromatography method in a Büchi Pure C-850 chromatograph and a Prep Pure C18 column (100 Å; 5 µm; 250×20 mm; Büchi, Switzerland). The isolation parameters included gradient elution mode and

Figure 1 Evaporating the blends

Table 1 Gradient elution

Column volumes	Solvent, vol. %		
	Hexane	Methylene chloride	Methanol
0.0	100.0	0.0	0.0
1.0 ± 0.2	80.0	20.0	0.0
1.0 ± 0.2	80.0	20.0	0.0
1.0 ± 0.2	60.0	40.0	0.0
1.0 ± 0.2	60.0	40.0	0.0
1.0 ± 0.2	40.0	60.0	0.0
1.0 ± 0.2	40.0	60.0	0.0
1.0 ± 0.2	20.0	80.0	0.0
1.0 ± 0.2	20.0	80.0	0.0
1.0 ± 0.2	0.0	100.0	0.0
1.0 ± 0.2	0.0	100.0	0.0
0.0	0.0	100.0	0.0
1.0 ± 0.2	0.0	80.0	20.0
1.0 ± 0.2	0.0	80.0	20.0
1.0 ± 0.2	0.0	60.0	40.0
1.0 ± 0.2	0.0	60.0	40.0
1.0 ± 0.2	0.0	40.0	60.0
1.0 ± 0.2	0.0	40.0	60.0
1.0 ± 0.2	0.0	20.0	80.0
1.0 ± 0.2	0.0	20.0	80.0
1.0 ± 0.2	0.0	0.0	100.0
3.5 ± 0.2	0.0	0.0	100.0

40°C; for mobile phase: 0.1% eluent A, TFA in bi-distilled water, acetonitrile B, 10 mL/min flow rate; 254, 270, 280, and 320 nm analytical wavelengths.

The fractions collected after separating the large fractions of Blends 1 and 2 were analyzed and purified by HPLC on an LC-20AB Shimadzu Prominence analytical chromatograph with a binary pump (Shimadzu, Japan). To study and purify each bioactive substance isolated from the blend fraction, we used an SPD M20A diode-matrix detector and a Zorbax 300SB-C18 4.6 column (250 mm, 5 µm; Agilent, USA).

Drying the individual bioactive substances. The drying procedure involved a sublimation unit at the following constant lyophilization conditions: 0.3 mbar, –20°C at the final drying stage, –80°C cooler temperature [34].

RESULTS AND DISCUSSION

To study the metabolomic composition of the plant extracts, we used the following chromatographic standards: 3,4-dihydroxybenzoic acid (protocatechuic acid), hyperoside, gallic acid, apigenin-7-O-glucoside, quercetin 3-D-glucoside, luteolin-7-glucoside (cinaroside), (+)-catechin, astragalin (kaempferol-3-glucoside), chlorogenic acid, acacetin, ellagic acid, rutin hydrate, p-coumaric acid, caftaric acid, rosemarinic acid, chicoric acid, trans-caffeic acid, trans-ferulic acid, rutin, ferulic acid (3-methoxy-4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), genistin, daidzein, formononetin, oenothein B, morin (3,5,7,2',4'-pentahydroxyflavone), 2,5-dihydroxybenzoic acid, and neochlorogenic acid.

The chromatographic profiles obtained were summarized as a graph (Fig. 2).

The metabolome profile included astragalin, 2,5-dihydroxybenzoic acid, isoquercetin, chlorogenic acid isomer, hyperoside, and gallic acid. The maximal astragalin content belonged to lilac inflorescences (1230.88 µg/g) while its leaves contained the maximal value of isoquercetin (931.00 µg/g). High levels of astragalin were also found in different parts of hogweed, the leaves of which also contained 2,5-dihydroxybenzoic acid (1038.80 µg/g). Chlorogenic acid isomer was detected only in lungwort (350.00 µg/g). The herbal part of thyme and lilac inflorescences had almost the same quantity of gallic acid, i. e., 24.00 and 23.00 µg/g, respectively. The yarrow samples were rich in hyperoside (335.00 µg/g). Hogweed, lilac, and lungwort contained the highest volumes of bioactive substances.

