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RESEARCH ARTICLE

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Effect of ecological factors on the accumulation of phenolic compounds in *Iris* species from Latvia, Lithuania and Ukraine

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Abstract

Introduction: It is important to conduct studies on the influence of environmental factors on the accumulation of secondary metabolites in plants, as well as the cultivation of plants and harvesting of their raw material.

Objective: In this study, we examined the influence of habitat types, soil composition, climatic factors and altitude on the content of phenolic compounds in *Iris* species from different populations in Latvia, Lithuania and Ukraine.

Methodology: According to high-performance liquid chromatography (HPLC) analysis, 25 compounds (flavonoids, isoflavonoids, isoflavonoid glucosides, xanthones, phenolcarboxylic acids) were identified in the methanol extracts of 16 samples of *Iris* rhizomes. The quantitative data were further analysed by principal component analysis (PCA) to reveal the impact of environmental factors on the accumulation of compounds in plants.

Results: *Iris pseudacorus* from Latvia and Lithuania had a more diverse composition of phenolic compounds than samples from Ukraine. Sampled plants of the *Iris* subg. *Iris* had a higher content of the analysed compounds than those of *Iris* subg. *Limniris*. PCA results showed that the levels of phenolic compounds in *Iris* rhizomes were influenced by the content of soil nutrients. The phosphorus and potassium content had a significant impact on the levels of phenolic compounds, whereas the impact of nitrogen content was not significant. Meteorological factors had a small impact; however, sunshine duration had a significant positive effect and the amount of precipitation had a significant negative impact.

Conclusion: The results of this study suggest that rhizomes of *Iris* species may be an important source of pharmacologically active compounds such as flavonoids, isoflavonoids and xanthones. Studies on the effect of environmental factors on the production and accumulation of secondary metabolites in *Iris* species are important because they contribute to knowledge of quantitative parameters of secondary metabolites in medicinal plants and could be employed for the cultivation and harvesting of raw material for medicinal purposes.

KEYWORDS

HPLC, Iridaceae, meteorological conditions, principal component analysis, rhizomes, soil

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1 | INTRODUCTION

The accumulation of biologically active compounds (BACs) by plants and their relationship with environmental conditions have been widely studied.[1-3] The content of BACs in plants largely depends on abiotic factors, i.e. climatic and meteorological conditions,[4,5] geographical location[6,7] and ecological conditions (temperature, humidity, conditions of the microhabitats and soil composition).[4,6]

Phenolic compounds comprise a large subgroup of secondary metabolites and exhibit different pharmacological effects.[8-10] The composition of plant phenolic compounds is dependent on ontogeny and phylogenesis.[2,11] The BACs that are contained in the same plant species may differ in composition, content and proportions because of differences in environmental factors in their habitats.[12,13] There is a relationship between the flavonoid composition in plants and plant ecological and morphological characteristics. According to the results of several studies, an increase in temperature or precipitation[2] increases the accumulation of flavonoids in some plants (*Silene littorea*, *Crocus sativus*).[13,14] However, a reduction in temperature has a positive effect on the accumulation of flavonoids in other plants.[15] Similar effects have been found in relation to geographical location, altitude and other local environmental factors, e.g. illumination, air humidity and soil pH.[6,16]

Soil, as a source of nutrients and an environment with a specific chemical composition, is essential for plant growth[17,18] and influences plant metabolism.[19,20] It is known that plants growing on nutrient-poor soils accumulate more phenolic compounds than plants in more fertile soils.[20] In their theory of the Protein Competition Model (PCM), Jones and Hartley[21] proposed that restricting nitrogen and phosphorus could have different effects, because the nutrients are involved in various cellular metabolic processes.[20]

The dependence of the content and composition of flavonoids on the growing conditions of plants in the natural environment and cultivation has been established.[22,23] This leads us to the conclusion that flavonoid compounds are involved in a complex chain of reactions that determine the adaptation of the plant organism to environmental conditions. In addition, there is a regular dynamic of the flavonoid content depending on the season, age of the individual and even daily changes.[24] Thus, the quantitative and qualitative composition of BACs in raw plant material is affected by the agrochemical factors of the soil,[25] geographical location[12,13] and climatic conditions.[4] In this regard, it is relevant to study the dynamics of the accumulation of BACs in natural populations from different habitats.

The genus *Iris* (Iridaceae) has almost worldwide distribution in tropical, subtropical and temperate regions. The largest diversity of *Iris* species is concentrated in South Africa, followed by South America, Europe and temperate regions of Asia.[26] There are 16 *Iris* species occurring in the wild in Ukraine and four species in the Baltic countries.

Some *Iris* species are used in medicines and dietary supplements, such as Pancreophile ("Fytolynyya SmartMed", Ukraine), "Iris-plus" (Doctor N, Russian Federation), Mastodynon (Bionorica SE, Germany), "Orris (Iris) Herbasol Extract PG" (Lipoid Kosmetik, Switzerland), "Original grosser Bittner balsam" (Richard Bittner AG, Austria), "*Iris versicolor*" (Boiron, USA), etc. Species of this genus have an immense medicinal importance and are used in the treatment of cancer, inflammation and bacterial and viral infections.[27] Numerous results of studies on the variety of pharmacological effects of the BACs found in *Iris* species and the composition of isoflavones, flavones, terpenoids, xanthones or simple phenolic compounds in extracts from these plants have been published.[26,28] However, to our knowledge data on the effect of various environmental factors on the composition of BACs in these plants were not available.

Environmental factors such as soil composition, habitat illumination, altitude and climate have a combined effect on the qualitative and quantitative content of the phenolic compounds in species of the genus *Iris*.[29-31] Phenolic compounds are the compounds that have long proven themselves to have multilateral biological activities. They are used in the treatment of cardiovascular diseases due to their antioxidant and endothelioprotective effects, as well as additional agents in the treatment of cancer, inflammatory and many other diseases. Therefore, their identification and study are relevant. In addition, the chemical profile of secondary metabolites in plants is the basis for further chemophenetic studies. High-performance liquid chromatography (HPLC) fingerprint analysis, and different statistical analyses were introduced for quality assessment of *Iris* species from different geographic locations.[32-34] The aim of this study was to perform a comparative analysis of the qualitative and quantitative composition of the phenolic compounds, employing the HPLC method, in *Iris pseudacorus*, *Iris sibirica*, *Iris halophila* and *Iris germanica* harvested in different geographical areas and habitats from Latvia, Lithuania and Ukraine.

2 | MATERIALS AND METHODS

2.1 | Study species

Four species of the genus *Iris* L. belonging to two subgenera,[35] *Iris* subg. *Iris* (*Iris germanica* L.) and *Iris* subg. *Limniris* (Tausch) Spach (*Iris halophila* Pall., *Iris pseudacorus* L., *Iris sibirica* L.), were selected for this study. Voucher specimens from populations in Latvia and Lithuania, collected and identified by Dr Gudžinskas, were deposited at the Herbarium of the Institute of Botany of the Nature Research Centre in Vilnius, Lithuania (BILAS, voucher specimens No. 75995–76004). Ukrainian species were collected and identified by Dr Mykhailenko and the identification was verified by Yu. G. Gamulya. Ukrainian specimens were deposited at the Herbarium of V.M. Karazin Kharkiv National University, Ukraine (CWN, voucher specimens No. CWN0056543– CWN0056554).

1Populations of *Iris* species for this study were selected in Latvia, Lithuania and Ukraine (Table 1, Figure 1). Rhizomes of *Iris* plants were sampled during September–October 2017, mainly in natural habitats, except for two samples of *Iris sibirica* and one

TABLE 1 Location and geographical characteristics of the *Iris* species sampling sites in Latvia, Lithuania and Ukraine

Site code	Site name	Species	Administrative location	Altitude (m)	Geographical coordinates
IH1	Vinnytsia	*Iris halophila*	Ukraine, Vinnytsia region, Murovanokurilovets district, valley of irises natural park	266	48.83694°N 27.61139°E
IP2	Uman'	*Iris pseudacorus*	Ukraine, Cherkasy region, Uman', Sofiyivka national Dendrological park	157	45.08985°N 33.99887°E
IP3	Vinnytsia	*Iris pseudacorus*	Ukraine, Vinnytsia region, Murovanokurilovets district, valley of irises Natural Park	266	48.83694°N 27.61138°E
IP4	Kharkiv	*Iris pseudacorus*	Ukraine, Kharkiv region, village Borshevaya	111	50.08528°N 36.38472°E
IP5	Zakarpatsk	*Iris pseudacorus*	Ukraine, Zakarpatsk region Carpathian biosphere reserve, Khust, valley of Narcissus	185	48.18520°N 24.31880°E
IP6	Medumi	*Iris pseudacorus*	Latvia, Daugavpils district, east of Medumi	125	55.78063°N 26.40766°E
IP7	Spindžius A	*Iris pseudacorus*	Lithuania, Trakai district, 9 km east of Aukštadvaris, at Lake Spindžius	125	54.57204°N 24.66853°E
IP8	Spindžius B	*Iris pseudacorus*	Lithuania, Trakai district, 9 km east of Aukštadvaris, Lake Spindžius	125	54.57255°N 24.66959°E
IP9	Meškerinė	*Iris Pseudarocus*	Lithuania, Švenčionys district, 8 km northeast of Pabradė, Meškerinė	252	55.04278°N 25.85584°E
IP10	Lakajai	*Iris Pseudarocus*	Lithuania, Molėtai district, environs of Mindūnai village, Lake Baltieji Lakajai	152	55.22050°N 25.55068°E
IS11	Šiauliai	*Iris sibirica*	Lithuania, Šiauliai, Botanical Garden of Šiauliai University	151	55.93283°N 23.28415°E
IS12	Kyiv	*Iris sibirica*	Ukraine, Kyiv, N.N. Gryshko national botanical garden	187	50.43178°N 30.51638°E
IS13	Zakarpatsk	*Iris sibirica*	Ukraine, Zakarpatsk region Carpathian biosphere reserve, Rakhiv, valley of Narcissus	185	48.18520°N 24.31880°E
IG14	Zabarauskai	*Iris germanica*	Lithuania, Trakai district, 2.5 km south of Aukštadvaris, Zabarauskai village	170	54.55444°N 24.52151°E
IG15	Pabradė	*Iris germanica*	Lithuania, Švenčionys district, environs of Pabradė Village	124	54.99421°N 25.79469°E
IG16	Lausgeniai	*Iris germanica*	Lithuania, Trakai district, 10 km southeast of Aukštadvaris, Lausgeniai village	166	54.54911°N 24.66966°E

sample of *Iris pseudacorus*, which were sampled from cultivated plants. In total, nine populations of *Iris pseudacorus*, three populations of *Iris germanica*, three populations of *Iris sibirica* and one population of *Iris halophila* were sampled (Table 1).

