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Free amino acids in *Viola tricolor* in relation to different habitat conditions

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Abstract: The purpose of this study was to establish the free amino acids profile of *Viola tricolor* collected from different habitats in Poland. *Viola tricolor* (heartsease) is a very popular plant found worldwide, classified both as weed and medicinal plant. Based on a validated method, the following nineteen free amino acids were analyzed using liquid chromatography-electrospray ionization coupled to a triple quadrupole mass spectrometer (LC-ESI-MS/MS): alanine, glycine, leucine, valine, isoleucine, proline, phenylalanine, tryptophan, tyrosine, serine, threonine, methionine, asparagine, glutamine, lysine, arginine, histidine, aspartic acid, glutamic acid. The total free amino acids (TAA) ranged from 9938.0 to 11393.8 mg/kg of fresh weight. The variability of the investigated amino acids with respect to different habitat conditions was statistically assessed using the method of discriminant and cluster analysis. Alanine, valine, glutamine and aspartic acid were the most abundant free amino acids present in both localizations. The ratio of total essential amino acids (EAA) to TAA was 0.27 and 0.11 in Zagródki and Wrocław, respectively. Discriminant analysis has demonstrated that the investigated habitats significantly differentiated the free amino acids content of *Viola tricolor*. Only methionine showed a similar concentration in both *Viola tricolor* populations.

Keywords: free amino acids; heartsease (*Viola tricolor*); LC-MS/MS; MANOVA; discriminant analysis.

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1 Introduction

Despite a very intensive development of herbal science, there are hardly any publications concerning weed amino acids (AA) profile. Thus, our objective was to apply a method to detect free amino acids (FAA) using liquid chromatography-electrospray ionization coupled to a triple quadrupole mass spectrometer (LC-ESI-MS/MS) and to establish the free amino acid profile of a very common weed - heartsease (*Viola tricolor*) found in different types of habitat in the Lower Silesia region in Poland. This plant species was selected based on its frequency of occurrence, worldwide habitat, edibility, implications with alternative and modern medicine. Heartsease (*Viola tricolor* L.) from *Violaceae* family is a traditional medicinal plant and has been documented in the Pharmacopoeia of Europe [1,2,3]. Moreover, farmers recognize this plant as a weed as it can spread rapidly, the seeds have excellent adaptation to germination which can lead to a crop yield loss when the weeds compete with crops [4]. Over the past few years a number of herbicides have been discovered which inhibit amino acids metabolism; therefore, studies relating to weed amino acids profile are of paramount importance [5]. Rop et al. [6] observed that flowers of *Viola* x *wittrockiana* possess the highest level of mineral elements among 12 edible flowers that were investigated. *Viola tricolor* could turn out to be a suitable and cheap source of amino acids for consumption, the cosmetic industry, pharmaceutical applications. or medicine. Data exists that show its favorable effect on human health - to cure skin diseases, rheumatic pains, eczema, asthma and respiratory problems. Amino acids participate in the biosynthesis of polyphenols and alkaloids which contribute to antioxidant properties in plants [7,8,9]. It is also worth mentioning, that there are promising projects underway that study cyclotide isolation from *Viola tricolor* and other species from the *Violaceae* family which contain anti-tumur properties [10,11]. Tang et al. [12] isolated 14 cyclotides from *Viola tricolor*, including seven novel cyclotides, using tandem mass spectrometry and NMR spectroscopy, some of which show cytotoxic activities against five cancer cell lines.

Hellinger et al. [13,14] shed light on the unexplored variety of plant-derived cyclotides of the *Violaceae* family and discovered that an aqueous *Viola tricolor* extract contained bioactive cyclotides, with immunosuppressive activity.

