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Effect of Abiotic Factors on Nectar Quality and Secretion of Two Early Spring Species, *Galanthus nivalis* L. and *Helleborus niger* L.

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Abstract: Floral nectar is mainly a reward in the form of food for pollinators. In early spring, when snow can still be present, pollinators have trouble finding food. The composition and productivity of nectar in flowers play an important role in a pollinator's life. It is known that low temperatures and lower humidity cause lower nectar secretion. Some studies have also shown that the quality of nectar can differ because of lower temperatures. In our research, we analysed whether abiotic factors affect nectar secretion, as well as the nectar composition of the early spring plant species *Galanthus nivalis* L. and *Helleborus niger* L. in February 2024. The study was conducted in two locations in nature. Nectar from *H. niger* was sampled in Tomišelj, Slovenia, whereas nectar from *G. nivalis* was sampled in Ljubljana, Slovenia. On four different days at three different times of day, we sampled nectar from flowers using microcapillaries. In total, we sampled 48 nectar samples from one species. We analysed soil humidity and temperature, air temperature and humidity, and UVB radiation. Our results show that nectar productivity is highest in the morning for both species. *H. niger* has sucrose-dominant nectar, while *G. nivalis* has hexose-dominant nectar. Proline, which is an important amino acid for bees, has the highest level in both species, as does the phenolic compound rutin. Environmental factors do affect nectar secretion. Soil and air temperature affect *G. nivalis* nectar secretion, while soil humidity affects *H. niger* nectar secretion. Soil and air temperature also have an effect on higher levels of sugars in both researched nectars. UVB, air humidity, and air and soil temperature seem to have an effect on phenolic compounds, but abiotic factors do not affect amino acids.

Keywords: amino acids; environmental factors; *Galanthus*; *Helleborus*; nectar composition; nectar secretion; phenolic compounds; sugars

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1. Introduction

Plants provide food for pollinators, mainly in the form of pollen and nectar, which attracts pollinators and provides those nutrients [1–6]. The species *Galanthus nivalis* L. and *Helleborus niger* L. are early spring species and it has been known that they are visited by different pollinators. The reason for those visits depends on flower morphology as well as nectar and pollen content [7–9].

Nectar is secreted from nectaries [10] and contains water, sugars, amino acids, phenols, organic acids, pigments, essential oils, vitamins, minerals, lipids, and terpenoids. Alkaloids, non-protein amino acids, antioxidants, and other secondary compounds [11,12] can make nectar addictive to pollinators and/or have a repellent effect on certain pollinators [9] and can enable self-healing or function as deterrents for pollinators [13]. When studying nectar characteristics, we must not generalise that larger flowers are the ones with higher quality nectar. Even the nectar sampling can vary as it is influenced by the following factors: pollinator visits, abiotic factors and nectar characteristics [4,14–16]. Nectar quality varies

between different plant taxa, but similarities can also be found between unrelated species if they grow in the same conditions [17]. Otherwise, nectar quality and secretion can vary within a species, among populations, or even between individual plants and flowers on one plant [18,19]. Those variations can be affected by different abiotic factors, such as air humidity and temperature, soil humidity and temperature [18], time of day [19], and evaporation, particularly for more open flowers [11,15,16,20].

Different pollinators visit particular flowers, since each pollinator prefers different nectar chemistry Sugars, as a source of energy, have the biggest impact on pollinators' choice [21]. The three main sugars found in nectar are sucrose, fructose, and glucose. Sucrose is a disaccharide and fructose and glucose are monosaccharides, and in this article, we mostly call those two compounds together as hexoses. Glucose and fructose are produced from sucrose in the phloem sap or by synthesis within the nectaries with transglucosidase and transfructosidase [11]. In nectar, we can also find other sugars in lower concentrations, such as mannose, arabinose, xylose, maltose, and melibiose and even lower concentrations of sugars such as raffinose, stachyose, and melezitose. Sorbitol—sugar alcohol—has also been found [11,22]. Based on the ratio between the three main sugars found in nectar, we can divide nectar into four types: sucrose-dominant, sucrose-rich, hexose-rich, and hexose-dominant [23]. Different pollinators choose different nectars. Honey bees prefer higher sugar content—more concentrated nectar [24,25] with higher sucrose levels [23,26]. Moths and butterflies prefer nectar with high sucrose content but less concentrated nectar, while flies and bats prefer nectar with high hexose content [23]. Nectar concentration is the main reason why pollinators choose different nectar. Pollinators have different mouths as well as different drinking techniques. Bees are viscous dippers and hummingbirds and butterflies are suction feeders [23,25]. Nectar also contains other compounds. These are mainly alkaloids, non-protein amino acids, etc. [27]. Other important compounds are amino acids. The evolutionary and ecological significance of their presence in nectar are still being studied. The amount of amino acids in nectar is estimated to be less than 5% of organic matter [28,29]. Amino acids play an important role in bee development, especially in the early stages. In nectar, the most abundant amino acid is proline, which is the dominant amino acid in the hemolymph of insects. High concentrations of proline have been found in nectar, as it constitutes as much as 45–60% of all amino acids. Nectar also contains large amounts of aromatic amino acids such as phenylalanine and tyrosine, amides such as asparagine and glutamine, and serine [28]. In their study, Baker and Baker [5] found amino acids in almost all 266 nectar samples from various species. The twenty most commonly found amino acids in nectar were found in many different plant species, suggesting that essential amino acids are an important resource for pollinators [11]. Nectar can also contain phenols or phenolic compounds, which are also quite common and can make nectar toxic and have a repellent effect for some foragers [12,30].

Since nectar composition affects the preferences of specific pollinators, our research examined the nectar composition of two early spring melliferous plant species: *G. nivalis* and *H. niger*. We analysed the nectar secretion and composition of three main sugars and some amino acids and phenolic compounds in relation to environmental factors such as UV-B radiation, soil temperature and humidity, and air temperature and humidity. These two species are abundant during the late winter and early spring seasons and serve as important food sources for both wild bees and honey bees. By understanding the nectar sugar composition of these plants, we can determine if those species are important for pollinators during the early part of the year. Additionally, we can see how those selected environmental factors affect nectar chemistry.

2. Materials and Methods

2.1. Researched Plant Species

We chose two late winter/early spring flowering species that are common in Slovenia in all phytogeographic regions. *H. niger* is perennial and has white flowers. When flowers are pollinated, they obtain a red-pink colour [31,32]. All the plant species in the *Helleborus*

genus have very distinct nectaries, transformed from petals (Figure 1). Noticeable white flower leaves, which resemble petals, are sepals [7,33]. The nectaries are green and can also photosynthesise [7]. *H. niger* grows in bright forests, scrubby slopes, and stony grasslands from the lowland to the subalpine zone, flowers from January to April [33], and in some places even starts to grow in November. It grows on limestone or dolomite lime. In Slovenia, this species is protected [31,32].