2.2 | Plant sampling

In each selected locality, at least five *Iris* plants were sampled. One 5–10 cm long rhizome per plant was dug out, cleaned from the soil and placed into a labelled paper bag. The same or following day after collection, rhizomes were cleaned, removing roots and dead parts, then carefully washed under running water. Washed rhizomes were left for several hours to dry and then cut into ca. 0.5 cm thick pieces with a sharp knife, placed on clean paper and left for drying in a well aerated room, protected from direct sunlight at ambient temperature (18–20°C) for approximately 4 weeks. Dried samples were placed into labelled paper bags and stored at room temperature until analysis.

2.3 | Soil sampling and characteristics

Soil samples (except for water habitats) were taken for agrochemical analyses in the proximity of the sampled plants, from 0–30 cm depth. One mixed sample of soil (ca. 1 kg) was prepared in each sampling area and put into a labelled cloth bag. At the laboratory, soil samples were dried at ambient temperature and then stored in cloth bags until

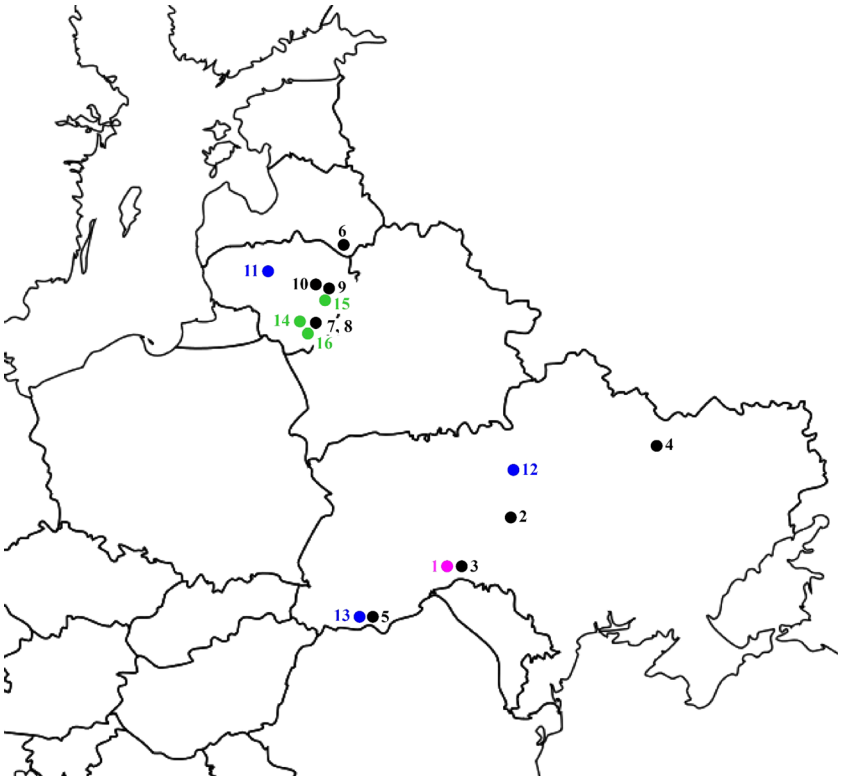


FIGURE 1 Map of rhizome sampling localities of *Iris halophila* (magenta), *Iris pseudacorus* (black), *Iris sibirica* (blue) and *Iris germanica* (green) in Latvia, Lithuania and Ukraine. Numbers next to the dots correspond with the population numbers in Table 1 [Colour figure can be viewed at wileyonlinelibrary.com]

analysis.[36] Agrochemical analyses of soil samples were performed at the Laboratory of Agrochemical Analyses of the Lithuanian Research Centre for Agriculture and Forestry (Kaunas, Lithuania) [37] and the Kharkiv Branch of the State Soil Protection Institute of Ukraine (Kharkiv, Ukraine).[36] The quantity of humus (%) and content of mobile phosphorus (P_2O_5) were determined by the photometric method using Specol 11 Kone Photometer. The content of exchange potassium (K_2O) was determined following the Chirikov method using Endorf Flame Photometer. Soil pH in water solution [mol/L potassium chloride (KCl) suspension] applying instrumental methods. The quantity of easy hydrolysable nitrogen (%) was determined according to the Kornfield method.[36,37] Water characteristics (conductivity and pH) were measured at the site using portable devices (AD32 for conductivity and AD12 for pH, produced by Adwa Instruments, Szeged, Hungary).

2.4 | Preparation of the extracts

At the first step of extract preparation, 0.1 mg of rhizomes pulverised by the electric mill were extracted with 10 mL of methanol in an ultrasonic bath at room temperature (20 ± 2°C) for 30 min. The solutions were filtered through a membrane filter (0.45 μm) prior to use. Aliquots of 20 μL were injected into the HPLC system for analysis. A standard solution of 25 reference compounds, at a concentration of 1.0 mg/mL for each compound, was prepared by dissolving in methanol and then used for calibration. All samples were kept at 4°C before use.

2.5 | HPLC conditions

Chromatographic separation of phenolic compounds was conducted using an ACE C18 column (250 mm × 4.6 mm, 5.0 μm; PA, USA). Elution was performed at a flow rate of 1 mL/min. The binary solvent system of the mobile phase consisted of solvent A (0.1% acetic acid in water) and solvent B (acetonitrile). All solvents were filtered through a 0.23 μm membrane filter after ultrasonic degassing. A linear gradient programme was applied as follows: 0–8 min, 5–15% B; 8–30 min, 15–20% B; 30–48 min, 20–40% B; 48–58 min, 40–50% B; 58–65 min, 50% B; 65–66 min, 50–95% B. The column temperature was held at a constant 25°C. Aliquots of 20 μL were injected into the HPLC system for analysis. The chromatograms were recorded at 269 nm (for mangiferin and isoflavones), at 270 nm and 320 nm (for phenol carboxylic acids), and at 340–350 nm (for flavones). The standard solutions, including 25 reference compounds (Table 4), were used for the calibration of a standard curve using an external standard method. The analyses were performed in duplicate. All reference compounds had a purity > 98% as detected by HPLC-UV using the area normalisation method. Reference compounds were purchased from ChromaDex (Santa Ana, CA, USA), Sigma-Aldrich (St Louis, MO,

USA), HWI Analytik GmbH and Roth GmbH (Karlsruhe, Germany). The chemical structures of these compounds are shown in Figure 2.

2.6 | Identification of peaks and peak purity

Identification of all constituents was performed by HPLC analysis by comparing the retention time (R_t), UV spectra of the peaks in samples with those of reference compounds. The purity of peaks was checked by a diode array detector (DAD) coupled to the HPLC system, comparing UV spectra of each peak with those of reference compounds and/or by examination of the UV spectra.

2.7 | Quantitative determination of constituents

The content of compounds in the plant extract was calculated (in mg/g) by the formula:

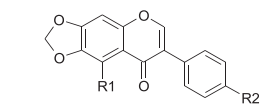
$$X = \frac{Sm_{st}V}{S_{st}mV_{st}}$$

where S is an average value of the area of phenolic compound peaks, calculated from parallel chromatograms of the sample solution; S_{st} is an average value of the area of reference compound peaks, calculated from parallel chromatograms of the standard solution; m weight amount of powdered raw materials (in grams), m_{st} the reference compound weight amount (in milligrams); V volume of volumetric flask of test extract of the raw material (in millilitres); V_{st} volume of volumetric flask of reference compounds (in millilitres).

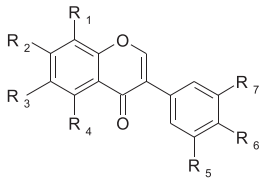
2.8 | Validation procedure for quantitative analysis

2.8.1 | Linearity, LOD, LOQ

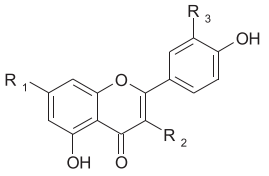
The linear relationships between the detector response and the different concentrations of compounds were confirmed as shown in



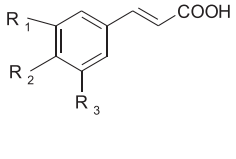
Isoflavones:
Nigricin-D-glu 13: R_1=OCH_3; R_2=O-Glu;
Nigricin 20: R_1=OCH_3; R_2=OH



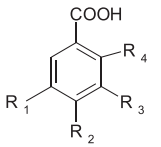
Isoflavones:
Tectoridin 10: R_1=R_5=R_7=H; R_4=R_6=OH; R_3=OCH_3; R_2=O-Glu;
Daidzein 12: R_1=R_3=R_4=R_5=R_7=H; R_2=R_6=OH;
Genistein 7-glu 15: R_1=R_3=R_5=R_7=H; R_2=O-Glu; R_6=OH; R_4=OH;
6,7-Dihydroxyisoflavone 17: R_1= R_4=R_5=R_6=R_7=H; R_2=R_3=OH;
Iristectorigenin B 19: R_2=R_4=R_6=OH; R_3=R_7=OCH_3; R_5=H;
Irigenin 21: R_3=R_5= R_6=OCH_3; R_2=R_4=R_7=OH;
7-Hydroxyisoflavone 22: R_1=R_3=R_4=R_5=R_6=R_7=H; R_2=OH;
Formononetin 23: R_1=R_3=R_4=R_5= R_7=H; R_2=OH; R_6=OCH_3;
5,6-Dihydroxy-7,8,3′,5′-tetramethoxyisoflavone 24:
R_1=R_2=R_5=R_7=OCH_3; R_3=R_4=OH; R_6=H;
5,7-Dihydroxy-4′-methoxyisoflavone 25: R_1=R_3=R_5=R_6=R_7=H; R_2=R_4=OH; R_6=OCH_3;



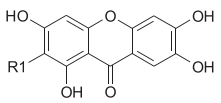
Flavones:
Hiperoside 8: R_1=OH; R_2= O-Gal; R_3=OH;
Isoquercitin 9: R_1=OH; R_2= O-Glu; R_3=OH;
Apigenin-7-glu 11: R_1= O-Glu; R_2=H; R_3=H;
Apigenin 16: R_1= OH; R_2=H; R_3=H



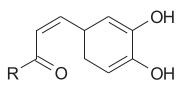
Caffeic acid 4: R_1=OH; R_2=OH; R_3=H
Coumaric acid 6: R_1=H; R_2=OH; R_3=H
Ferulic acid 7: R_1=OCH_3; R_2=OH; R_3=H
Cinnamic acid 14: R_1=H; R_2=H; R_3=H



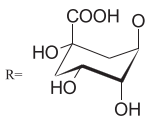
Gallic acid 1: R_1=OH; R_2=OH; R_3=OH; R_4=H;



Xanthones:
Mangiferin 5: R_1 = C-β-D-Glc



Chlorogenic acid 3:



Neochlorogenic acid 2:

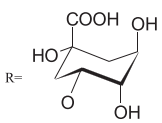


FIGURE 2 Structural formulas of 25 compounds identified in *Iris* species

Table 4. To determinate the linearity of this method, eight different concentrations of reference compounds were used for all substances. All calibration curves showed good results within the tested ranges. The sensitivity values of the method were calculated for each compound.