Metabolite profiling aimed at analyzing a small number of known metabolites belonging to the same compound classes is one of the three general approaches to analyze small molecules, among metabolic fingerprinting and metabolomics. Tandem mass spectrometry (MS/MS) allows interfering signals to be filtered out after molecular ions are broken into fragments between steps of mass analysis. Mass spectrometry coupled with ultra high-performance liquid chromatography (UHPLC) is a powerful tool enabling accurate and reliable metabolite analysis in a short time [15]. Also, the sample preparation is very important, and the choice of the drying method could be a goal itself when analyzing plants used for herbal medicine. Zhu et al. [16] used UHPLC-TQ-MS coupled with multivariate statistical analysis to characterise amino acids, nucleosides and nucleobases in *Angelicae Sinensis Radix* was obtained under different drying methods.

The evaluation of amino acids as well as other metabolites present in plants grown in different geographical locations has been a subject of interest for different authors [17,18].

Sami et al. [17] found 17 amino acids in the okra plant that were collected from four different regions in Egypt, 11 of which were essential. Authors found that the major amino acid was aspartic acid, which dry weight concentrations were 2.91–4.92 g/100 g. However, the authors did not provide information about the locations; therefore, no conclusions could be drawn between the relation between amino acid profile and habitat conditions. Sun et al. [18] selected 13 wild edible mushrooms species found in China and analyzed their free amino acid composition using reversed phase liquid chromatography The total free amino acid (TAA) content ranged from 1462.6 mg/100 g to 13,106.2 mg/100 g on a dry weight basis. Furthermore, the authors did a principal component analysis and cluster analysis to show that essential amino acids composition and content might be an important parameter in separating the mushroom species.

This work identifies (both qualify and quantify) the composition of 19 FAA in a *Viola tricolor* matrice for the first time, due to important biological functions and a possible value for industry; it also shows free amino acid variability related to different environmental conditions using the discriminant and cluster analysis method.

2 Materials and Methods

2.1 Sampling

Samples of heartsease (*Viola tricolor*) used in this study were collected from a field in Zagródki (A) N50°59' 4.9943" E17°6' 47.3708" and Wrocław (B) N51°4' 40.2254" E17°2' 35.0415 at the end of June 2015. The aboveground biomass was obtained at stage five (flower development), with both stems and leaves. Three specimens per habitat were collected, immediately transported to the laboratory; the samples were frozen and ground using pestle and liquid nitrogen.

2.2 Determination of Free Amino Acids Composition

2.2.1 Sample preparation

The samples (0.5 g) were extracted using LC-MS grade water, followed by a 15 min sonification in an ultrasonic bath. Homogenates were centrifuged at 11000 rpm for 15 min at 4ºC to obtain supernatants. EZ:faast(TM) Free Amino Acid kit (Phenomenex, Torrance, CA, USA) was used for AA analysis. The procedure was performed according to the optimized and validated method presented by Dziągwa-Becker et al. [19]. In short, the procedure consisted of solid phase extraction, where the plant extract was passed through the sorbent tip that bound the amino acids, derivatization using propyl chloroformate and liquid-liquid extraction [20].

2.2.2 Determination of free amino acids by high performance liquid chromatography with electrospray tandem mass spectrometry (LC-MS/MS)

The analysis was conducted using a high-performance liquid chromatograph Shimadzu 8030 (Shimadzu, Kyoto, Japan) with a binary solvent manager, autosampler and column oven. An EZ:faast(TM)4u AAA-MS column, 3 µm, 250 × 2.0 mm (Phenomenex, Torrance, CA, USA) at a flow rate of 0.25 mL min-1 was used for the chromatographic separation. The column temperature was maintained at 35°C. The mobile phase consisted of water/methanol (A/B) gradient both 10 mM ammonium formate where the methanol percentage was changed linearly as follows: 0 min, 68%; 13 min, 83%; 13.01 min, 68%; 18 min, 68%. The abovementioned chromatographic conditions were applied to all analyzed samples. All AAs were analyzed