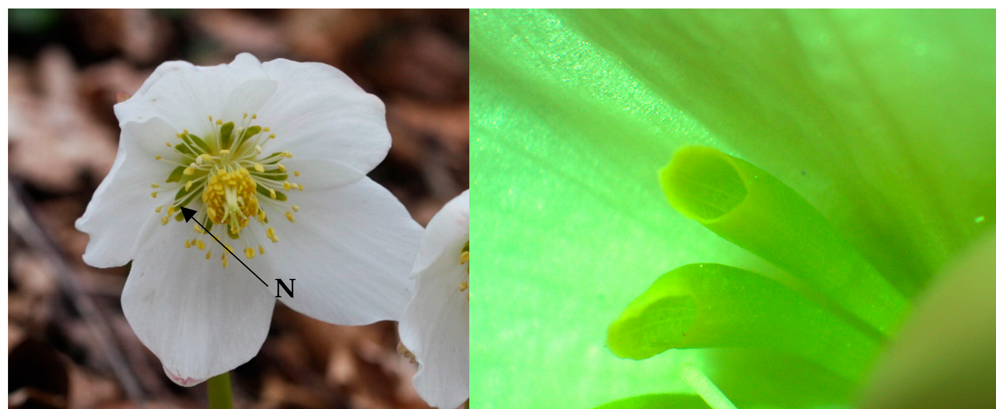

Figure 1. An *H. niger* flower with visible nectaries (N).

G. nivalis is also perennial and has a stem with one flower that is facing down [33]. Sometimes, it is possible that there are two or three flowers [34]. It can be reproduced by bulbs that reproduce in a Fibonacci sequence. In a few years, you can have more plants together in one clump [35]. This species also plays an important role in bees' lives. It is known that this species has a lot of pollen, but it is not so known for its nectar. Few studies have discussed nectaries and nectar in the *Galanthus* genus. Some older studies reported [36] that nectar is secreted from a nectary pad on the top of an inferior ovary and that *Galanthus* has two types of nectaries: one on the inside of perigon flowers (epidermal cells) (Figure 2) and the other in the shape of a disc on the flower base (Figure 3). Otherwise, the latest studies only confirmed one type. On the top of the inferior ovary, between the base of the perigon leaves and the style, the nectariferous layer forms a bright area in contrast with a green receptacle fused with the ovary [34,36]. *G. nivalis* grows in forests, on forest edges, between bushes, and on meadows from the lowlands to the lower mountains. It flowers from December to May and it is a protected species [33,35].

Figure 2. *G. nivalis* nectar and possible place for nectaries under a microscope.

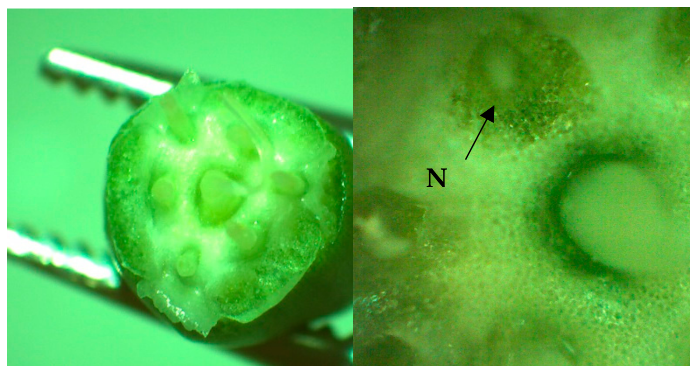

Figure 3. *G. nivalis* nectaries (N) under a microscope.

2.2. Sampling Locations

We sampled *G. nivalis* nectar in the University Botanic Gardens, Ljubljana, Slovenia, from bushes and meadows, and *H. niger* nectar in the forest at Tomišelj, Slovenia. Tomišelj is a small town near Ljubljana with similar climatic conditions as Ljubljana. Both locations are natural, which also means that the population of *G. nivalis* in the garden is a native one. Nectar was first sampled from January to March 2023. Those data were only used for knowledge about the flowering phase (the best time to collect nectar). For data used in this article, we collected nectar in February 2024. The average air temperature was 7.6 °C, while the average amount of precipitation was 58.5 mm, with 0 days of snow cover. Slovenia combines alpine, continental, and Mediterranean climates, while Ljubljana has a moderate continental climate [37].

2.3. Nectar Sampling

Before we sampled the flowers, we needed to cover the flowers with a light blanket to prevent insects' visits a day before. We sampled nectar at three different times within one day (9 a.m., 12 noon, 3 p.m.). We chose flowers at the same flowering stage. Based on previous field observations from last year and some previous studies conducted by other researchers [8,36], we used closed *G. nivalis* flowers and *H. niger* with open flowers. We sucked nectar with microcapillaries from one flower of *H. niger* per sample, and for *G. nivalis*, we used flowers that grew from the same divided bulb. On each day, we chose four plants and we collected nectar from the same plants all day. For both species, we repeated sampling four times within one month, but every other day, we chose four other plants, since the previous ones were not in the same growth phase all month. We used the same sampling method for both species—nectar sucking with microcapillaries, where nectar is drawn up using capillary action [10]. This way, the flowers were not damaged by our repeatable sampling, and there was less contamination. For nectar sampling, we used 1 µL microcapillaries (Vitrex Medical, Herlev, Denmark). For each plant species, we collected four samples at each chosen time. This included 48 samplings for each species on 16 different specimens. Latex gloves were worn when handling microcapillaries. After sampling, the nectar samples were put into the refrigerator at −20 °C. In the field, we also measured the height of the nectar in the microcapillary and calculated the nectar volume.

2.4. Abiotic Factors Measurements

For each sampling hour, we measured abiotic factors (temperature and moisture of the soil, temperature and moisture of the air, and UV-B radiation). Soil parameters were measured at a depth of 7 cm (the length of the sensors of the measuring instrument), and air parameters were measured at the height of the flowers of the sampled species. For measuring soil humidity and temperature, we used a 3001-SCY-PT device (JXCT, Weihai, China); for air humidity and temperature, we used an Onset HOBO® MX2302A device (Bourne, MA, USA); and for measuring UVB (ultraviolet) radiation, we used a Solarmeter

device, serial number 07564 (Glenside, PA, USA). Since the devices measured relative humidity, we converted the relative humidity data to absolute humidity.