The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected but not necessarily quantified. The limit of quantification (LOQ) is the lowest amount of analyte that can be determined with suitable precision and accuracy. These parameters were estimated using the formulae: LOD = 3.3*(σ)/S and LOQ = 10*(σ)/S, where (σ) is a standard deviation of the response (peak area) and S is the slope of the calibration curve.

2.8.2 | Precision and accuracy

The relative standard deviation (RSD) for intra-day and inter-day measurements are shown in Table 5. According to the International Conference for Harmonisation (ICH) guidelines, the results of intermediate precision were obtained by different analysts and on different days.[38]

2.8.3 | Stability

There were no significantly different changes in analyte composition during 24 h of storage. The mean RSD between peak areas for the samples stored under refrigeration (8 ± 1°C) showed suitable results (Table 5). This indicated that solutions could be stored without any degradation for the duration of the internal studies.

2.8.4 | Specificity

The resolution between the peaks of the main constituents found in the methanol extract of *Iris* species was determined by the analysis of chromatograms of the standard solution and the sample solution. The data are presented in the Supporting Information Table S1.

2.9 | Apparatus

Liquid chromatography (LC) separation was performed using the Shimadzu Nexera X2 LC-30 AD HPLC system (Shimadzu, Kyoto, Japan), composed of a quaternary pump, an on-line degasser, a column temperature controller, the SIL-30 AC autosampler (Shimadzu), the CTO-20 AC thermostat (Shimadzu) and the SPD-M20A DAD. Other instruments, such as the Ultrasonic Cleaner Set for ultra-sonication (Wise Clean WUC-A06H, Witeg Labortechnik GmbH, Wertheim, Germany); Libra UniBloc AUW120D (Shimadzu Analytical Scale, Kyoto, Japan); pH-meter – Knick Electronic Battery-operated pH Meter 911 PH (Portamess, Berlin Germany) and class A analytical vessels were used in the investigation.

2.10 | Statistical analyses

All data processing was carried out using LabSolutions Analysis Data System (Shimadzu). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with the software package Prism v.5.04 (GraphPad Software

TABLE 2 Environmental characteristics of the *Iris* species sampling sites in Latvia, Lithuania and Ukraine

Site code	Species	Habitat	Coverage (%)			Water depth (m)
			Trees	Shrubs	Herbs	
IH1	*Iris halophila*	Dry steppe	0	10	80	–
IP2	*Iris pseudacorus*	Cultivation	0	0	50	–
IP3	*Iris pseudacorus*	Dry steppe	0	10	80	–
IP4	*Iris pseudacorus*	Edge of dry pine forest	30	10	60	–
IP5	*Iris pseudacorus*	Open steppe wet meadows	0	0	70	–
IP6	*Iris pseudacorus*	Alluvial alder forest	60	40	60	–
IP7	*Iris pseudacorus*	Alluvial alder forest	50	40	70	–
IP8	*Iris pseudacorus*	Mesotrophic lake	0	0	60	0.2–0.3
IP9	*Iris pseudacorus*	Edge of dry pine forest	20	10	70	–
IP10	*Iris pseudacorus*	Mesotrophic lake	0	0	50	0.3–0.4
IS11	*Iris sibirica*	Cultivation	0	0	60	–
IS12	*Iris sibirica*	Cultivation	0	0	50	–
IS13	*Iris sibirica*	Open steppe meadows	0	10	80	–
IG14	*Iris germanica*	Edge of Mesic pine forest	40	40	50	–
IG15	*Iris germanica*	Dry pine forest	40	10	60	–
IG16	*Iris germanica*	Dry grassland	0	20	70	–

Inc., La Jolla, CA, USA). The value of $P < 0.05$ was taken as the level of significance. The Bray–Curtis similarity index, according to the presence of phenolic compounds, was calculated for the studied species. The data on quantity of phenolic compounds were transformed to reflect only the presence (1) or absence (0) of certain phenolic compounds. The dendrogram was created applying the UPGMA algorithm and Bray-Curtis distance. For the principal component analysis (PCA), phenolic compounds were grouped in classes and data were analysed applying a correlation matrix. Statistical calculations were performed, and dendrograms and PCA graphs were prepared, employing PAST 3.2 software.

3 | RESULTS AND DISCUSSION

3.1 | Site and habitat characteristics

In each sampling site the habitat characteristics were recorded (vegetation type, habitat type, water depth). The population of *Iris halophila* was sampled in well illuminated dry steppe habitats with a sparse shrub layer (Table 2). Samples of *Iris pseudacorus* were collected in three types of contrasting habitats. One population in Latvia and one in Lithuania were selected in alluvial forest habitats dominated by grey (*Alnus incana*) and black (*Alnus glutinosa*) alders. These habitats were significantly shaded by tree and shrub layers (Table 2). The other two populations of *Iris pseudacorus* were sampled in mesotrophic lakes in Lithuania. During the sampling, plants grew in water at 0.2–0.4 m depth. In both lake habitats, *Iris pseudacorus* occurred in moderately dense stands (coverage 50–60%) of heliophytes dominated by common reed (*Phragmites australis*) (Table 2). One population of this species in Lithuania was sampled in a dry habitat, which was situated at the edge of a dry pine forest, in sandy soil.

Samples of *Iris sibirica* were taken from cultivated and wild plants area. In Lithuania, plant rhizomes were collected in the Botanical Garden of Šiauliai University. This accession of *Iris sibirica* at the Botanical Garden was cultivated since 2009 and originated from a wild population occurring in the environs of the Truskava village (Kėdainiai district, central Lithuania). In Ukraine, *Iris sibirica* were collected in Kyiv, in N.N. Gryshko National Botanical Garden and in an open steppe meadow in Zakarpatsk region (Table 1). *Iris sibirica*, in both places of cultivation and in the natural habitat, grew in open, well illuminated mesic sites (Table 2).

Iris germanica, which in Lithuania is a naturalised alien species,[39] was sampled in three localities (Table 1). Two sampling sites were in partial shade, in sparse pine forest (Pabradė site) or at the edge of mesic pine forest (Zabarauskai site) with quite an abundant shrub layer (Table 2). The third population (Lausgeniai site) was situated in a dry grassland with quite a sparse shrub layer.

3.2 | Physical and chemical soil analysis results

Results of agrochemical soil analyses revealed broad variation of the studied soil properties among sampling sites (Table 3). Soil pH in habitats of the studied species in Lithuania and Latvia was close to neutral, i.e. slightly acidic (pH 6.1–6.5), neutral (pH 6.6–7.3) or slightly alkaline (pH 7.4–7.8), whereas in Ukraine soil pH in most cases was strongly (pH 5.1–5.5), very strongly (pH 4.5–5.0) or extremely acidic (pH 3.5–4.4). Soils with very low (< 50 mg/kg) and low (50–100 mg/kg) content of phosphorus prevailed in sites of studied populations in Lithuania and Latvia, however, soils in study

TABLE 3 Soil characteristics of the *Iris* species sampling sites in Latvia, Lithuania and Ukraine

Site code	Species	pH (Mol/l)	P_2O_5 (mg/kg)	K_2O (mg/kg)	Nitrogen (%)	Humus (%)	Water conductivity (μS)	Water pH
IH1	*Iris halophila*	4.9	48	97	0.08	1.98	–	–
IP2	*Iris pseudacorus*	6.5	207	166	1.10	4.80	–	–
IP3	*Iris pseudacorus*	5.1	46	100	0.10	2.05	–	–
IP4	*Iris pseudacorus*	5.6	95	98	1.05	3.40	–	–
IP5	*Iris pseudacorus*	4.2	81	165	1.24	5.32	–	–
IP6	*Iris pseudacorus*	7.2	115	63	0.11	5.74	–	–
IP7	*Iris pseudacorus*	7.6	105	126	0.23	6.41	–	–
IP8	*Iris pseudacorus*	–	–	–	–	–	565	7.6
IP9	*Iris pseudacorus*	6.8	38	342	0.26	2.60	–	–
IP10	*Iris pseudacorus*	–	–	–	–	–	351	7.2
IS11	*Iris sibirica*	7.3	85	187	0.19	2.21	–	–
IS12	*Iris sibirica*	5.7	406	248	1.04	5.30	–	–
IS13	*Iris sibirica*	4.3	90	150	1.17	4.85	–	–
IG14	*Iris germanica*	6.3	65	192	0.14	2.68	–	–
IG15	*Iris germanica*	6.0	35	68	0.23	2.41	–	–
IG16	*Iris germanica*	7.4	187	35	0.05	1.63	–	–