in a positive ionization mode, showing an abundant $[M + H]^+$ ion for each derivatised amino acid. The sample volume injected into the HPLC system was 1 µL. The tandem mass spectrometer LCMS-8030 (Shimadzu, Kyoto, Japan) with ultra fast polarity switching and ultra fast Multiple Reaction Monitoring (MRM) transitions was used for analysis. Nitrogen was drying as well as nebulising gas, obtained from pressurized air in a N2 LC-MS pump, working at a flow rate 15 L min-1 and 3 L min-1, respectively. The desolvation line temperature was 250°C and the heat block temperature was 400°C. The collision-induced dissociation gas (CID) was argon 99.999% (Linde, Wrocław, Poland) at a pressure of 230 kPa. A dwell time of 10 ms was selected. LabSolution Ver. 5.6 (Shimadzu, Kyoto, Japan) software was used to process the quantitative data. LC-MS grade solvents were used, obtained from Fluka Analytical (St. Louis, MO, USA) [19].

2.3 Soil properties determination

The physico-chemical proprieties of the soils were determined as follows: soil pH was tested potentiometrically in 1 M KCl, potassium and phosphorus content was tested according to the Egner-Riehm method, the granulomertic composition was conducted using the sieve method by Casagrande in Prószyński modification. Granulometric group was specified according to USDA. The organic carbon content was determined according to Tiurin's method, soil organic matter (SOM) was performed on the basis of C_{org} content [21,22] (Table 2).

2.4 Meteorological data

Meteorological data came from two IUNG weather stations. Temperature measurements were made at a standard shelter height (2m) in accordance with established on-site meteorological guidelines (Table 3).

2.5 Data Analysis

Assessing the variability of the free amino acid content in *Viola tricolor* populations in Wrocław and Zagródki, multivariate analysis of variance (MANOVA) and discriminant analysis method was performed using the statistical data analysis program STATISTICA 6 (StatSoft Polska, Poland), laid down by Morrison [23], Caliński and Chudzik [24], Krzysko [25]. The analysis enabled the assessment of the amino acid content in a dimension created by two variables - different habitats. Cluster analysis was conducted using Ward's method to compare the achieved results.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results

3.1 Viola tricolor amino acids profile

All 19 free amino acids were found in *Viola tricolor* samples collected from two locations in Poland - Zagródki and Wrocław. Their concentrations are exhibited in Table 1. The total free amino acids (TAA) content in the analyzed samples ranged from 9938.0 to 11393.8 mg/kg of fresh weight. To the best of our knowledge, this is the first work that shows the presence of 19 FAA belonging to the group of 23 proteinogenic amino acids. Plants can synthesize all proteinogenic amino acids. Among them. 9 amino acids are known to be essential, meaning humans can them from their diet. All essential AA are present in *Viola tricolor* and their content amounts of 2703.9 and 1202.3 mg/kg in Zagródki and Wrocław respectively. The ratio between EAA and TAA was 0.272 and 0.106 for Zagródki and Wrocław, respectively. As shown in Table 1, Figure 1 and 2, it was possible to determine all t19 free amino acids: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, asparagine, andglutamine.

3.2 Habitat conditions

Both soils originated from farmland. The soil from Wrocław was slightly acidic with light texture and classified as sandy loam (USDA Soil Texture Calculator https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/survey/?cid=nrcs142p2_054167). This soil that was very high in phosphorus, potassium and Soil Organic Matter (SOM) resulted from intensive cultivation and fertilization. Also, the pH level was controlled by intensive limning. Sandy loam soils are susceptible to over-drying.

The soil from Zagródki had a high content of clay fraction and was classified as sandy clay loam [26]. This type of soil is not intensively cultivated and has lower phosphorus and potassium content than Wrocław, but a slightly higher SOM content and similar pH level. Soils with sandy clay loam texture are susceptible to poor drainage and gley properties (Table 2).