2.5. Nectar Analysis

After some time, the frozen nectar samples were thawed and dissolved. We dissolved the samples in 150 µL of distilled water per sample in a 2 mL centrifuge tube. Centrifuged samples were then placed in a centrifuge three times for 1 min each. After the final centrifugation (3 min), the supernatant was transferred to an HPLC vial for further analysis.

2.5.1. Sugar Analysis

Stock solutions of three main sugars (Merck Millipore, Darmstadt, Germany) were prepared by dissolving 100 mg of each sugar in 10 mL of deionised water (MilliQ, Merck Millipore). The stock solutions were then combined and diluted in order to obtain a working standard solution of 1 mg/mL for each sugar. Properly diluted nectar samples were analysed with a Vanquish (Thermo Scientific, San Jose, CA, USA) UHPLC system coupled with a charged-aerosol detector (CAD) and data acquisition software Chromeleon 7.2 SR4 (Thermo Scientific). The separation column was Nucleogel Sugar Ca with dimensions 300 mm × 6.5 mm i.d. (Macherey-Nagel, Düren, Germany) with the temperature set to 90 °C. The mobile phase was water under isocratic conditions and the flow rate was 0.7 mL/min. The CAD detector source temperature was 90 °C. The analysis run time was 13 min. Sample vials were thermostatted at 10 °C. The flush solvent was water. Injection volumes were 5 µL and 15 µL for standard and sample solutions, respectively. For more information about the descriptive statistics of the sugar analysis method see Table 1.

Table 1. Descriptive statistics of the sugar analysis method.

Analyte	Repeatability (% RSD; n = 5)	Reproducibility (% RSD; n = 3)	LOD (µg) *	LOQ (µg) *	Linearity Range (µg *; r)
Sucrose	2.24	3.63	0.17	0.56	0.6–15; 0.9999
Glucose	3.07	4.73	0.22	0.72	0.7–15; 0.9998
Fructose	3.46	4.02	0.25	0.82	0.8–15; 0.9994

* The values refer to the analyte amount injected onto the column; LOD—limit of detection; LOQ—limit of quantification.

2.5.2. Phenolic Compound Analysis

Stock solutions of phenolic analytes (chlorogenic acid, rutin, quercetin, quercitrin, isoquercitrin and hyperoside; Extrasynthese, Genay, France) were prepared by dissolving 5 mg of each standard in 10 mL of methanol (Merck, Darmstadt, Germany). The stock solutions were then combined and diluted with 50% (*v*/*v*) methanol in order to obtain a working standard solution of 20 µg/mL for each analyte. Properly diluted nectar samples were analysed with a Vanquish (Thermo Scientific, San Jose, CA, USA) UHPLC system coupled with a UV detector (detection wavelengths set at 330 nnm and 360 nm) and data acquisition software Chromeleon 7.2 SR4 (Thermo Scientific). The separation column was Hypersil Gold C18 with dimensions 100 mm × 2.1 mm i.d., 3 µm particle size (Thermo Scientific), with the temperature set to 40 °C. Solvents for gradient elution were water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The elution program consisted of isocratic elution from 0 to 7 min at 10% solvent B, then gradient elution from 7 to 14 min from 10% to 60% solvent B. Column conditioning consisted of isocratic elution from 14.1 min to 19 min at 10% solvent B. Flow rate was constant at 0.25 mL/min. The analysis run time was 19 min. Sample vials were thermostatted at 10 °C. The flush solvent was water with 10% methanol (*v*/*v*). Injection volumes were 5 µL and 10 µL for standard and sample solutions, respectively. For more information about the descriptive statistics of the phenolic compound analysis method see Table 2.

Table 2. Descriptive statistics of the phenolic compound analysis method.

Analyte	Repeatability (% RSD; n = 5)	Reproducibility (% RSD; n = 3)	LOD (ng) *	LOQ (ng) *	Linearity Range (ng *; r)
Chlorogenic acid	0.45	0.72	1.1	3.6	3.6–250; 0.9999
Rutin	0.29	1.64	1.4	4.9	4.9–250; 0.9999
Quercitrin/isoquercitrin	1.38	4.94	1.9	6.6	6.6–250; 0.9998
Hyperoside	1.47	4.21	2.2	7.2	7.2–250; 0.9995
Quercetin	0.76	3.27	3.7	12.2	12.2–250; 0.9997

* The values refer to the analyte amount injected onto the column; LOD—limit of detection; LOQ—limit of quantification.

2.5.3. Amino Acid Analysis

An amino acid standard solution was purchased from Sigma Aldrich (St. Louis, MO, USA), containing 10 µg/mL of each amino acid. Properly diluted nectar samples were analysed with an Accela 600 (Thermo Scientific, San Jose, CA, USA) HPLC system coupled with a TSQ Quantum mass spectrometer with an electrospray ion source (Thermo Scientific) and data acquisition software Xcalibur 2.1 (Thermo Scientific). Detection was performed in single ion monitoring, positive ionisation mode. For a given set of amino acids analysed, m/z values of M+1 were monitored and quantified. The separation column was Hypercarb with dimensions of 50 mm × 2.1 mm i.d., 3 µm particle size (Thermo Scientific), with the temperature set to 55 °C. Solvents for gradient elution were water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B). The elution program consisted of isocratic elution from 0 to 2 min at 100% solvent A, then gradient elution from 2 to 5 min from 100% to 10% solvent A, followed by a second isocratic elution from 5 to 9.4 min at 10% solvent A. Column conditioning consisted of isocratic elution from 9.4 min to 14 min at 100% solvent A. Flow rate was constant at 0.25 mL/min. The analysis run time was 14 min. Sample vials were thermostatted at 8 °C. The flush solvent was water with 10% methanol (*v*/*v*). Injection volumes were 5 µL for both standard and sample solutions. For more information about the descriptive statistics of the amino acids analysis method see Table 3.

Table 3. Descriptive statistics of the amino acids analysis method.

Analyte	Repeatability (% RSD; n = 5)	Reproducibility (% RSD; n = 3)	LOD (ng) *	LOQ (ng) *	Linearity Range (ng *; r)
Proline	2.82	5.37	0.7	2.3	2.3–200; 0.9993
Leucine/Isoleucine	2.07	3.71	0.9	3.0	3.0–200; 0.9995
Methionine	4.74	6.39	1.9	6.4	6.4–200; 0.9988
Alanine	0.58	4.38	0.1	0.5	0.5–200; 0.9958
Tyrosine	1.96	2.53	0.2	0.5	0.5–200; 0.9992

* The values refer to the analyte amount injected onto the column; LOD—limit of detection; LOQ—limit of quantification.