TABLE 4 Calibration curves of 25 quantified reference standard compounds

Compound		Calibration curve[a]	Correlation coefficient r^2 (n = 6)	Linear range (μm/mL)	RSD (%)	LOD[b] (ng/mL)	LOQ[c] (ng/mL)
1	**Gallic acid**	***f*(*x*) = 32880.6**x* – 612.983**	**0.9999718**	**0.48–61.08**	**1.31**	**30**	**100**
2	Neochlorogenic acid	*f*(*x*) = 30834.6**x* – 613.100	0.9999594	0.38–48	1.18	30	80
3	Chlorogenic acid	*f*(*x*) = 29930.2**x* – 538.361	0.9999502	0.36–46	1.29	20	70
4	Caffeic acid	*f*(*x*) = 57646.8**x* – 3853.48	0.9999218	0.72–91.92	1.56	20	60
5	Mangiferin	*f*(*x*) = 29263.5**x* + 13863.9	0.9997952	0.28–145.00	1.32	310	940
6	*p*-Coumaric acid	*f*(*x*) = 55638.6**x* + 519.752	0.9999693	0.31–39.25	1.14	30	80
7	Ferulic acid	*f*(*x*) = 54955.4**x* – 638.345	0.9999592	0.44–56.5	1.60	30	80
8	Hiperoside	*f*(*x*) = 22498.4**x* + 2508.57	0.9998647	0.21–27.04	1.19	75	230
9	Isoquercitrin	*f*(*x*) = 24139.7**x* + 3904.44	0.9998941	0.35–44.56	1.02	73	220
10	Tectoridin	*f*(*x*) = 76104.4**x* + 114152	0.9995802	0.51–260.00	0.55	130	400
11	Apigenin 7-glucoside	*f*(*x*) = 38477.5**x* + 4025.41	0.9999249	0.25–32	0.82	53	160
12	Daidzein	*f*(*x*) = 63650.3**x* + 3897.03	0.9998269	0.09–12.02	0.71	4.98	20
13	Nigricin-4′-D-glucoside	*f*(*x*) = 60944.8**x* + 123042	0.9993218	0.58–298.00	0.46	50	160
14	*trans*-Cinnamic acid	*f*(*x*) = 78502.3**x* + 0	1.000000	0.3–38.75	0.12	30	90
15	Genistein 7-D-glucoside	*f*(*x*) = 21546.1**x* + 1464.76	0.9998262	0.09–12.02	0.59	70	210
16	Apigenin	*f*(*x*) = 50138.3**x* + 5722.97	0.9998899	0.2–25.76	0.53	25	80
17	6,7-Dihydroisoflavone	*f*(*x*) = 58103.6**x* + 3915.34	0.9998860	0.1–13.02	0.46	16.4	50
18	Kaempherol	*f*(*x*) = 29888.8**x* + 1814.27	0.9999240	0.14–18.32	0.90	37	110
19	Iristectorigenin B	*f*(*x*) = 109562**x* + 68062.7	0.9996806	0.23–120.00	0.85	50	150
20	Nigricin	*f*(*x*) = 89415.4**x* + 103288	0.9994037	0.35–181.00	0.30	40	130
21	Irigenin	*f*(*x*) = 81832.6**x* + 137668	0.9994881	0.54–277.00	0.64	50	160
22	7-Hidroxyisoflavone	*f*(*x*) = 37826.1**x* + 6991.56	0.9998489	0.25–31.6	0.80	17.9	50
23	Formononetin	*f*(*x*) = 68012.0**x* + 6377.56	0.9998138	0.13–15.96	0.67	3.82	10
24	5,6-Dihydroxy-7,8,3′,5′-tetramethoxyisoflavone	*f*(*x*) = 86268.5**x* + 59193.5	0.9996879	0.26–132.00	0.54	70	210
25	5,7-Dihydroxy-4′-methoxyisoflavone	*f*(*x*) = 66391.3**x* + 6173.76	0.9998366	0.15–18.76	1.12	32	100

[a]x, concentration of compound (mg/mL); *y*, peak area.
[b]LOD, limit of detection (signal/noise = 3).
[c]LOQ, limit of quantification (signal/noise = 10).

sites of Ukraine ranged from very low (< 50 mg/kg) to high content (> 200 mg/kg) of phosphorus (Table 3). Soils with moderate (101–150 mg/kg) and sufficient (151–200 mg/kg) content of potassium prevailed in the study sites of *Iris* species in all countries (Table 3), although in several sites soils were with very low (< 50 mg/kg) and low content of potassium (51–100 mg/kg) and in two cases the content of potassium was very high (> 200 mg/kg). The content of nitrogen in the soil samples varied in a broad range, however, in samples from Ukraine it tends to be higher (ranged from 0.08% to 1.24%) than in soils from Lithuania and Latvia (ranged from 0.05% to 0.26%). Humus content in the soil of most study sites in Ukraine was moderate (3–4%) or high (> 4%), only in two study sites it was low (< 3%), whereas study sites in Lithuania and Latvia it was low (< 3%), except two sites of *Iris pseudacorus* occurring in alluvial forest habitats (Table 3) correlate.

3.3 | Validation of the HPLC procedure

A validation study was conducted to demonstrate the applicability of the developed analytical method. The validation was done in terms of specificity, linearity, LOD, LOQ, precision and recovery according to ICH Q2 (R1).[38] The results are given in Tables 4 and 5.

The specificity is the ability of a method to discriminate the study analytes and other constituents in the sample. It was demonstrated by separation of the analytes from other interfering compounds. Determination of main compounds in the test solutions was done by comparing retention times of the peaks and UV-spectrum obtained from the chromatogram of the standard solution (Table S1).

Reproducibility and repeatability of the method were evaluated by analysing two injections of the same sample solution and 16 replicates of *Iris* sample extraction, respectively. Precision of retention

TABLE 5 Precision and stability of 25 quantified compounds

			Precision				Repeatability	
			Intra-day (n = 3)		Inter-day (n = 3)			
Compound		Concentrate (μg/mL)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	Recovery (%)	RSD (%)
1	Gallic acid	7.65	0.57	99.81	0.75	101.37	101.07	0.65
		30.35	0.78	99.56	0.24	102.14	99.69	0.56
		61.20	1.02	101.53	0.38	101.32	100.09	0.94
2	Neochlorogenic acid	6	0.87	100.35	0.88	101.16	100.17	0.55
		24	1.25	99.17	0.97	102.37	98.58	0.57
		48	1.22	100.25	0.71	101.27	100.14	1.01
3	Chlorogenic acid	5.75	1.31	101.12	0.38	98.40	100.69	0.86
		23	0.42	99.08	0.73	99.43	99.58	1.05
		46	0.96	100.27	0.48	98.24	101.91	0.97
4	Caffeic acid	11.49	1.05	102.02	0.52	98.49	100.01	0.46
		45.96	1.08	98.78	0.67	99.73	99.39	0.99
		91.92	0.64	100.35	0.95	98.17	100.17	0.37
5	Mangiferin	9.06	0.33	100.46	0.29	100.41	100.29	0.25
		36.25	0.24	99.66	0.32	100.45	100.03	0.39
		145	0.22	100.32	1.10	98.45	99.58	0.99
6	*p*-Coumaric acid	4.91	0.72	100.30	0.34	99.22	100.15	0.65
		19.63	0.94	99.30	0.82	99.20	99.65	0.49
		39.25	0.67	100.20	0.76	98.98	100.10	0.77
7	Ferulic acid	7.06	0.68	100.22	0.90	98.29	99.11	0.69
		28.25	0.93	98.20	0.29	99.31	99.60	0.57
		56.5	1.22	100.24	0.46	98.28	100.12	0.49
8	Hiperoside	3.433	0.99	101.06	0.86	98.95	100.78	0.90
		13.715	0.50	101.04	0.70	99.07	100.72	0.71
		26.915	0.42	99.53	0.80	100.97	99.77	0.43
9	Isoquercitrin	5.57	0.86	100.26	0.41	100.23	100.13	0.69
		22.28	1.12	101.27	0.98	99.24	99.64	0.90
		44.56	0.80	99.58	0.91	100.92	97.79	0.49
10	Tectoridin	16.25	1.35	101.93	1.57	102.24	101.39	0.98
		65	1.13	101.92	0.72	101.03	100.98	0.95
		260	0.30	99.57	0.03	99.96	99.84	0.23
11	Apigenin 7-D-glucoside	4	0.46	100.75	0.90	98.76	100.38	0.53
		16	0.45	100.89	0.64	98.62	100.45	0.63
		32	0.62	100.70	0.20	99.03	102.35	0.85
12	Daidzein	1.5	0.83	100.2	0.60	98.32	100.60	0.84
		6.01	1.04	100.44	0.32	99.28	100.72	0.72
		12.02	0.33	99.48	0.62	100.02	99.74	0.67
13	Nigricin-4′-D-glucoside	18.62	0.65	100.92	0.16	100.23	100.38	0.48
		74.5	1.07	101.52	1.50	102.15	101.22	0.99
		298	0.64	99.09	0.93	98.69	99.26	0.68
14	*t*-Cinnamic acid	4.77	0.68	100.41	0.62	100.09	99.41	0.29
		21.01	1.09	99.55	0.80	100.97	99.55	0.31
		38.795	1.10	98.99	0.47	101.53	99.99	0.56
15	Genistein 7-D-glucoside	1.5	0.48	101.20	0.81	98.32	100.60	0.84

(Continues)

TABLE 5 (Continued)

Compound		Concentrate (μg/mL)	Precision				Repeatability	
			Intra-day (n = 3)		Inter-day (n = 3)			
			RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	Recovery (%)	RSD (%)
		6.01	0.66	100.51	0.61	98.02	100.76	0.66
		12.02	0.87	99.48	0.49	100.02	99.74	0.37
16	Apigenin	4	0.74	100.75	0.57	98.76	100.56	0.79
		16	0.89	100.89	0.29	98.62	98.96	0.71
		32	0.88	100.70	0.70	98.03	99.80	1.02
17	6,7-Dihydroisoflavone	1.63	1.11	100.22	0.65	98.30	100.61	0.86
		6.51	0.36	101.07	0.86	98.44	100.54	0.76
		13.02	0.82	99.58	0.28	99.92	99.79	0.39
18	Kaempherol	2.29	0.48	100.69	0.44	99.81	100.35	0.49
		9.16	0.77	100.97	1.01	99.54	100.49	0.68
		18.32	1.14	99.66	1.12	100.84	99.83	0.34
19	Iristectorigenin B	7.5	1.23	101.76	1.64	102.35	101.36	0.97
		30	1.01	102.88	1.23	101.76	101.54	1.01
		120	0.07	99.90	0.33	99.53	99.81	0.25
20	Nigricin	11.31	1.19	101.70	1.21	101.73	101.14	0.98
		45.25	0.37	99.47	1.19	101.70	100.39	0.96
		181	0.57	99.19	0.48	99.33	99.50	0.43
21	Irigenin	17.31	1.08	101.54	1.29	101.84	101.12	0.98
		69.25	0.80	101.14	1.16	101.65	100.93	0.84
		277	0.33	99.53	0.20	99.71	99.74	0.24
22	7-Hydroxyisoflavone	3.95	0.89	100.65	0.82	98.85	99.33	0.46
		15.8	0.92	101.24	0.44	98.09	100.72	0.58
		31.6	0.54	99.51	0.84	99.99	99.76	1.03
23	Formononetin	1.99	0.71	101.25	0.55	97.88	100.83	0.66
		7.98	0.36	101.50	0.60	98.03	100.75	0.36
		15.96	0.30	99.47	0.77	100.03	99.74	0.47
24	5,6-Dihydroxy-7,8,3′,5′-tetrametoxyisoflavone	8.25	0.43	100.61	0.77	101.09	100.56	0.54
		33	0.06	100.08	0.52	100.74	100.27	0.41
		132	0.18	99.74	0.80	98.88	99.54	0.59
25	5,7-Dihydroxy-4′-methoxyisoflavone	2.35	0.61	100.72	0.45	98.79	99.36	0.51
		9.38	0.80	100.47	0.60	98.06	100.74	0.53
		18.76	0.67	99.49	0.77	100.00	99.75	0.66

times and peak areas of 25 reference compounds for replicated injections were in the range 0.3–1.34% of RSD (n = 2). To confirm the accuracy of the method, a recovery experiment was performed by mixing quantified samples with specific quantities of reference compounds. The average percentages of recovery of 25 compounds ranged from 98.25 ± 5.27% to 103.61 ± 3.12%. In addition, the RSD varied from 0.12% to 5.38% (n = 2). The LOD (signal/noise = 3) and LOQ (signal/noise = 10) of the all compounds varied within the range 3.82–360 ng/mL and 10–940 ng/mL. The stability of 25 compounds in the sample solution was evaluated by determining their relative peak area (RPA) after storage for 0 to 24 h, respectively. The RSDs of the retention times and peak areas were both less than 3%. The results demonstrated that conditions for the fingerprint analysis were repeatable and accurate.