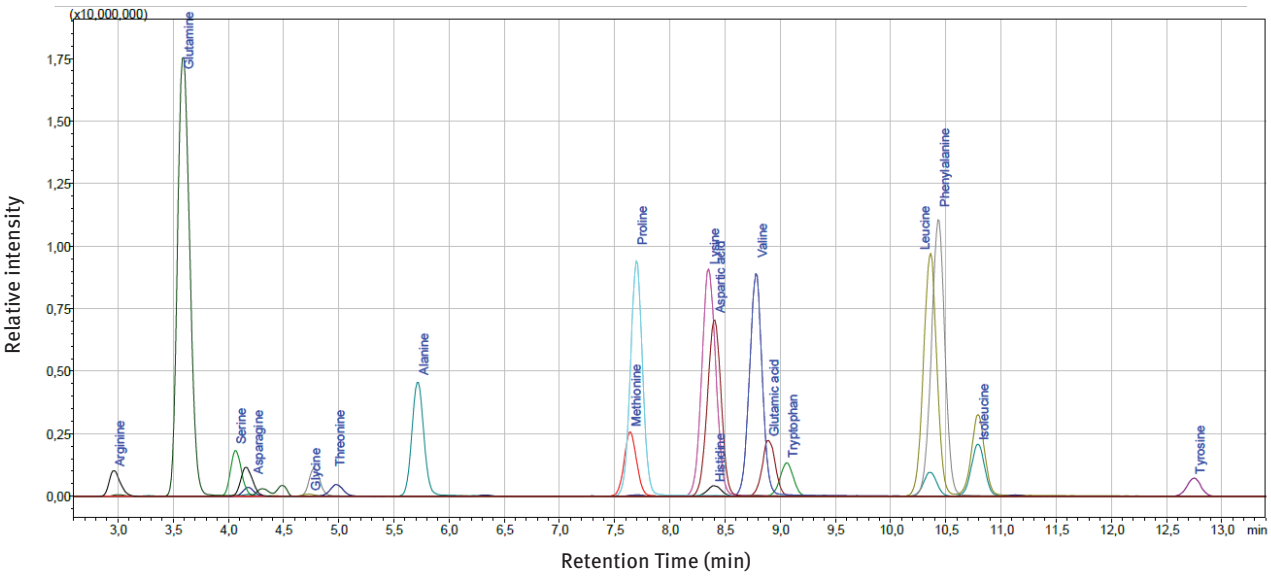

Figure 1: A chromatogram of free amino acids in *Viola tricolor.*

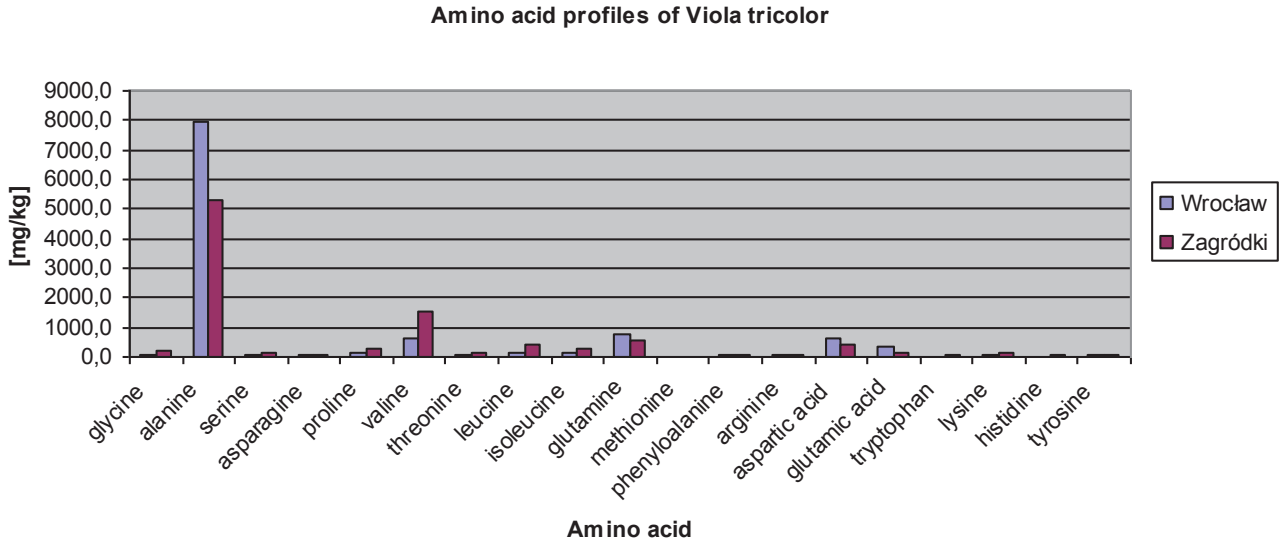

Figure 2: Amino acids profiles of *Viola tricolor.*

The second quarter of 2015 was warm, with high precipitation levels. Especially in June, there was heavy rain (Table 3).

4 Discussion

4.1 Amino acids profile

Ala is the most abundant AA found in both habitats. Its ratio to the TAA content was 0.535 and 0.700. The divergence of FAA content in *Viola tricolor* from two locations can be a result of many conditions interfering, co-affecting and interchanging the AA profile. Among them are the use of pesticides, weather conditions, soil properties, microorganisms, harvesting time and growth conditions. No one single factor can change the amino acids profile, but more importantly, the group of interrelating factors can lead to enormous alterations. Furthermore, *Viola tricolor* has a tendency to crossbreed with other species from the *Violaceae* family and form hybrids [27]. Therefore, the different AA profile from two habitats can be a proof of genetic variability.

4.2 Statistical Analysis of Variability

The variability of the investigated amino acids with respect to different habitat conditions was statistically assessed using the methods of discriminant and cluster analysis. Statistical analysis showed significant differences among the free amino acid content in the investigated habitats (Table 1). Only Met showed similar concentrations in both *Viola tricolor* populations. The majority of amino acids showed a higher concentration in Zagródki. Only the content of Ala, Gln, Glu and Asp was higher in Wrocław habitat in comparison to Zagródki. A multidimensional analysis of variance (MANOVA) showed a significant differentiation of free amino acids in *Viola tricolor* in relation to different habitats (Table 4).