The metabolomic analysis made it possible to compile two blends of the plants (Table 2). The blends consisted of the same plant materials in the same proportions but had different extractants.

Figure 3 shows the blends obtained as a result of extracting plant materials with whey and 70% water-ethanol extractant.

Figures 4, 5 and Table 3 summarize the phytochemical composition of Blends 1 and 2. We identified the components by their retention times and the spectra of such standard substances as 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, hyperoside, gallic acid, apigenin-7-O-glucoside (cosmosiin), catechin, astragalin, ferulic acid, caffeic acid, chlorogenic acid, neochlorogenic acid, acacetin, quercetin-3D-glucoside, ellagic acid, rutin, luteolin-7-glucoside (cinaroside), p-coumaric acid, caftaric acid, rosemarinic acid, chicoric acid, oenothein B, genistein, daidzein, formononetin, and morin. The concentration was calculated using calibration equations (3–5% mean error).

According to the HPLC analysis, the greatest number of phenolic components belonged to Blend 1. Onnonin and formononetin were not detected.

Figure 4 and Table 3 show that Blend 1 contained significant amounts of chlorogenic acid (637.92 mg/kg), ferulic acid (507.43 mg/kg), catechin (416.38 mg/kg), and coumaric acid (93.05 mg/kg).

Traces of 3,4-dihydroxybenzoic and rosemarinic acids were below the detection limit (BLD) in Blend 2 (Fig. 5, Table 3). However, Blend 2 proved to be rich in phenolic acids, e. g., chlorogenic acid (380.45 mg/kg), caffeic acids (265.91 mg/kg), and rutin (104.08 mg/kg). Blend 2 also contained coumaric acid (2.34 mg/kg) and astragalin (3.09 mg/kg).

To summarize, the phytochemical composition studied by the HPLC method showed that Blend 1 contained a lot of catechin, chlorogenic acid, coumaric acid, and ferulic acid while Blend 2 was rich in chlorogenic acid, caffeic acid, and rutin.

The HPLC fractionation demonstrated that the qualitative and quantitative profiles of fractions in Blend 1 (Fig. 6, Table 4) were quite diverse, as represented by