3.4 | Phenolic compounds in rhizomes of *Iris* from different locations

During this study we identified and quantified 25 phenolic compounds in the methanol extract of 16 samples of *Iris* rhizomes (Table 6) from different ecological areas. The relative retention time of each common peak in each of the HPLC prints is consistent with the relative retention time of the remaining compounds (Figures 3 and 4),

TABLE 6 Content of 25 phenolic compound in the rhizomes of *Iris* species (μg/g)

		R_t	Subg. *Limniris* (site number)					
	Compounds		**IH1**	**IP2**	**IP3**	**IP4**	**IP5**	**IP6**
1	Gallic acid	**5.96**	–	–	60.9 ± 1.045	35.6 ± 1.18	–	–
2	Neochlorogenic acid	9.36	–	–	–	–	–	–
3	Chlorogenic acid	11.66	–	–	–	–	–	–
4	Caffeic acid	**14.18**	–	11 ± 0.35	7.3 ± 0.165	6.1 ± 0.105	–	13.6 ± 0.36
5	Mangiferin	**14.18**	–	–	–	–	–	11.8 ± 0.095
6	*p*-Coumaric acid	20.39	–	–	–	–	6.6 ± 0.24	–
7	Ferulic acid	23.16	–	–	–	–	–	–
8	Hiperoside	23.89	–	–	–	–	–	–
9	Isoquercitin	24.83	–	–	–	–	–	–
10	Tectoridin	**29.89**	–	–	–	–	–	8.9 ± 0.245
11	Apigenin-7-glucoside	33.49	–	–	–	–	–	–
12	Daidzein	40.96	–	–	–	–	1.5 ± 0.052	28.9 ± 0.54
13	Nigricin-4′-glucoside	**39.56**	3.4 ± 0.10	–	1.2 ± 0.02	–	–	2.2 ± 0.07
14	*trans*-Cinnamic acid	45.22	0.7 ± 0.02	1.1 ± 0.027	–	–	2.2 ± 0.09	1.5 ± 0.057
15	Genistein-7-glucoside	**46.07**	–	–	15.3 ± 0.165	–	33 ± 0.63	–
16	Apigenin	47.90	–	–	–	–	–	–
17	6,7-Dihydroxyisoflavone	48.10	–	–	13.2 ± 0.46	–	8.5 ± 0.254	–
18	Kempferol	48.99	–	–	–	–	–	–
19	Iristectorigenin B	49.15	7.4 ± 0.27	0.9 ± 0.021	–	–	–	–
20	Nigricin	49.50	–	2.7 ± 0.032	2.5 ± 0.105	–	4.2 ± 0.12	–
21	Irigenin	**50.03**	5.6 ± 0.18	15.4 ± 0.17	9.2 ± 0.24	–	–	5.3 ± 0.161
22	7-Hydroxyisoflavone	52.68	–	–	–	–	–	–
23	Formononentin	53.00	–	–	–	–	–	–
24	5,6-Dihydroxy-7,8,3′,5′-tetramethoxyisoflavone	56.03	4.8 ± 0.014	–	–	0.3 ± 0.07	1.3 ± 0.045	1.9 ± 0.059
25	5,7-Dihydroxy-4′-methoxyisoflavone	60.66	–	0.3 ± 0.011	–	–	–	–

TABLE 6 Content of 25 phenolic compound in the rhizomes of *Iris* species (μg/g)

	Subg. *Limniris* (site number)							Subg. *Iris* (site number)		
	IP7	IP 8	IP9	IP10	IS11	IS12	IS13	IG14	IG15	IG16
1	59.5 ± 1.579	81.3 ± 2.065	69.3 ± 1.651	62.3 ± 1.115	35.2 ± 0.76	–	–	–	–	–
2	–	17.9 ± 0.598	–	–	–	–	–	–	–	–
3	–	28.6 ± 0.43	–	–	–	–	8.1 ± 0.205	–	–	–
4	–	–	11 ± 0.27	–	–	183.9 ± 4.591	–	414.2 ± 10.17	145.8 ± 2.97	374.3 ± 5.187
5	–	12.7 ± 0.356	–	–	–	267.2 ± 6.133	31.2 ± 0.65	714.6 ± 15.73	2700.4 ± 35.02	1169.6 ± 28.58
6	–	–	–	–	–	–	30.6 ± 0.08	–	–	–
7	–	–	–	–	–	–	–	188.7 ± 3.954	198.5 ± 5.299	87.4 ± 3.47
8	–	–	2.5 ± 0.015	–	–	–	–	–	345.7 ± 8.572	–
9	–	–	–	–	–	–	–	905.1 ± 15.52	–	–
10	–	8.5 ± 0.254	–	–	4.9 ± 0.145	38.3 ± 12.19	131.2 ± 3.61	2000.2 ± 35.01	2217.6 ± 81.11	1779.7 ± 28.589
11	–	–	–	–	–	–	279.5 ± 3.795	–	–	–
12	–	6.2 ± 0.13	–	0.1 ± 0.002	0.1 ± 0.001	–	–	–	–	–
13	2.5 ± 0.025	1.3 ± 0.056	2.2 ± 0.03	2 ± 0.01	1.9 ± 0.059	12.3 ± 0.156	28.4 ± 0.92	53.2 ± 1.62	528.8 ± 16.44	40.2 ± 2.01
14	–	–	–	–	–	19.9 ± 0.599	–	16 ± 0.05	279.4 ± 7.193	–
15	–	28.9 ± 0.544	–	–	–	374.7 ± 7.358	–	2571.7 ± 28.581	9672.3 ± 83.615	3112 ± 15.6
16	0.2 ± 0.001	–	0.6 ± 0.01	–	34.7 ± 0.537	3.2 ± 0.06	–	–	28 ± 0.41	155.8 ± 4.79
17	–	4.3 ± 0.152	–	–	18.7 ± 0.593	–	53.8 ± 0.1.69	–	134.2 ± 2.67	–
18	–	–	–	–	–	–	–	–	15.9 ± 0.579	10.1 ± 20.5
19	1.1 ± 0.017	–	0.2 ± 0.004	–	0.3 ± 0.004	–	–	113.7 ± 2.568	111.3 ± 2.556	96.2 ± 1.81
20	–	–	–	–	2 ± 0.08	–	–	41.3 ± 1.065	42.5 ± 1.252	3 ± 0.05
21	25.2 ± 0.16	0.7 ± 0.015	0.6 ± 0.01	2.7 ± 0.035	11.2 ± 0.28	69.1 ± 1.255	8.9 ± 0.145	2105.4 ± 27.105	1885.6 ± 28.94	2027 ± 0.58
22	–	–	–	–	–	–	–	–	–	37 ± 0.81
23	–	–	–	0.1 ± 0.002	–	–	–	–	–	–
24	23.1 ± 0.551	9.1 ± 0.155	0.6 ± 0.015	1 ± 0.03	1.2 ± 0.02	–	–	547.1 ± 17.553	440.4 ± 11.22	458.2 ± 19.22
25	–	0.3 ± 0.05	–	–	–	–	–	759.8 ± 17.99	548.3 ± 7.514	–