Wilk's lambda distribution for the total discriminant, calculated as the ratio of determinant matrix variance and intra-group covariance to determinant matrix variance and total covariance shows that the hypothesis of centroid equality of the two habitats should be rejected at p<0,0001 significance level. Partial Wilk's lambda and F test results indicate that the Zagródki habitat affected the amino acids variability more than the Wrocław habitat; however, in Wrocław the differences between AA were also significant. Table 5 shows squared Mahalanobis distances between two amino acids formed by two habitats. The Mahalanobis distance is similar to the standard Euclidean distance, however it additionally shows the correlation between the two variables. The larger the distances shown in the table, the farther the amino acids are located and also the bigger discriminant power has the proposed model in order to differentiate the examined amino acids.

Mean concentration values of the investigated amino acids from two habitats varied significantly in most of the cases. Insignificant Mahalanobis distances were found between Asn and Phe, Phe and Arg. No significant differences were calculated between Arg and Asn, Phe, His and Tyr. Considerable similarities were found between the concentration of His and Tyr in the investigated locations. Cluster analysis was performed to compare the concentration of 19 AA in the dimension formed by two habitats. The closer the location of AA, the higher similarity between groups of the investigated plants. While analyzing the aggregation of the control subject, four clusters could be distinguished (Figure 3).

The first cluster was formed by 10 amino acids - Gly, Thr, Ser, Lys, Asn, Phe, Met, Trp, Arg and His. They were characterized by a significant Euclidean distance in comparison to the second cluster formed by four amino acids - Pro, Ile, Leu and Glu. The two clusters were in a considerable distance from the third cluster, formed by Val,

Table 1: Content (mg/kg) of Free Amino Acids in *Viola tricolor*.