2.6. Statistics Analysis

Concentration calculations were made in Excel 2010. The calculation was performed using the external standard method (calibration solutions). In Excel, chromatographic peak areas were used as a parameter for concentration calculations, which are linearly proportional to the concentration of each compound (analyte). Basic statistical analyses (median, standard deviation, average) were performed in Microsoft Excel 2010, and Pearson's correlation coefficient ($p < 0.05$) between single abiotic factors and the nectar quantity of the species *G. nivalis* and *H. niger* was carried out in Statistica 8.0 software (Statsoft Inc. 2007, Tulsa, OK, USA).

3. Results

3.1. *G. nivalis and H. niger* Nectar Secretion through the Day

By calculating the cumulative median for three different hours (9 a.m., 12 noon, 3 p.m.) of all samples (entire sampling season), we obtained the results for *G. nivalis* (Figure 4) and *H. niger* (Figure 5), which show that nectar secretion is highest in the morning and lowest in the afternoon. The quantity of nectar secretion is always lower in *G. nivalis*.

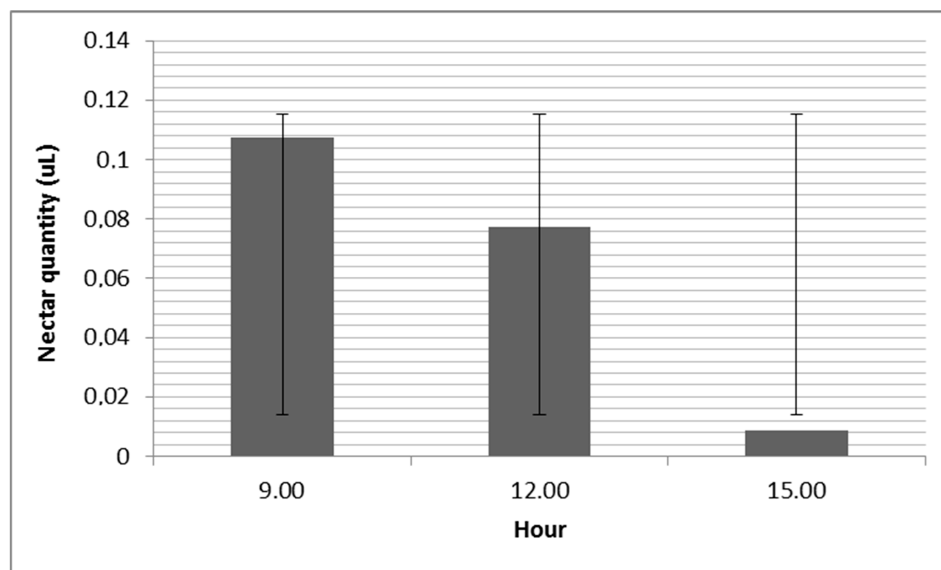

Figure 4. Median of nectar secretion through the day in *G. nivalis*.

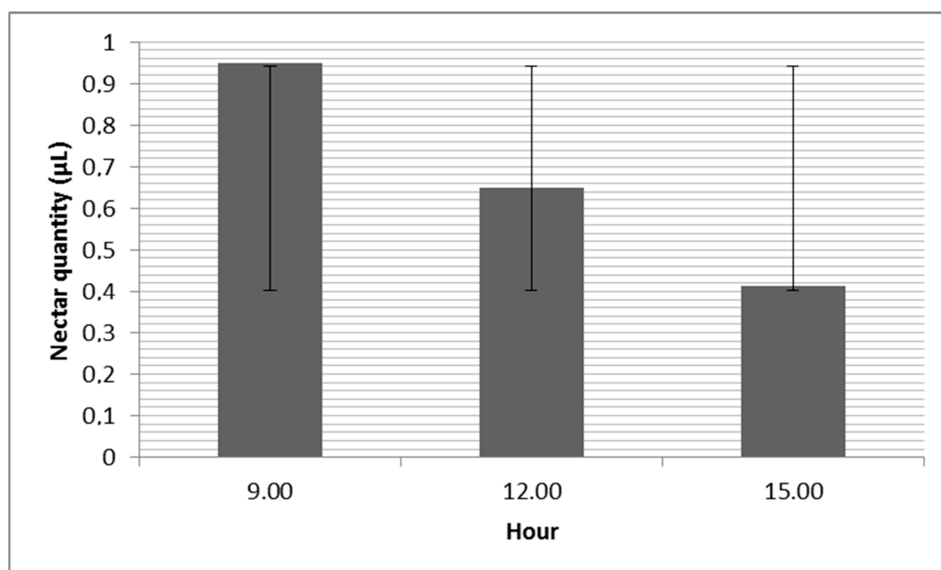

Figure 5. Median of nectar secretion through the day in *H. niger*.

3.2. *Abiotic Factors' Influence on Nectar in G. nivalis and H.niger*

By measuring the abiotic factors (air temperature, air humidity, soil temperature, soil humidity, and UVB radiation) we obtained the results of nectar secretion and nectar composition effects. We calculated the Pearson's correlation coefficient for all abiotic factors and quantity, sugars, phenolic compounds, and amino acids for *G. nivalis*. We obtained the correlations for compounds and abiotic factors, which can be seen in Table 4. For other phenolic compounds and abiotic factors, we did not obtain any correlations, nor did we obtain any correlations between abiotic factors and amino acids.

Table 4. The Pearson's correlation coefficient of abiotic factors on nectar quantity, sugars, and phenolic compounds for *G. nivalis*.

Abiotic Factor	Quantity	Sucrose	Glucose	Fructose	Hyperoside
UVB					
Soil T	−0.54	−0.43	−0.36	−0.40	−0.35
Soil hum					
Air T	−0.35	−0.29			
Air hum					

The negative Pearson's correlation coefficient for soil and air temperature versus quantity shows that when soil temperature (Figure 6) and air temperature (Figure 7) are increased, the nectar secretion is decreased. Pearson's correlation coefficient is −0.54 for the effect of soil temperature, indicating a moderate correlation, while the coefficient of −0.35 for air temperature (−0.35) indicates a weak correlation.