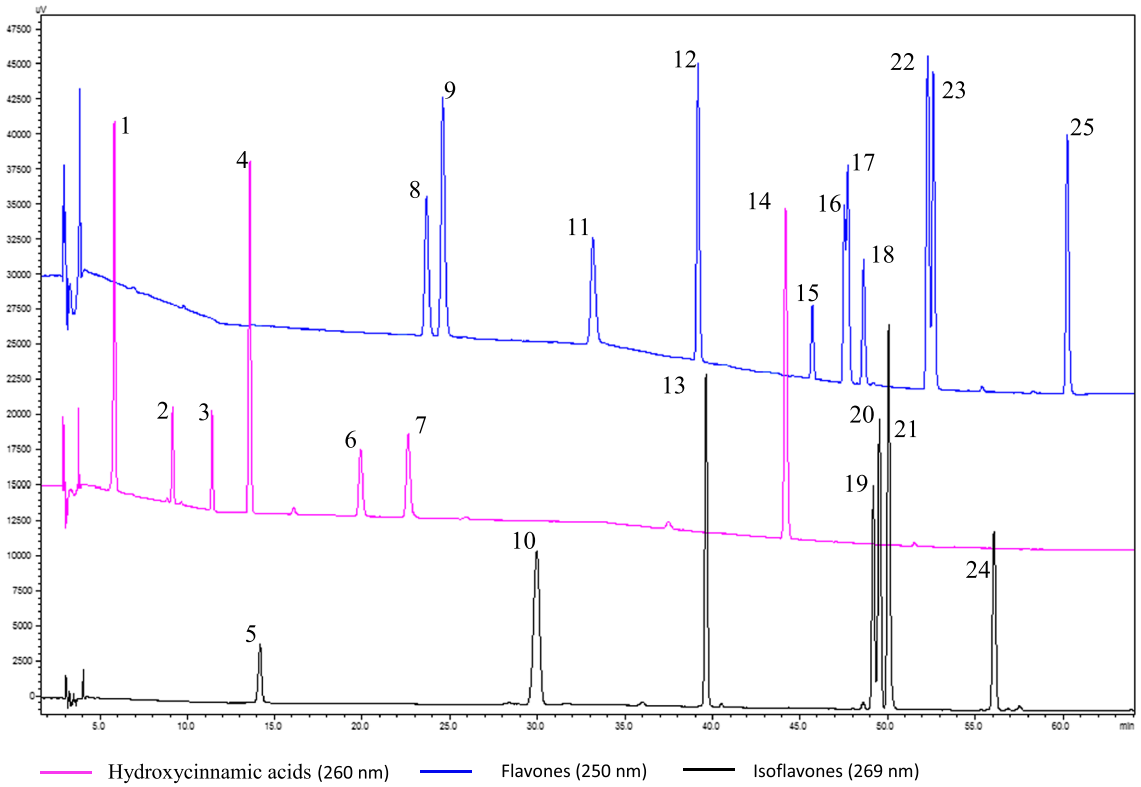


FIGURE 3 HPLC chromatograms of mixed reference compounds: **1**, galic acid; **2**, neochlorogenic acid; **3**, chlorogenic acid; **4**, caffeic acid; **5**, mangiferin; **6**, *p*-coumaric acid; **7**, ferulic acid; **8**, hiperoside; **9**, isoquercetine; **10**, tectoridin; **11**, apigenin 7-glucoside; **12**, daidzein; **13**, nigricin-4′-D-glucoside; **14**, *trans*-cinnamic acid; **15**, genistin 7-glucoside; **16**, apigenin; **17**, 6,7-dihydroisoflavone; **18**, kaemferol; **19**, iristectorigenin B; **20**, nigricin; **21**, irigenin; **22**, 7-hydroxyisflavove; **23**, formononetin; **24**, 5,6-dihydroxy-7,8,3′,5′-tetramethoxyisoflavone; **25**, 5,7-dihydroxy-4′-methoxyisoflavone [Colour figure can be viewed at wileyonlinelibrary.com]

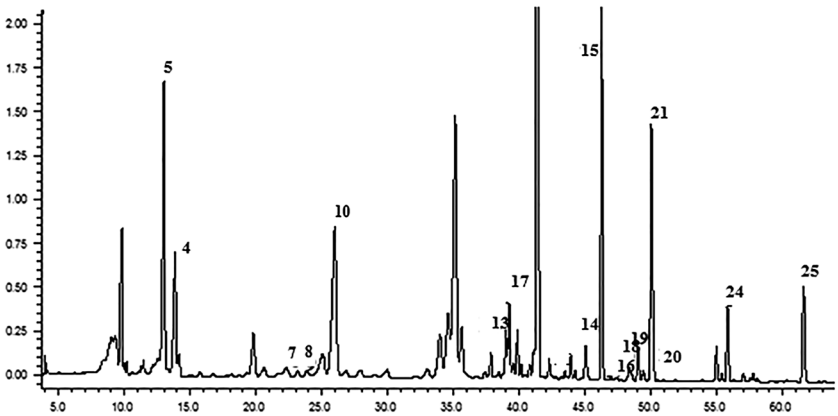


FIGURE 4 Typical chromatogram of *Iris germanica* rhizome (IG15) methanol extract obtained by HPLC separation at 269 mn. Caffeic acid **4**; ferulic acid **6**; *trans*-cinnamic acid **7**; mangiferin **8**; hiperoside **9**; kaemferol **10**; tectoridin **12**; nigricin-4′-D-glucoside **14**; genistein 7-D-glucoside **16**; apigenin **17**; 6,7-dihydroisoflavone **18**; iristectorigenin B **19**; nigricin **20**; irigenin **21**; 5,6-dihydroxy-7,8,3′,5′-tetramethoxyisoflavone **24**; 5,7-dihydroxy-4′-methoxyisoflavone **25**

although the relative peak areas vary considerably. From the number of BACs of *Iris*, we determined the content of flavones, isoflavones, isoflavone glycosides, phenol carboxylic acids and xanthones as the main active compounds. The chromatograms of reference compounds are shown in Figure 3.

The phenol carboxylic acids (gallic acid, neochlorogenic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid and *trans*-cinnamic acid) were determined in all studied *Iris* species in different compositions and quantitative contents. The composition of the acids in the subg. *Limniris* was more diverse and consisted of six acids. Gallic acid was found in seven samples of 16 and it dominated among all the acids recorded in the rhizomes of the subg. *Limniris*. In particular, a high content of gallic acid was recorded in samples of *Iris pseudacorus* from Lake Spindžius (IP8, 0.08 mg/g) and Meškerinė (IP9, 0.07 mg/g). Caffeic acid had the second largest accumulation in *Iris* rhizomes of this subgenus (from 0.01 to 0.2 mg/g). It should be noted that in the

representatives of the subg. *Limniris*, ferulic acid was not detected. Among all the samples of the subg. *Limniris*, the flavonoid hyperoside (0.003 mg/g) was identified only in the *Iris pseudacorus* from Meškerinė (IP9), apigenin-7-glucoside (0.3 mg/g) was found only in *Iris sibirica* from Zakarpatsk (IS13) and formononetin (in trace amounts) was found in *Iris pseudacorus* from Lakajai (IP10). Isoquercetin, kaempferol and 7-hydroxisoflavone were not detected in the samples of the subg. *Limniris*.

Among the phenol carboxylic acids in the samples of the subg. *Iris*, only caffeic, ferulic (0.1–0.2 mg/g) and cinnamic acids (0.02–0.3 mg/g) were identified. It should be noted that, caffeic acid was present in a large quantity (0.1–0.4 mg/g) in all samples of *Iris germanica*. Among the flavonoids, hyperoside, kaempferol, quercetin 3-glucoside (0.9 mg/g in the sample of *Iris germanica* from Zabarauskai (IG14)), apigenin-7-glucoside and apigenin were identified. Isoquercetin was detected in only one sample of *Iris germanica* from Zabarauskai (IG14) and 7-hydroxisoflavone (0.04 mg/g) was found in *Iris germanica* from Lausgeniai (IG16). Plants of the genus *Iris* accumulate particularly diverse isoflavonoids among the secondary metabolites, and in this study, we identified 12 of them (Table 6). The most common isoflavonoids were nigricin-4′-glucoside (found in 13 *Iris* samples), irigenin (in 14 samples) and 5,6-dihydroxy-7,8,3′,5′-tetramethoxyisoflavone (in 12 samples). Irigenin was found nearly in all tested samples and its amount ranged from 0.01 to 2.11 mg/g. Comparison of the content of phenolic compounds revealed that irigenin was predominant in *Iris germanica* (ranged from 1.89 to 2.11 mg/g), while mangiferin was predominant (2.70 mg/g) in *Iris germanica* from Pabradė (Lithuania) (Figure 4). The highest content of genistein-7-glucoside was also found in the sample of *Iris germanica* from Pabradė (IG15, 9.67 mg/g), and slightly less in *Iris sibirica* from Kyiv (IS12) and *Iris germanica* from Zabarauskai (IG14) and Lausgeniai (IG16) (0.3 mg/g, 2.57 mg/g and 3.11 mg/g, respectively).

The glucosyl xanthone mangiferin was identified in seven samples, with an average content ranging from 0.2 to 2.7 mg/g. Species of the subg. *Iris* had a higher content of all compounds compared with subg. *Limniris*. The analysis revealed that the number of phenolic compounds in the methanol extracts of the rhizomes of *Iris pseudacorus* from Latvia and Lithuania was larger than in samples from Ukraine. It should be noted that the amount of the xanthone mangiferin and genistein 7-glucoside in *Iris* samples was the highest compared with other compounds. The dendrograms of the distribution of compounds in subgenera of *Iris* are presented in, Figures S1 and S2 in the Supporting Information. The analysis showed an uneven quantitative distribution of phenolic compounds in the studied *Iris* samples and the differences were significant between and within species as well as sampling sites.

The obtained results correlated with the data of chemical composition of *Iris adriatica*[32] from Croatian, where the compound with the 6,7-methylendioxy group (irisolone, irigenin, iristectorigenin A, nigricin-4′-glucoside) are dominant. However, authors investigation only 6,7-methylendioxy derivatives, benzophenones and some xanthones by ultra-high-performance liquid chromatography photodiode array electrospray ionisation mass spectrometry (UHPLC-PDA-ESI-MS) analysis. According to other authors,[33] investigation of the chemical profile of *Iris humilis*, *Iris pumila* and *Iris variegata* from Serbia by the UHPLC-Orbitrap MS analysis, showed the presence of derivates of iriflophenone, luteolin glycosides, apigenin glycosides, isoflavones derivates of iristectorigenin, irilone. However, not one of the earlier studies reported on the quantitative content of the identified compounds. If we compare the compounds that are considered in previously published works[32-34] and the data obtained, it can be seen that the common identified compounds are only mangiferin, tectoridin, nigricin, nigricin-4′-glucoside and irigenin.

The results showed that *Iris* species from Ukraine, Lithuania and Latvia are characterised by a special pattern of metabolites such as isoflavones, flavonoids, phenol carboxylic acids and xanthones. *Iris* rhizomes also exhibit differences in their metabolic profiles according to the ecological factors. *Iris pseudacorus* was distinguished by the presence of the phenol carboxylic acids (gallic acid, neochlorogenic acid, chlorogenic acid, coumaric acid), while ferulic acid was detected only in *Iris germanica*. At the same time, *Iris germanica* showed a high level of some isoflavones (tectoridin, nigricin, nigricin-4′-glucoside, genistein 7-glucoside, iristectorigenin B, irigenin) in rhizomes. The determination of the phenol carboxylic acids and flavonoids for these *Iris* species was carried out for the first time, and these data are chemotaxonomic significant at the species level. Thus, the obtained data can be used for the genus *Iris* plant chemophenetics studies,[40] which describe the composition of the secondary metabolites in each given taxon.

The discovery in *Iris* raw material of caffeic acid opens up new possibilities in the use of the plant, since it has been found that this acid is effective in reducing cytotoxic damage, and has antioxidant, anti-inflammatory, antimicrobial and antidiabetic effects.[41] Caffeic acid and its derivatives (chlorogenic and neochlorogenic acids) are the most common acids observed in plants.[22,41] Cinnamic acids are involved in the synthesis of flavonoids and isoflavones.