Amino acid number	*Viola tricolor* Zagródki [mg/kg]	*Viola tricolor* Wrocław [mg/kg]	standard deviation Zagródki	standard deviation Wrocław
1. Gly	185.7**a**	90.0**b**	0.41	8.96
2. Ala	5318.7**a**	7979.2**b**	15.77	19.39
3. Ser	107.1**a**	72.8**b**	2.81	10.22
4. Asn	84.1**a**	72.2**b**	0.52	5.42
5. Pro	291.5**a**	153.8**b**	0.21	12.35
6. Val	1565.8**a**	654.4**b**	7.02	5.61
7. Thr	134.4**a**	104.6**b**	3.15	3.74
8. Leu	391.4**a**	148.4**b**	2.57	25.94
9. Ile	281.2**a**	106.3**b**	2.03	5.65
10. Gln	542.9**a**	773.5**b**	3.33	13.95
11. Met	16.8**a**	15.2**a**	0.33	2.85
12. Phe	84.6**a**	52.5**b**	1.63	10.52
13. Arg	73.7**a**	42.7**b**	5.28	8.68
14. Asp	418.6**a**	654.5**b**	4.49	12.49
15. Glu	144.0**a**	314.5**b**	0.94	3.06
16. Trp	36.7**a**	30.8**b**	0.15	0.70
17. Lys	125.5**a**	56.9**b**	1.49	7.86
18. His	67.4**a**	33.1**b**	0.53	2.13
19. Tyr	67.9**a**	38.2**b**	0.24	6.12
TAA	**9938.0**	**11393.8**		
EAA	**2703.9**	**1202.3**		
EAA/TAA	**0.272**	**0.106**		

Gly: glycine; Ala: alanine; Ser: serine; Asn: asparagine; Pro: proline; Val: valine; Thr: threonine; Leu: leucine; Ile: isoleucine; Gln: glutamine; Met: methionine; Phe: phenylalanine; Arg: arginine; Asp: aspartic acid; Glu: glutamic acid; Trp: tryptophan; Lys: lysine; His: histidine; Tyr: tyrosine. EAA - essential AA were calculated as the total content of Val, Thr, Leu, Ile, Met, Phe, Trp, Lys, His
TAA - total AA content a,b - heterogeneous groups

Table 2: Physicochemical properties of the tested soils.

Soil	Location	pH [1M KCl]	SOM [%]	P_2O_5 [mg/ 100g soil]	K_2O [mg/100 g soil]	Texture of soils [%] 2000-50 µm	50-2 µm	< 2µm	Texture classes/granulometric group USDA [by PTG 2008]
A	Zagródki	**5.2**	**3.2**	**24.6**	**18.5**	**48**	**25**	**27**	**Sandy clay loam**
B	Wrocław	**5.8**	**3.01**	**52**	**21**	**71**	**24**	**5**	**Sandy loam**

Table 3: Meteorological data.

Soil	Localisation	average temperature [°C]			precipitation totals [mm]		
		April	May	June	April	May	June
A	Zagródki	8.9	13.4	16.6	14.7	19.5	12.7
B	Wrocław	9.02	13.5	16.7	11.8	28.5	54.8

Table 4: Discriminant analysis results.

Wilk's lambda = 0,00001; approximate F = 3335,8 p<0,00001				
Habitat	**Wilk's lambda**	**Partial Wilk's lambda**	**F**	**Level p**
Zagródki (A)	0,004279	0,000089	23191,85	p<0,00001
Wrocław (B)	0,000010	0,038364	51,52	p<0,00001