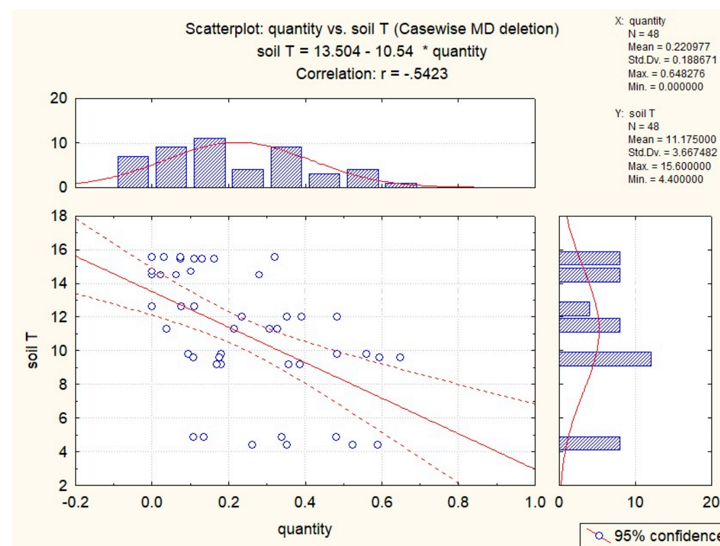

Figure 6. Scatterplot diagram of soil temperature's influence on the nectar secretion of *G. nivalis*.

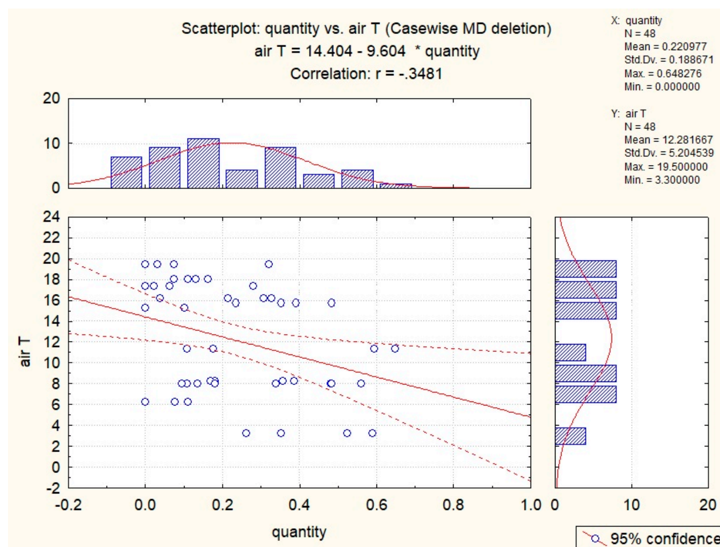

Figure 7. Scatterplot diagram of air temperature's influence on the nectar secretion of *G. nivalis*.

Based on our results, soil (Figure 8) and air temperature (Figure 9) also affect sucrose. When soil and air temperatures increase, the level of sucrose decreases. Soil temperature and sucrose quantity have a moderate correlation (−0.43), while the correlation between air temperature and sucrose is weak (−0.29). When soil temperature increases, the levels of glucose (Figure 10), fructose (Figure 11), and hyperoside (Figure 12) also seem to decrease. Pearson's correlation coefficient for glucose and hyperoside versus soil temperature shows a weak correlation while showing a moderate correlation for fructose versus soil temperature.

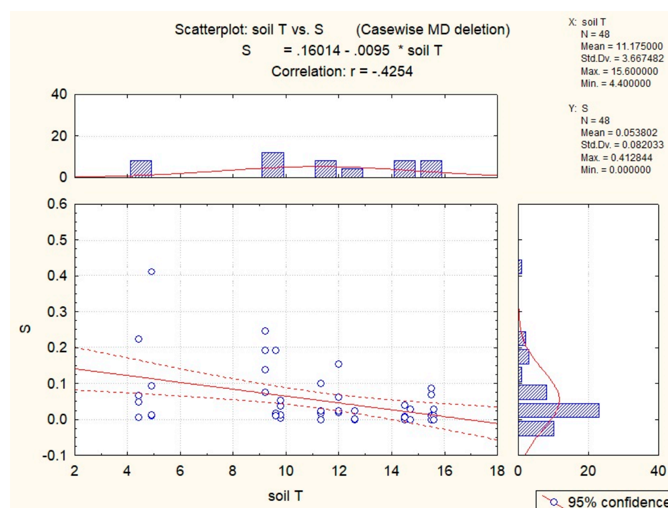

Figure 8. Scatterplot diagram of soil temperature's influence on sucrose in *G. nivalis* nectar.

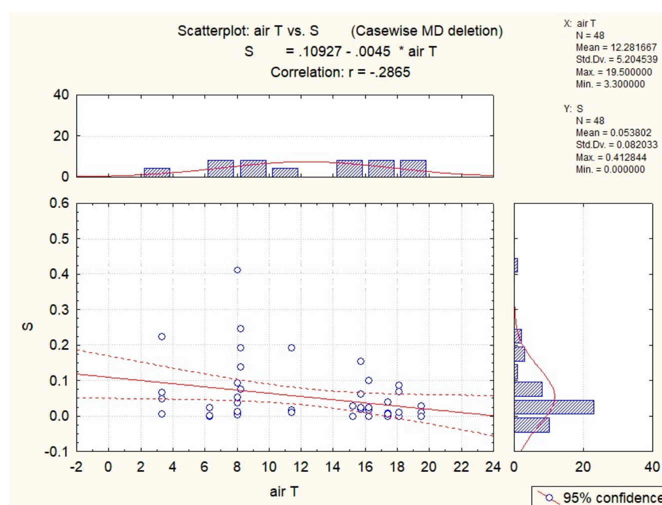

Figure 9. Scatterplot diagram of air temperature's influence on sucrose in *G. nivalis* nectar.

We also calculated Pearson's correlation coefficient for *H. niger*. We obtained correlations for compounds and abiotic factors, which can be seen in Table 5. For the other two sugars and phenolic compounds versus abiotic factors, we did not obtain any correlations, nor did we obtain any correlations between abiotic factors and amino acids.

The positive Pearson's correlation coefficient for quantity, glucose, chlorogenic acid, and hyperoside shows that when the level of abiotic factors increases, the level of those compounds also increases. Pearson's correlation coefficient for soil humidity versus glucose (Figure 13) shows that there is a weak to moderate correlation (0.38). Soil temperature (Figure 14) and air temperature (Figure 15) do affect glucose, but there is a weak correlation for both (0.35). Air temperature (Figure 16) and air humidity (Figure 17) seem to affect chlorogenic acid, but the correlation is weak, as is also the case for UVB (Figure 18) and hyperoside (0.34).