3.5 | Qualitative variation of phenolic compounds in the studied species

Analysis of the detected phenolic compounds in the studied samples revealed that only gallic acid, frequent in the subg. *Limniris*, was absent in the rhizomes of the representatives of the subg. *Iris*. Several other compounds (gallic acid, neochlorogenic acid, chlorogenic acid, coumaric acid, isoquercitin, apigenin-7-glucoside, daidzein, formononetin), that were not recorded in samples of the subg. *Iris* were also recorded only occasionally in species of the subg. *Limniris*. However, ferulic acid, registered in all samples of the subg. *Iris*, was not recorded in species of the subg. *Limniris*. Kaempferol was not registered in the rhizomes of the subg. *Limniris*, but was present in the rhizomes from two populations of the subg. *Iris* (of three sampled populations). Although *Iris germanica* (subg. *Iris*), applying the Bray–Curtis similarity index, nested among other studied species, all its populations composed of a well-defined cluster (Figure 5). It should be noted that *Iris sibirica* and *Iris halophila* nested among *Iris*

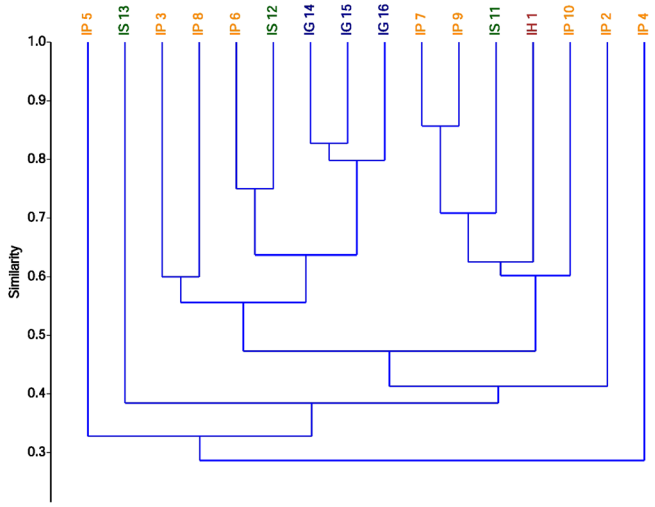


FIGURE 5 Dendrogram of the sampled *Iris* populations according to the diversity of the analysed phenolic compounds applying the Bray–Curtis distance. Abbreviations for populations and species correspond with those presented in Table 1 [Colour figure can be viewed at wileyonlinelibrary.com]

pseudacorus and all sampled populations of the subg. *Limniris* were very diverse both according to the diversity of phenolic compounds and according to their content. The highest similarity index was found between the two populations of *Iris pseudacorus* sampled from aquatic habitats and these populations differed only by the presence of two compounds (caffeic acid and hyperoside). Furthermore, differences among studied populations of *Iris pseudacorus* from Ukraine were much higher than differences among populations of *Iris pseudacorus* from Latvia and Lithuania (Figure 5).

Correlation analysis of the quantity of phenolic compounds grouped in classes revealed statistically significant positive relationships between them (Table 7). The strongest relationships were revealed between the quantity of isoflavonoids and xanthones (r_s = 0.84, P = 0.000) as well as between the quantity of phenol carboxylic acids and xanthones (r_s = 0.75, P = 0.001). Moderate and statistically reliable relationships were found between other pairs of the classes of phenolic compounds (Table 5).

3.6 | Effect of environmental conditions on the quantity of phenolic compounds

PCA based on the quantity of phenolic compounds in classes and meteorological data revealed that meteorological factors [first principal component (PC1)] explain 80.07% of the variation (loadings for duration of the sunshine 0.76; for annual precipitation −0.64) (Figure 6).

Analysis of the effect of soil properties on the accumulation of phenolic compound by classes revealed a strong effect of phosphorus and potassium (Figure 7). The first two principal components explain 52.35% and 43.07% of variation, respectively. In both PC1 and PC2 loading for phosphorus and potassium was equal, i.e. 0.99.

Analysis of the effect of geographical factors (longitude, latitude and altitude) revealed that the first two axes explain 80.57% and 8.94% of the variation (Figure 8). In PC1 loading for the altitude was 0.99, whereas in PC2 loading for the latitude was −0.62 and for the longitude it was 0.68.

3.7 | Relationships with environmental factors

The results of the correlation analysis between the quantity of phenolic compounds in rhizomes of *Iris* species grouped in classes and the analysed meteorological factors were quite unexpected. No reliable correlations were revealed between the quantity of the studied compounds and mean annual temperature as well as between the annual precipitation (Table 8). However, a moderate negative and statistically reliable correlation was found between the quantity of phenol carboxylic acids and duration of the sunshine (r_s = −0.62; P = 0.0110). Furthermore, negative, though weak and not statistically reliable, relationships were found between the duration of the sunshine and other classes of the studied phenolic compounds (Table 8).

An analysis of the relationships between meteorological parameters and quantity of individual phenolic compounds in rhizomes of the studied *Iris* species revealed a few statistically reliable correlations. A significant strong negative correlation was found between the quantity of 5,6-dihydroxy-7,8,3′,5′-tetramethoxyisoflavone and duration of sunshine (r_s = −0.75; P = 0.0009) and a moderate negative correlation was revealed between the quantity of hyperoside and mean annual temperature (r_s = −0.58; P = 0.0189). Moderate and reliable positive correlations were found between the quantity of hyperoside and annual precipitation (r_s = 0.57; P = 0.0190) as well as between the quantity of *p*-coumaric acid and mean annual temperature (r_s = 0.58; P = 0.0188).

No statistically reliable relationships were found between agrochemical properties of the soil and the quantity of phenolic compounds grouped in classes. However, several moderate statistically reliable relationships were found between agrochemical properties of

TABLE 7 Results of Spearman's correlation analysis between the content of phenolic compounds by classes (P values are above the diagonal, r_s values are below the diagonal)

Compound classes	Phenol carboxylic acids	Xanthones	Flavonoids	Isoflavonoids
Phenol carboxylic acids	–	0.001	0.014	0.019
Xanthones	0.75	–	0.013	0.000
Flavonoids	0.60	0.61	–	0.004
Isoflavonoids	0.58	0.84	0.68	–

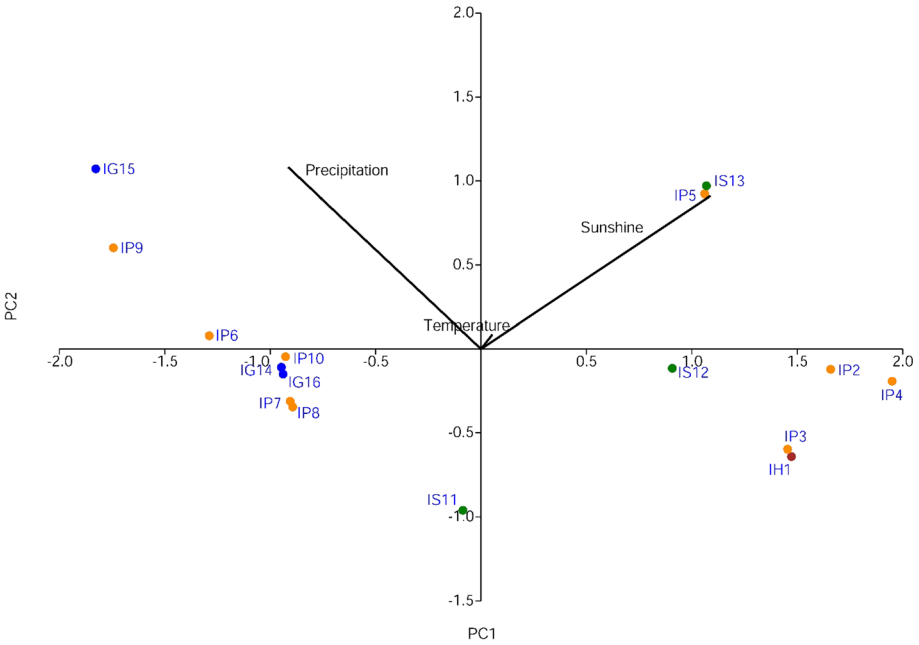


FIGURE 6 Principal component analysis of the studied *Iris* species based on the content of phenolic compounds grouped into classes and meteorological conditions (annual precipitation, mm; duration of the sunshine, hours; mean annual temperature, °C). The first two axes explain 80.07% and 18.63% of the variation, respectively. Abbreviations for populations and species correspond with those presented in Table 1 [Colour figure can be viewed at wileyonlinelibrary.com]

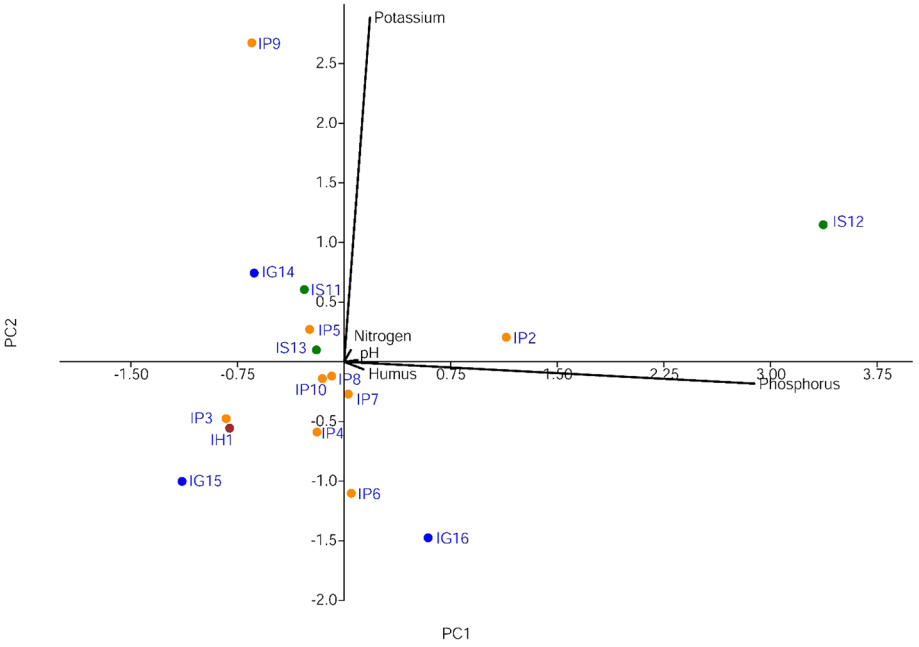


FIGURE 7 Principal component analysis of the studied *Iris* species based on the content of phenolic compounds grouped into classes and agrochemical soil properties (content of potassium, phosphorus, nitrogen and humus, soil pH). The first two axes explain 52.35% and 43.07% of the variation, respectively. Abbreviations for populations and species correspond with those presented in Table 1 [Colour figure can be viewed at wileyonlinelibrary.com]

the soil and the quantity of individual phenolic compounds. A moderate positive correlation was revealed between the quantity of *p*-coumaric acid and content of nitrogen in the soil (r_s = 0.57; P = 0.0209). Moreover, moderate negative correlations were revealed between the soil pH and the quantity of *p*-coumaric acid (r_s = −0.57; P = 0.0210), between the quantity of hyperoside and phosphorus content (r_s = −0.58; P = 0.0196), between the quantity of kaempferol and content of potassium (r_s = −0.53; P = 0.0359) and quantity of 5,6-dihydroxy-7,8,3′,5′-tetramethoxyisoflavone and content of nitrogen (r_s = −0.52; P = 0.0380). It should be noted that the correlation between the content of humus and content of individual phenolic compounds as well as the content of phenolic compounds according to classes was very weak or weak and statistically unreliable.