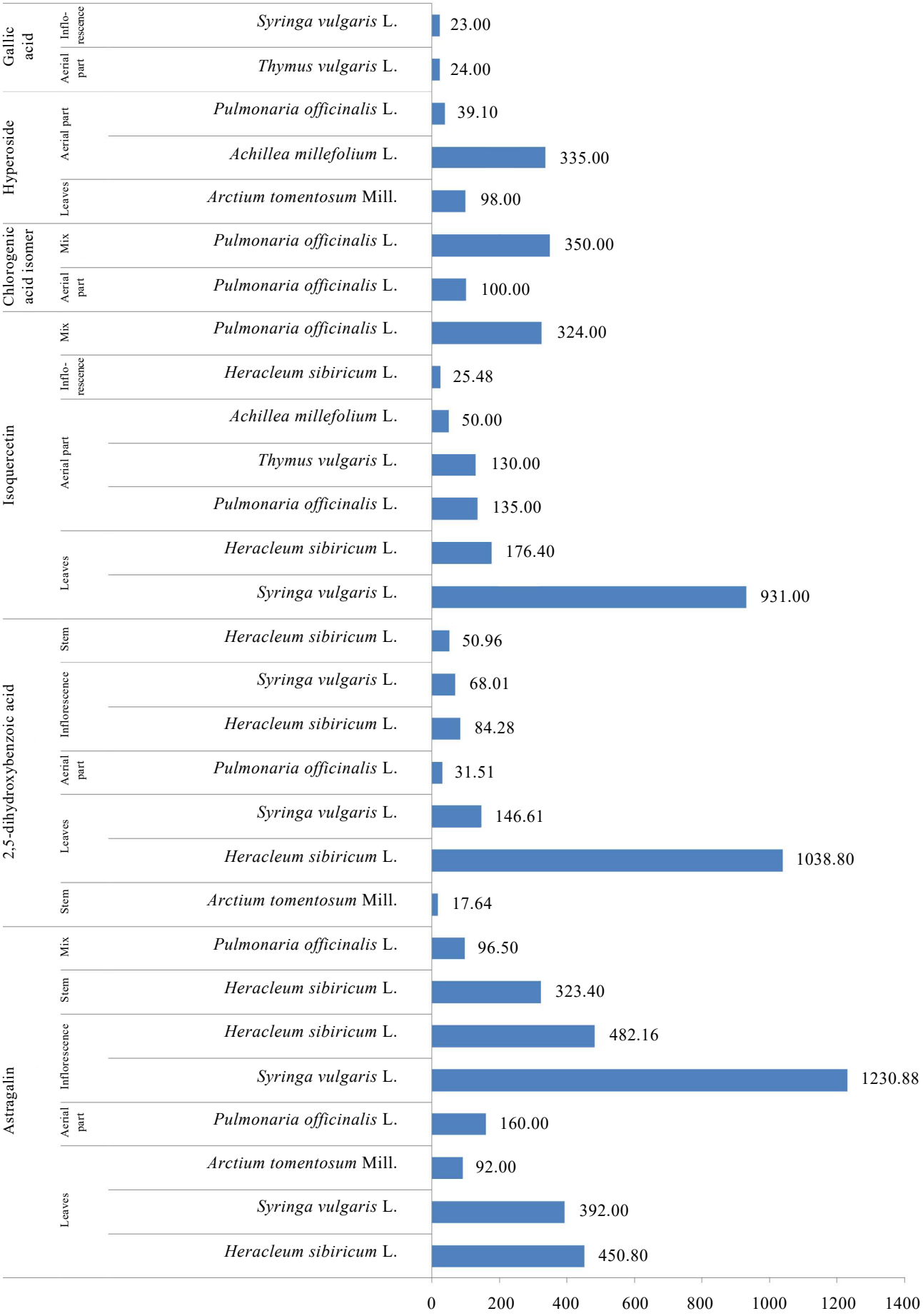

Figure 2 Metabolomic analysis of bioactive substances in plant extracts, μg/g

Table 2 Composition of blends

Blend	Extractant	Composition
1	70% water-ethanol	Hogweed, lilac, burdock, yarrow, thyme, lungwort
2	Whey	

Figure 3 Blends for phytochemical composition analysis

coumaric acid (fraction 2, № 4–6), chlorogenic acid (fraction 2, № 4–5), formononetin (fraction 5), catechin (fractions 5–8), ferulic acid (fractions 6–9), astragalin (fractions 7–8), and rosemarinic acid (fraction 9).

Fraction 2 contained chlorogenic acid in very small quantities; fraction 5 contained coumaric acid and formononetin below the detection limit.

Fraction 5, Blend 1 had the highest amount of catechin (5.20 ± 0.23 mg/kg); in fraction 6, it was below the detection limit. The biggest amount of catechin was contained in fraction 7 (12.91 ± 1.53 mg/kg) and fraction 8 (6.77 ± 1.48 mg/kg).

Fractions 6–9 were rich in ferulic acid: from 2.30 ± 0.08 mg/kg in fraction 8 to 8.26 ± 0.29 mg/kg in fraction 7. Fraction 6 contained 5.36 ± 0.17 mg/kg ferulic acid whereas fraction 9 had 5.78 ± 0.23 mg/kg ferulic acid.

Fractions 7 and 8 contained 2.43 ± 0.12 mg/kg and 3.24 ± 0.12 mg/kg astragalin, respectively.

Rosemarinic acid was detected nowhere but in fraction 9, where it was below the detection limit.

Fractions 1, 3–5, 8, and 10 in Blend 1 contained some unidentified compounds.