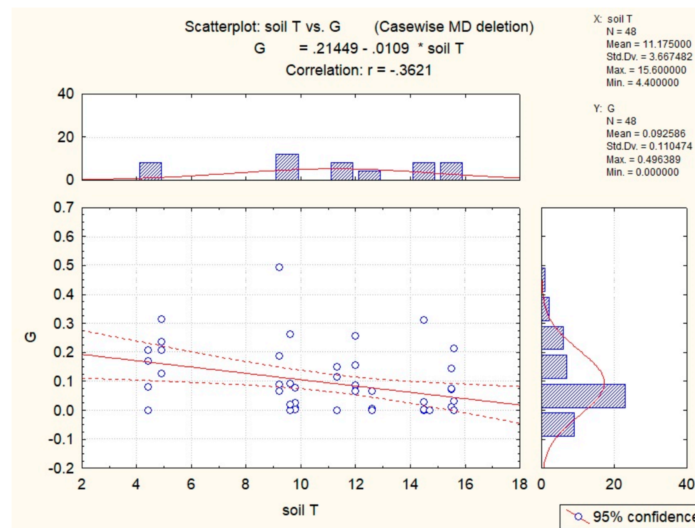

Figure 10. Scatterplot diagram of soil temperature's influence on glucose in *G. nivalis* nectar.

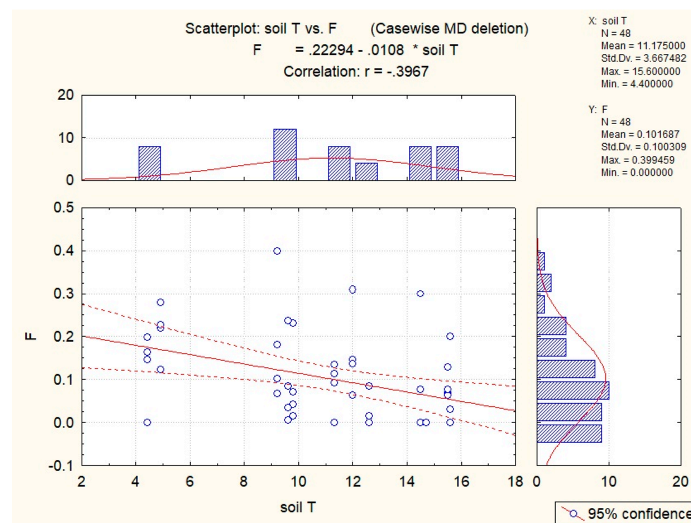

Figure 11. Scatterplot diagram of soil temperature's influence on fructose in *G. nivalis* nectar.

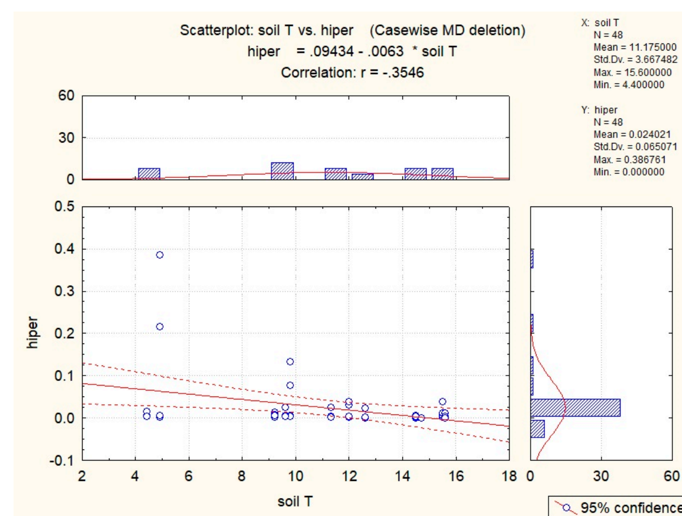

Figure 12. Scatterplot diagram of soil temperature's influence on hyperoside in *G. nivalis* nectar.

Table 5. Pearson's correlation coefficient of abiotic factors on nectar quantity, sugars, and phenolic compounds for *H. niger*.

Abiotic Factor	Quantity	Glucose	Chlorogenic Acid	Hyperoside
UVB				0.34
Soil T		0.35		
Soil hum	0.38			
Air T		0.35	0.35	
Air hum			0.35	

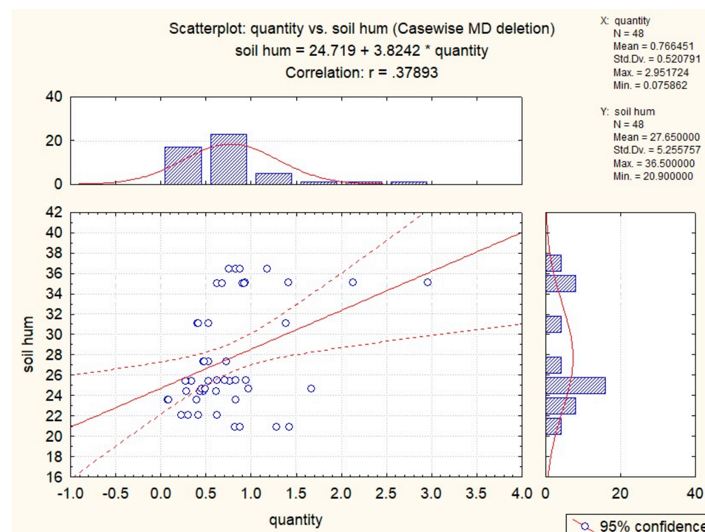

Figure 13. Scatterplot diagram of soil humidity's influence on the nectar secretion of *H. niger*.

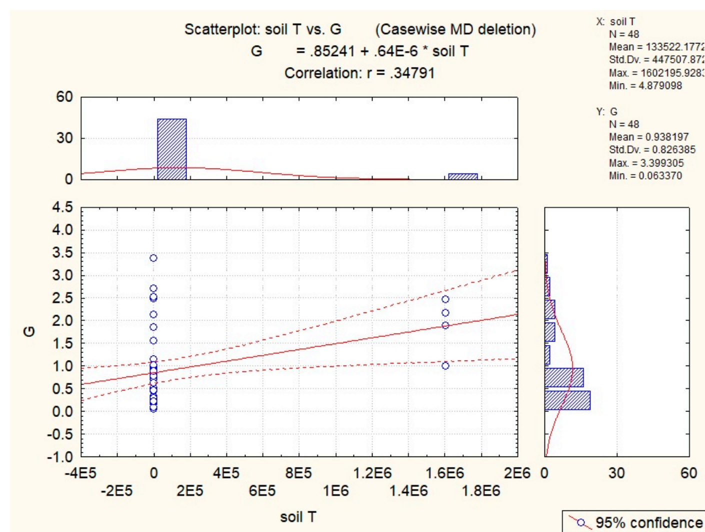

Figure 14. Scatterplot diagram of soil temperature's influence on glucose in *H. niger* nectar.