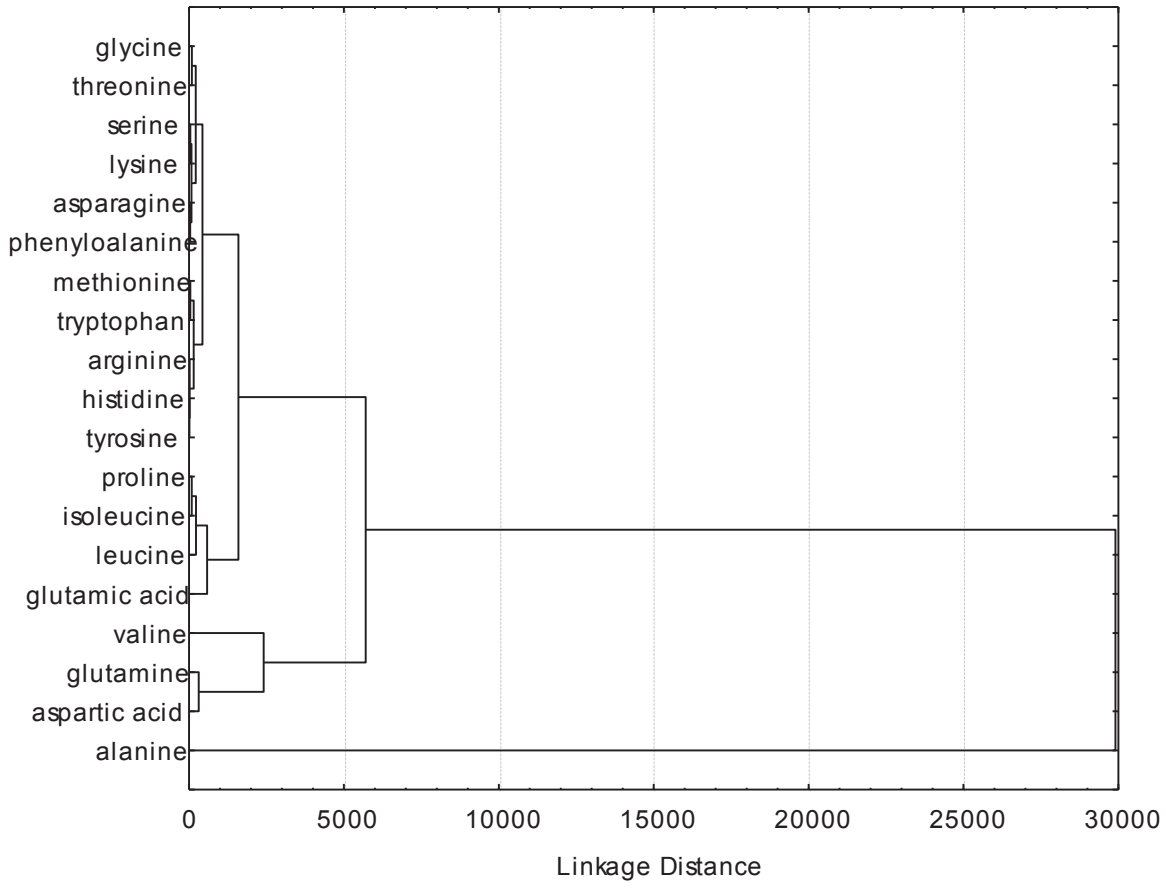

Figure 3: Tree diagram of the cluster analysis for *Viola tricolor* from 2 habitats.

Gln, Asp. Interestingly, Ala formed the fourth one-element cluster. Ala was present in a far bigger concentration compared to the other analyzed compounds. The tree diagram shows the Euclidean distances between the AA in two dimensional spaces. However, it does not include the correlation between the two habitats (for example similar temperature and precipitation range during *Viola tricolor's* vegetation). Therefore, some of the Mahalanobis distances do not illustrate the distance of each AA in the Euclidean space. However, there is some tendency that

Table 5: Squared Mahalanobis distances between free amino acids in two environment.