The qualitative and quantitative analysis of bioactive substances in the fractions of Blend 2 (Fig. 7, Table 5) revealed that fraction 1 contained traces of 3,4-dihydroxybenzoic acid (below the detection limit), as well as a number of unidentified compounds. Unidentified compounds were also detected in fractions 4 (vials 14–18, fraction yield 1.68 ± 0.07 g/kg), fraction 6 (vials 23–25, fraction yield 9.19 ± 0.28 g/kg), and fraction 8 (vials 28–35, fraction yield 12.38 ± 0.60 g/kg).

In fraction 2, we identified only two compounds, i. e., chlorogenic and caffeic acids. The content of chlorogenic acid in fraction 2 was 38.42 ± 1.58 mg/kg. Caffeic acid was present in trace amounts.

Fraction 3, Blend 2 was a mix of phenolic acids, i. e., chlorogenic, caffeic, and coumaric acids. The content of chlorogenic acid was 24.56 ± 1.10 mg/kg while that of

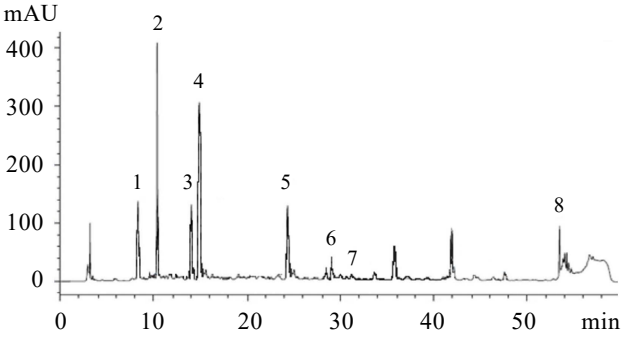

Figure 4 Blend 1, HPLC chromatogram: (1) catechin; (2) chlorogenic acid; (3) coumaric acid; (4) ferulic acid; (5) astragalin; (6) rosemarinic acid; (7) daidzein; and (8) formononetin

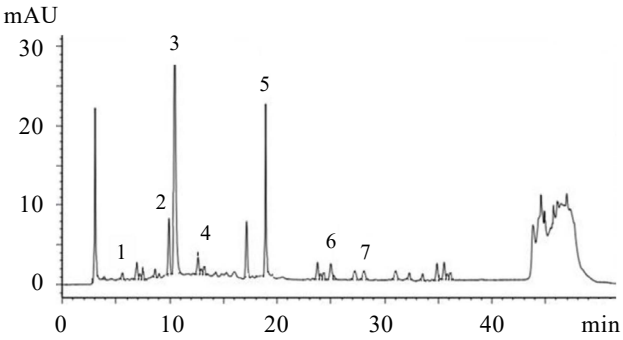

Figure 5 Blend 2, HPLC chromatogram: (1) 3,4-dihydroxybenzoic acid; (2) caffeic acid; (3) chlorogenic acid; (4) coumaric acid; (5) rutin; (6) astragalin; and (7) rosemarinic acid

Table 3 Phytochemical composition of blends

Bioactive substance	Retention time, min	Content, mg/kg	
		Blend 1	Blend 2
3,4-dihydroxybenzoic acid	5.10	–	traces
Catechin	8.99	416.38	–
Chlorogenic acid	10.35	637.92	380.45
Caffeic acid	10.76	–	265.91
Coumaric acid	14.56	93.05	2.34
Ferulic acid	15.44	507.43	–
Rutin	19.70	–	104.08
Astragalin	24.58	53.12	3.09
Rosemarinic acid	28.24	traces	b.l.d.
Daidzein	31.80	b.l.d.	–
Formononetin	53.87	b.l.d.	–

b.l.d. – below the detection limit

caffeic acid was as low as 5.89 ± 0.21 mg/kg. Coumaric acid was present in fraction 3 in trace amounts.

The HPLC analysis of fractions 4 and 5 revealed caffeic acid (1.29 ± 0.05 and 86.67 ± 3.07 mg/kg, respectively) and coumaric acid (0.98 ± 0.04 and 0.52 ± 0.02 mg/kg, respectively). Fraction 4 was rich in chlorogenic acid (5.73 ± 0.28 mg/kg).