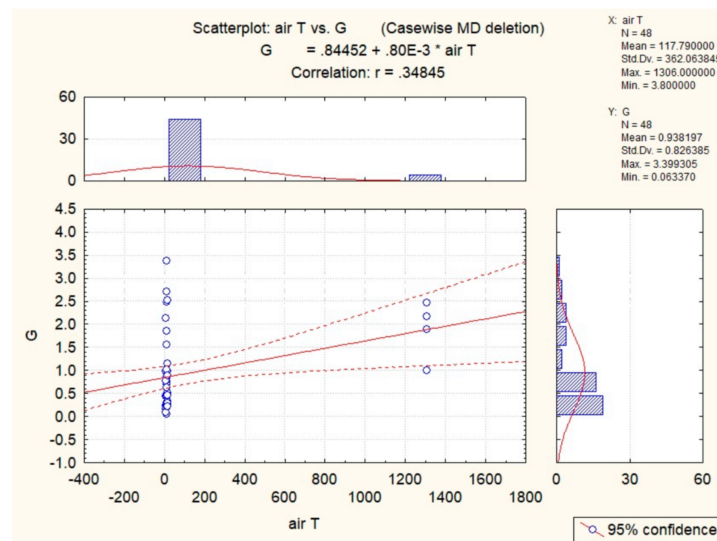

Figure 15. Scatterplot diagram of air temperature's influence on glucose in *H. niger* nectar.

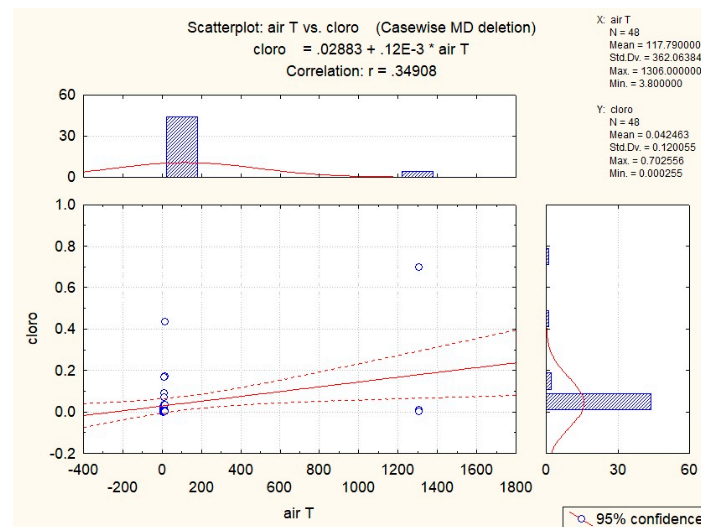

Figure 16. Scatterplot diagram of air temperature's influence on chlorogenic acid in *H. niger* nectar.

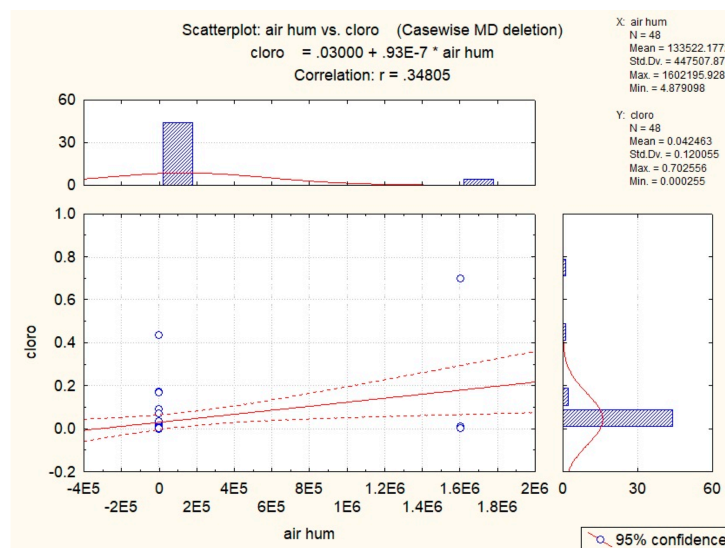

Figure 17. Scatterplot diagram of air humidity's influence on chlorogenic acid in *H. niger* nectar.

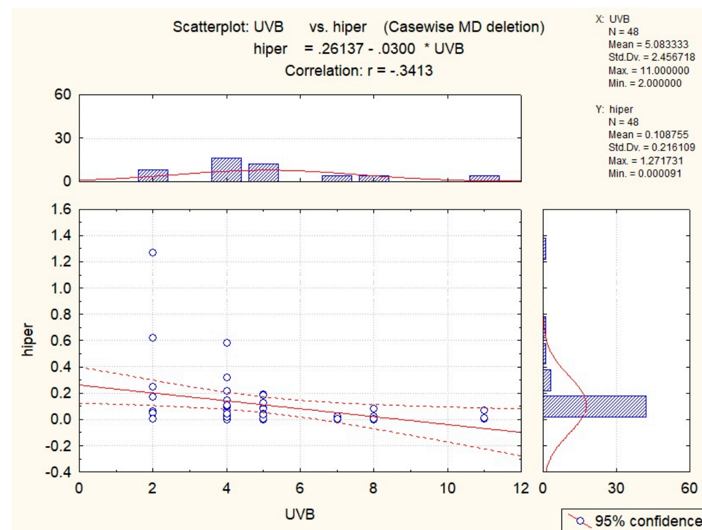

Figure 18. Scatterplot diagram of UVB radiation's influence on hyperoside in *H. niger* nectar.

3.3. Frequency of Occurrence of the Most Represented Sugar in Nectar Samples of G. nivalis and H. niger

Our analysis of the three main sugars (sucrose, glucose, fructose) in nectar samples of *G. nivalis* (Figure 19) and *H. niger* (Figure 20) shows that the most common sugar in nectar samples of *G. nivalis* are hexoses (glucose in 48.7% samples and fructose in 20.5% samples), while sucrose was found in 30.8% samples, and the most common sugar in nectar of *H. niger* is sucrose (in 83.3% samples), while glucose was found in 14.6% samples and fructose only in 2.1% samples.