nr Amino acid	1	2	3	4	5	6	7	8	9
1	0	4640289	1022	1705	1885	317832	425	7018	1503
2	4640288	0	4779016	4819793	4455156	2529919	4729467	4286503	4474848
3	1022	4779016	0	87	5684	354906	129	13398	5004
4	1705	4819793	87	0	7174	366086	427	15640	6408
5	1885	4455156	5684	7174	0	270763	4100	1629	22
6	317832	2529919	354906	366086	270763	0	341499	230391	275626
7	425	4729467	129	427	4100	341499	0	10897	3526
8	7018	4286503	13398	15640	1629	230391	10897	0	2026
9	1503	4474848	5004	6408	22	275626	3526	2026	0
10	22874	4011595	33562	37059	11631	170256	29528	4566	12658
11	4773	4942634	1377	773	12657	400503	2350	23367	11632
12	1703	4819726	86	0,01	7171	366063	427	15635	6405
13	2098	4839652	191	20	7960	371570	634	16790	7151
14	9959	4220363	17357	19896	3184	215359	14493	272	3733
15	221	4703631	302	710	3382	334631	38	9703	2864
16	3707	4906239	836	384	10879	390190	1622	20927	9930
17	610	4747305	53	275	4640	346295	17	11767	4028
18	2344	4851187	270	51	8434	374770	773	17475	7601
19	2321	4850137	263	48	8390	374479	760	17412	7560

	10	11	12	13	14	15	16	17	18	19
1	22874	4773	1703	2098	9959	221	3707	610	2344	2321
2	4011595	4942634	4819726	4839652	4220363	4703631	4906239	4747305	4851187	4850137
3	33562	1377	86	191	17357	302	836	53	270	263
4	37059	773	0,01	20	19896	710	384	275	51	48
5	11631	12657	7171	7960	3184	3382	10879	4640	8434	8390
6	170256	400503	366063	371570	215359	334631	390190	346295	374770	374479
7	29528	2350	427	634	14493	38	1622	17	773	760
8	4566	23367	15635	16790	272	9703	20927	11767	17475	17412
9	12658	11632	6405	7151	3733	2864	9930	4028	7601	7560
10	0	48535	37053	38819	2648	27520	44989	30953	39858	39763
11	48535	0	774	542	28511	2962	67	1970	427	437
12	37053	774	0	21	19892	710	385	274	51	48
13	38819	542	21	0	21191	971	228	445	7	6
14	2648	28511	19892	21191	0	13095	25809	15497	21961	21890
15	27520	2962	710	971	13095	0	2137	105	1141	1125
16	44989	67	385	228	25809	2137	0	1309	155	161
17	30953	1970	274	445	155497	105	1309	0	526	551
18	39858	427	51	7	21961	1141	155	562	0	0,01
19	39763	437	48	6	21890	1125	161	551	0,01	0

- no significant difference p=0,05; nr amino acid see table 1

can be observed. In Table 5 as well as in the tree diagram Ala is far distant from the other AA. The insignificant differences in the content of Asp and Phe as well as in Tyr and His are confirmed by Mahalanobis distances and by the tree diagram.

5 Conclusions

Plants grown in both habitats contained all of the 19 analyzed amino acids, some of which had significant concentrations, like Ala, Val, Asp and Gln. It cannot be unequivocally determined whether the results of the study on the influence of habitat conditions on the amino acids composition of *Viola tricolor* are permanent. Therefore, it is necessary to continue the research.

1. Discriminant analysis demonstrated that the investigated habitats significantly differentiated the free amino acids content of *Viola tricolor*. Only Met showed similar concentration in both *Viola tricolor* populations.
2. Zagródki habitat is far more diverse with regard to free amino acid content in comparison to Wrocław.
3. No significant difference with regard to free amino acid content in *Viola tricolor* was found between Asn and Phe, Tyr and His and also between Tyr and Arg.
4. Ala is by far the most abundant amino acid in *Viola tricolor*.
5. *Viola tricolor* is prone to genetic variability and FAA content can be one of the evidence.

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Conflict of interest: Authors state no conflict of interest.

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