Fraction 6 also contained rutin (67.92 ± 2.40 mg/kg) and astragalin (below the detection limit).

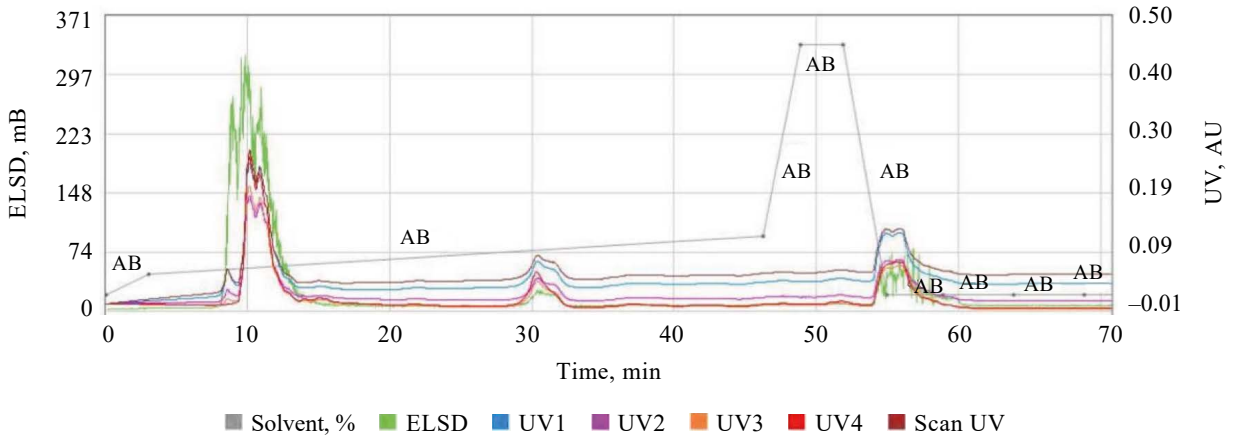

Figure 6 Preparative separation in Blend 1, chromatogram: UV1 λ = 254 nm; UV2 λ = 270 nm; UV3 λ = 280 nm; and UV4 λ = 320 nm

Table 4 Composition of fractions in Blend 1

Fraction	Vial	Yield of fraction, g/kg	Bioactive substances	Yield, g/kg
1	2	8.93 ± 0.41	n.i.	n.d.
2	3–4	11.79 ± 0.53	Coumaric acid Chlorogenic acid	33.74 ± 1.25 b.l.d.
3	5–7	14.94 ± 0.53	n.i.	n.d.
4	8–10	13.45 ± 0.46	n.i. Chlorogenic acid n.i. Coumaric acid	n.d. 18.29 ± 0.54 n.d. 14.33 ± 0.51
5	12–16	7.19 ± 0.26	Catechin n.i. Coumaric acid n.i. n.i. Formononetin	5.20 ± 0.23 6.95 ± 0.21 b.l.d. n.d. n.d. b.l.d.
6	16–20	3.41 ± 0.11	Catechin Ferulic acid n.i. Coumaric acid	b.l.d. 5.36 ± 0.17 n.d. 13.98 ± 0.64
7	21–25	2.11 ± 0.08	Ferulic acid Astragalin Catechin	8.26 ± 0.29 2.43 ± 0.12 12.91 ± 1.53
8	26–29	28.19 ± 1.02	n.i. n.i. Catechin Ferulic acid Astragalin	n.d. n.d. 6.77 ± 1.48 2.30 ± 0.08 3.24 ± 0.12
9	30–36	12.57 ± 0.43	Rosemarinic acid Ferulic acid	b.l.d. 5.78 ± 0.23
10	37–41	16.23 ± 0.58	n.i.	n.d.

n.i. – not identified; n.d. – not detected; and b.l.d. – below the detection limit

In Fraction 7 was revealed nothing but rutin (38.18 ± 1.52 mg/kg).