Statistically reliable relationships were not revealed between the quantity of individual phenolic compounds, as well as between the quantity of phenolic compounds grouped by classes, and geographical location of the rhizome sampling sites (both longitude and latitude) or the elevation of the sampling sites above sea level.

There are several theories (Nutrient Balance Hypothesis, PCM, etc.) that provide different explanations for the change in the concentration of phenolic compounds in plants[20,21,42] depending on the nutrients in the soil. Accelerated biomass growth and a reduction in the release of secondary metabolites based on carbon are observed in plants that receive more nutrients, due to the lack of a carbohydrate substrate.[43] According to Wright *et al.*,[20] nitrogen could have a greater impact on the production of phenolic compounds than

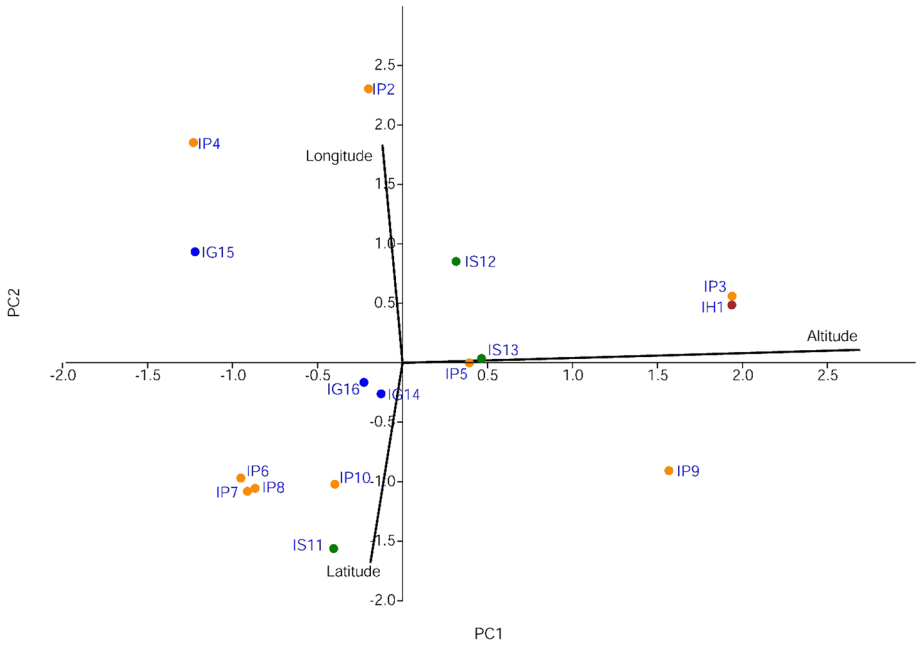


FIGURE 8 Principal component analysis of the studied *Iris* species based on the content of phenolic compounds grouped into classes and the geographical factors (altitude, longitude and latitude). The first two axes explain 80.57% and 8.94% of the variation, respectively. Abbreviations for populations and species correspond with those presented in Table 1 [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 8 Results of Spearman's correlation analysis between the content of phenolic compounds by classes and the analysed meteorological parameters

Compound classes	Mean annual temperature (°C)	Annual precipitation (mm)	Duration of sunshine (h)
Phenol carboxylic acids	r_s = –0,24; P = 0.3661	r_s = 0.46; P = 0.0738	r_s = –0.62; P = 0.0110
Xanthones	r_s = 0.08; P = 0.7747	r_s = 0.21; P = 0.4259	r_s = –0.38; P = 0.1494
Flavonoids	r_s = –0.03; P = 0.8979	r_s = 0.35; P = 0.1853	r_s = –0.38; P = 0.1579
Isoflavonoids	r_s = 0.27; P = 0.3110	r_s = 0.28; P = 0.2924	r_s = –0,47; P = 0.0690

phosphorus availability, since restricting nitrogen reduces protein production and, therefore, competition for phenylalanine. Considering these theories, we decided to analyse how the nutrients of the soil affected the content of phenolic compounds in different *Iris* species.

According to the hypothesis of Wright *et al.*,[20] *Iris* species growing in relatively phosphorus-rich soils should have similar concentrations of the phenolic compounds as *Iris* rhizomes growing in phosphorus-depleted soils. However, according to our results, PCA revealed a significant effect of phosphorus content in the soil on the quantity of phenolic compounds grouped in classes. (Figure 7). The phosphorus content in the soils of studied populations in Lithuania and Latvia was very low (< 50 mg/kg) and low (50–100 mg/kg), however, soils in study sites of Ukraine had a high content (> 200 mg/kg) of phosphorus (Table 3). An especially high concentration of phosphorus in the soil was noted for samples that were cultivated, where fertiliser was added to the soil.[44] Although *Iris pseudacorus*, as well as *Iris sibirica* and *Iris halophila* (subg. *Limniris*), were sampled in different geographical areas, minor differences were found in the soil composition of their habitats.

The potassium content in the soil was quite high (on average, from 97 to 248 mg/kg), due to its high mobility[45] and rapid absorption by the plant. Various researchers have reported that *Iris pseudacorus*, *Iris galatica*, *Iris histrioides*, *Iris sari*, *Iris aphylla* and others prefer soils with a moderate quantity of potassium for biomass formation.[28,29,31,44] We found a significant effect of potassium content in the soil on the quantity of phenolic compounds grouped in classes (Figure 7). *Iris germanica* and *Iris pseudacorus* samples collected from soil with a medium and low potassium content had a more diverse composition of phenolic compounds and their content was higher.

The study of the chemical composition of flavonoids in *Iris* species in connection with the ecology of some species showed that the qualitative composition of compound groups characteristic in plant species growing in different environmental conditions (various forest formations, valleys, height of terrain above sea level, slope exposure), as a rule, does not change. The quantitative content of flavonoids in all studied species varies depending on environmental factors, and fluctuations in the content of compounds are greater when sharper differences in growth conditions are observed. For example, it was found that with an increase in the height of growth above sea level, the number of flavonoids in plants increases (one example of subg. *Iris* for IG16). This study revealed that the contents of phenolic compounds in *Iris* rhizomes was influenced by the soil nutrients. Phosphorus and potassium had a very significant impact, whereas the content of nitrogen was insignificant. Similarly, climatic factors had a small impact, but

the duration of sunshine had a significant positive impact, whereas an increase in precipitation had a significant negative impact.

The qualitative composition of flavonoids has a specific character and is formed over a long time as a result of species phylogenesis. The quantitative content of plant flavonoids is formed during ontogeny under the influence of climatic and edaphic environmental factors and depends on the phase of plant development. The positive or negative influence of one or another factor on the content of flavonoids is a specific feature. This study revealed significant differences between the content of bioactive compounds in the rhizomes of *Iris* subg. *Iris* and *Iris* subg. *Limniris*. In *Iris* species rhizomes, we identified phenol carboxylic acids (caffeic acid, ferulic acid, *trans*-cinnamic acid), glycosides and flavonoid aglycones (hyperoside, kaempferol, apigenin, apigenin-7-glucoside) and glycosides and aglycones of isoflavones (tectoridin, nigricin-4′-glucoside, genistein 7-glucoside, 6,7-dihydroisoflavone, iristectorigenin B, nigricin, irigenin, 5,6-dihydroxy-7,8,3′,5′-tetramethoxyisoflavone, 5,7-dihydroxy-4′-methoxyisoflavone) as well as xanthone (mangiferin). The content of phenolic compounds in the raw material of *Iris* subg. *Iris* was significantly higher compared to *Iris* subg. *Limniris*.

The present study on the effect of environmental factors on the production and accumulation of phenolic compounds in the rhizomes of *Iris* species belonging to two subgenera, i.e. *Iris* subg. *Iris* (*Iris germanica*) and *Iris* subg. *Limniris* (*Iris pseudacorus*, *Iris sibirica* and *Iris halophila*), collected in natural and artificial habitats in Latvia, Lithuania and Ukraine, revealed quite intricate relationships. We found that *Iris germanica* accumulates more diverse phenolic compounds and their quantity tend to be higher than in other studied species. We found positive and statistically reliable relationships between the accumulation of phenolic compounds of all compound subclasses (phenol carboxylic acids, xanthones, flavonoids and isoflavonoids). An analysis of meteorological conditions revealed a positive effect of sunshine duration and a negative effect of the amount of annual precipitation on the quantity of phenolic compounds. However, when the duration of the sunshine was analysed as a separate variable, reliable negative relationships with the quantity of phenol carboxylic acids in rhizomes of *Iris* species were found. Analysis of the effect of soil properties revealed that the content of phosphorus and potassium have a significant positive effect on the quantity of phenolic compounds, whereas content of nitrogen in the soil has no significant effect on the accumulation of the analysed substances. Several statistically reliable moderate relationships were found between the agrochemical properties of the soil and the quantity of individual phenolic compounds in the rhizomes of studied *Iris* species. An analysis of geographical factors revealed that the most significant positive effect on the content of phenolic compounds grouped by classes was the altitude of the locality, whereas latitude had negative effect.

The results of this study suggest that rhizomes of *Iris* species may be an important source of pharmacologically active compounds such as flavonoids, isoflavonoids and xanthones. We conclude that *Iris germanica*, accumulating the largest quantities of phenolic compounds, is the most promising species for further pharmacological studies. Since *Iris pseudacorus* is a widespread and quite common species with large resources of raw material in natural habitats, it is necessary to conduct further studies on the relationships between the accumulation of phenolic compounds and the time of the harvesting the raw material.

Studies on the effect of environmental factors on the production and accumulation of secondary metabolites in *Iris* species are important because they contribute to the knowledge of quantitative parameters of secondary metabolites in medicinal plants and could be employed for the cultivation and harvesting of raw material of *Iris* rhizomes for medicinal purposes.

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Geographical coordinates and elevation above the sea level were identified using global positioning system (GPS) devices (Prestigio GeoVision 5056 and Garmin eTrex Touch 35).

Note: Site number corresponds the number presented on the map in Table 1 and Figure 1

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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