The total of ten fractions in Blend 1contained coumaric (fraction 2), chlorogenic (fraction 4), and ferulic acids (fractions 8–9). Blend 2 had eight fractions which contained rutin (fraction 7) and caffeic acid (fraction 5).

Fractions 2, 4, 8, and 9, Blend 1 were selected to isolate individual bioactive substances. Coumaric acid was isolated from fraction 2, and chlorogenic acid was isolated from fraction 4. Since fractions 8 and 9 in Blend 1 were rich in ferulic acid, we decided to isolate it from these fractions. Fractions 5 and 7, Blend 2 were used to isolate caffeic acid and rutin. Table 6 demonstrates the isolation results for individual bioactive substances.

Thus, Blend 1 yielded chlorogenic acid (90.0% mass fraction), ferulic acid (92.0%), and coumaric acid (88.0%)

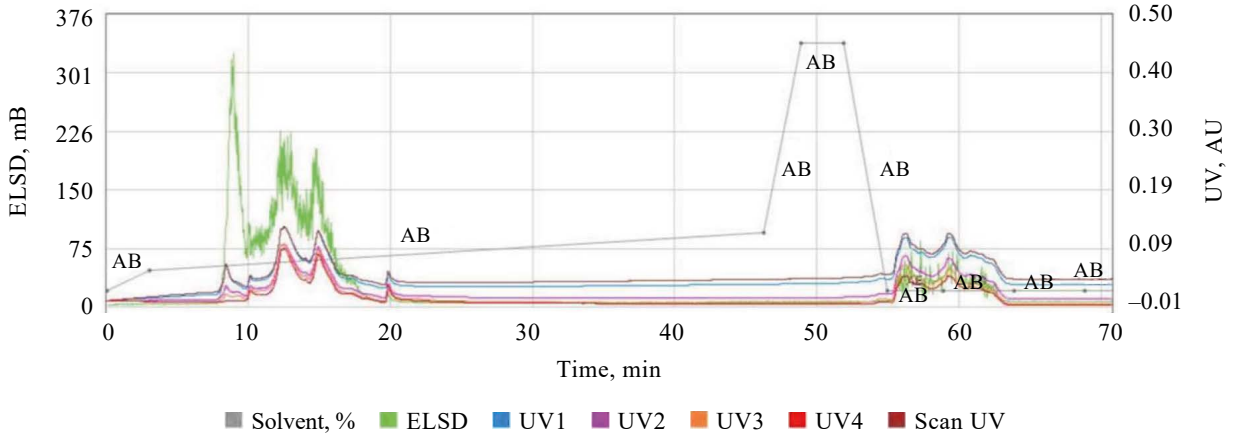

Figure 7 Preparative separation in Blend 2, chromatogram: UV1 λ = 254 nm; UV2 λ = 270 nm; UV3 λ = 280 nm; and UV4 λ = 320 nm

Table 5 Composition of fractions in Blend 2

Fraction	Vial	Yield, g/kg	Bioactive substances	Yield, g/kg
1	1–3	1.77 ± 0.04	3,4-dihydroxybenzoic acid	b.l.d.
			n.i.	n.d.
			n.i.	n.d.
			n.i.	n.d.
2	4–7	1.18 ± 0.05	Chlorogenic acid	38.42 ± 1.58
			Caffeic acid	b.l.d.
3	8–12	7.73 ± 0.37	Chlorogenic acid	24.56 ± 1.10
			Caffeic acid	5.89 ± 0.21
			Coumaric acid	b.l.d.
4	14–18	1.68 ± 0.07	Chlorogenic acid	5.73 ± 0.28
			Caffeic acid	1.29 ± 0.05
			Coumaric acid	0.98 ± 0.04
			n.i.	n.d.
5	19–22	33.17 ± 1.32	Caffeic acid	86.67 ± 3.07
			Coumaric acid	0.52 ± 0.02
6	23–25	9.19 ± 0.28	n.i.	n.d.
			n.i.	n.d.
			Rutin	67.92 ± 2.40
			Astragalin	b.l.d.
7	25–28	0.57 ± 0.02	Rutin	38.18 ± 1.52
8	28–35	12.38 ± 0.60	n.i.	n.d.

n.i. – not identified; n.d. – not detected; and b.l.d. – below the detection limit

Table 6 Bioactive substances isolated from blends

Blend	Bioactive substance	Concentration, vol. % (purity)
1	Chlorogenic acid	90.0 ± 3.5
	Ferulic acid	92.0 ± 4.0
	Coumaric acid	88.0 ± 4.0
2	Caffeic acid	84.5 ± 4.2
	Rutin	78.0 ± 3.8

in individual form. Blend 2 yielded caffeic acid (84.5%) and rutin (78.0%).

Individual bioactive substances were obtained as white crystalline powder by freeze-drying (Fig. 8). All bioactive substances had a low moisture content (Table 7), which means they could retain their biological properties during long-time storage.

Sublimation made it possible to obtain individual bioactive substances as solids with a moisture content of 3.8–4.5%. A comparative analysis of the obtained extractants proved that Blend 1 (70% water-ethanol solution) contained more biologically active substances than Blend 2 (whey). Unlike Blend 1, Blend 2 contained rutin and caffeic acid. Unlike Blend 2, Blend 1 had traces of catechin, ferulic acid, and some other bioactive substances. Figure 9 compares bioactive substances isolated from the two blends.

The samples with 70% water-ethanol extractant yielded more bioactive substances than the whey extractant: chlorogenic acid (637.92 vs. 380.45), coumaric acid (93.05 vs. 2.34), astragalin (53.12 vs. 3.09), catechin (416.38 vs. 0), ferulic acid (507.43 vs. 0). However, the whey extractant made it possible to isolate some

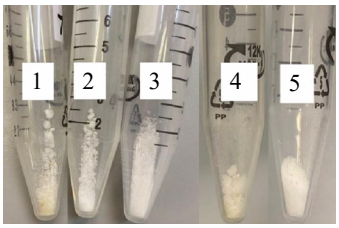

Figure 8 Lyophilized bioactive substances: (1) rutin; (2) chlorogenic acid; (3) coumaric acid; (4) caffeic acid; and (5) ferulic acid

Table 7 Mass fraction of moisture in fractions

Bioactive substance	Moisture mass fraction, %
Chlorogenic acid	4.0 ± 0.1
Caffeic acid	3.8 ± 0.1
Ferulic acid	4.3 ± 0.2
Coumaric acid	4.5 ± 0.2
Rutin	4.2 ± 0.2

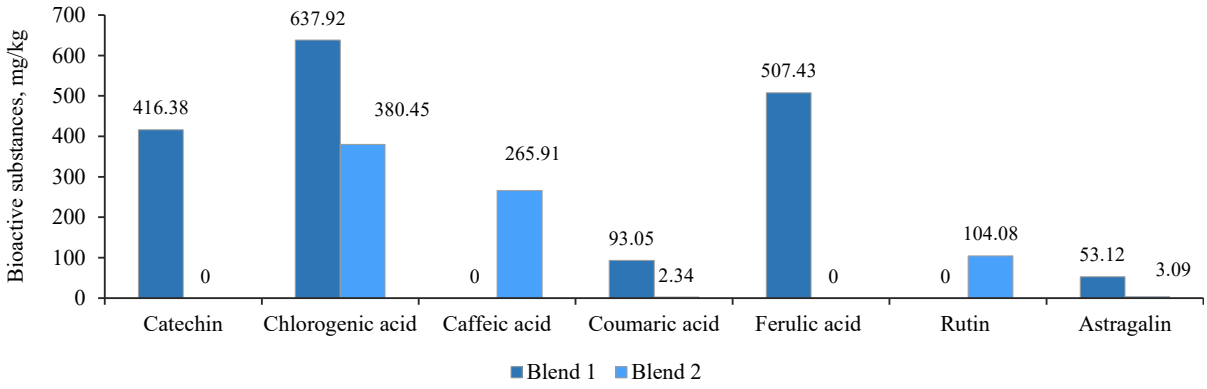

Figure 9 Quantities of bioactive substances in Blend 1 (70% water-ethanol extractant) and Blend 2 (whey)

substances that the 70% water-ethanol extractant failed to isolate, i. e., caffeic acid (265.91) and rutin (104.08).

To summarize, the water-ethanol extractants proved more effective in extracting bioactive substances in larger volumes. Yet, the industrial use of way might still be feasible, given that whey is a massive waste product of dairy production and its drain causes significant harm to the environment. In addition, whey was able to isolate some biologically active substances (rutin and caffeic acid) that the water-ethanol solution failed to extract.

CONCLUSION

The focus of phytochemical extraction research is shifting towards more efficient but environmentally friendly and scalable methods. While maximizing the yield and purity of bioactive substances, these methods ensure the sustainability of the extraction process.

Ethanol extraction is effective due to its wide solubility range. It is a neutral solvent capable of extracting a wide range of compounds, including both hydrophilic (water-soluble) components and lipophilic ones. Its antimicrobial properties reduce the risks of contamination and preserve the quality of extracts. Ethanol is relatively safe and economical. It evaporates after extraction, thus facilitating the concentration processes. These characteristics make ethanol a popular extractant of bioactive ingredients, especially those with high antioxidant activities [35].

Whey remains an unconventional extractant. However, its status as a major pollutant makes it a prospective candidate for industrial recycling. Today, whey wastes are recycled for the biologically active substances they contain, which may complicate the purification of the finished products. But whey can also be used to extract bioactive substances from plant materials. While few studies report whey as an effective extractant, some do feature the whey extraction of terpenoid fractions, phenolic compounds, vitamin C, and rutin.

We studied the phytochemical composition of plant blends that had the same component composition but different extractants, i. e., 70% water-ethanol solution (Blend 1) and whey (Blend 2). Blend 1 contained large amounts of catechin, chlorogenic acid, coumaric acid, and ferulic acid. Blend 2 was rich in chlorogenic acid, caffeic acid, and rutin. We also fractionated the blends. Blend 1 yielded ten fractions, which mainly contained coumaric acid (fraction 2), chlorogenic acid (fraction 4), and ferulic acid (fractions 8–9). Blend 2 yielded eight fractions, some of which contained rutin (fraction 7) and caffeic acid (fraction 5).

We also isolated some individual bioactive substances from the fractions obtained. Blend 1 yielded chlorogenic acid (90.0%), ferulic acid (92.0%), and coumaric acid (88.0%). Blend 2 yielded caffeic acid (84.5%) and rutin (78.0%).

The bioactive substances underwent freeze-drying to a moisture mass fraction of 3.8–4.5%, which makes them a valuable ingredient with a long-term shelf-life to be used in feed for live-stock animals.

The alcohol extractant proved superior to the whey extractant in terms of quantity and diversity of bioactive

substances extracted. However, the whey extractant was able to isolate substances that the water-ethanol solution failed to affect. The fact that whey was able to extract caffeic acid and rutin proved its efficiency as a solvent and rendered it with some prospects for future studies. Both ethanol and whey proved industrially relevant as extractants of biologically active substances from plant materials.

CONTRIBUTION

The authors were equally involved in writing the manuscript and bear equal responsibility for any potential plagiarism.

CONFLICT OF INTEREST

The authors declared no conflict of interest regarding the publication of this article.

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ORCID IDs
Anna A. Stepanova https://orcid.org/0000-0001-7774-8859
Alice D. Mitina https://orcid.org/0009-0004-7824-3982
Natalia S. Velichkovich https://orcid.org/0000-0002-9061-1256
Oksana V. Kozlova https://orcid.org/0000-0002-2960-0216
Timothy A. Larichev https://orcid.org/0000-0003-0166-2527
Larisa A. Proskuryakova https://orcid.org/0000-0002-9583-9161
Vladimir V. Martirosyan https://orcid.org/0000-0002-4026-5789