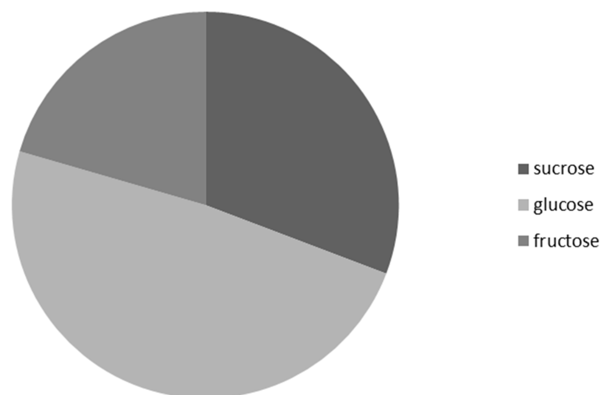

Figure 19. Frequency of the occurrence of the most represented sugar in nectar samples of *G. nivalis*.

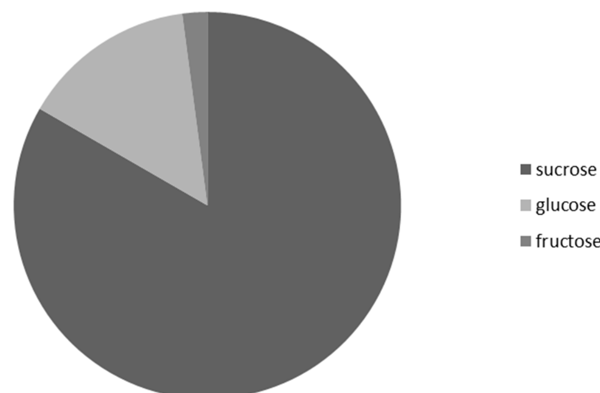

Figure 20. Frequency of the occurrence of the most represented sugar in nectar samples of *H. niger*.

3.4. Frequency of Occurrence of Selected Phenolic Compounds in Nectar Samples of G. nivalis and H. niger

The analysis of selected phenolic compounds shows that rutin is the most present phenolic compound in both species' nectar samples. Rutin is followed by isoquercetin and hyperoside. The difference between rutin and the other two compounds is not as high for *G. nivalis* (Figure 21) as for *H. niger* (Figure 22). The less abundant phenolic compound is chlorogenic acid for *G. nivalis* and quercetin for *H. niger*.

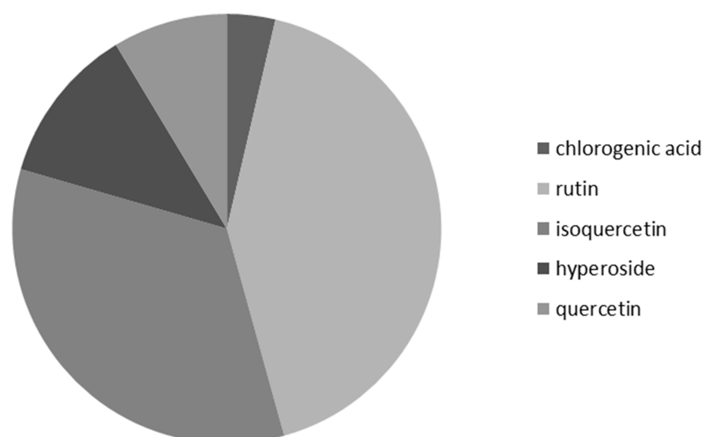

Figure 21. Frequency of the occurrence of the most represented selected phenolic compounds in nectar samples of *G. nivalis*.

The results show that the average quantity of selected phenolic compounds in nectar samples of *G. nivalis* (Figure 21) is rutin (0.085 μg/mL) > isoquercetin (0.068 μg/mL) > hyperoside (0.024 μg/mL) > quercetin (0.017 μg/mL) > chlorogenic acid (0.007 μg/mL).

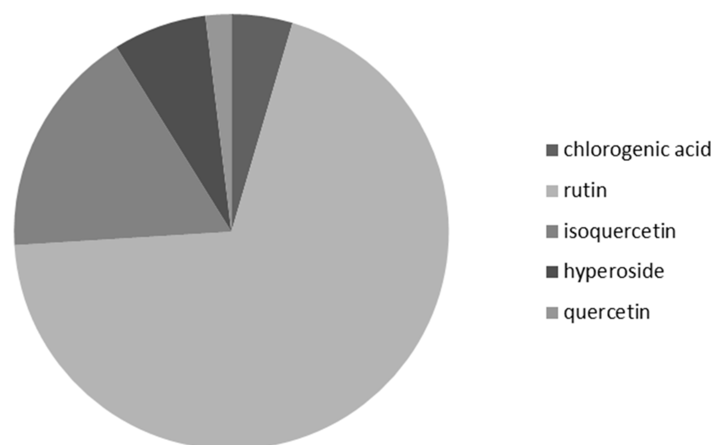

Figure 22. Frequency of the occurrence of the most represented selected phenolic compounds in nectar samples of *H. niger*.

The results show that the average quantity of selected phenolic compounds in nectar samples of *H. niger* (Figure 22) is rutin (1.178 μg/mL) > isoquercetin (0.290 μg/mL) > hyperoside (0.118 μg/mL) > chlorogenic acid (0.077 μg/mL) > quercetin (0.032 μg/mL).

3.5. Frequency of Occurrence of Selected Amino acids in Nectar Samples of G. nivalis and H. niger

The analysis of selected amino acids shows that proline is the predominant amino acid in both species' nectar samples. Leucine is the next for *H. niger* (Figure 23) but is the least abundant amino acid for *G. nivalis* (Figure 24). The least abundant amino acids are alanine for *G. nivalis* and tyrosine for *H. niger*.

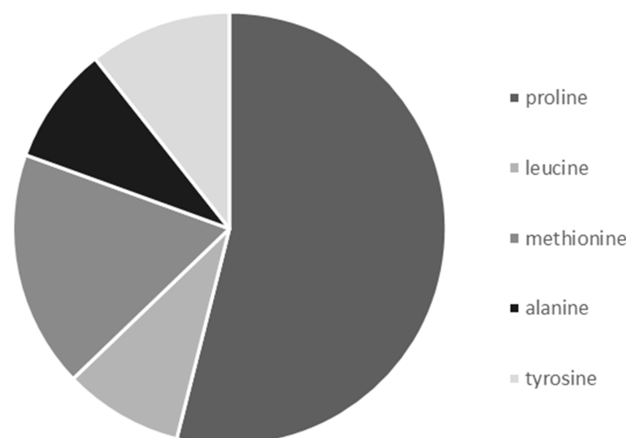

Figure 23. Frequency of occurrence of the most represented selected amino acids in nectar samples of *G. nivalis*.

The results show that the average quantity of selected amino acids in nectar samples of G. nivalis (Figure 23) is proline (1.130 µg/mL) > methionine (0.371 µg/mL) > tyrosine (0.223 µg/mL) > leucine (0.187 µg/mL) > alanine (0.185 µg/mL).

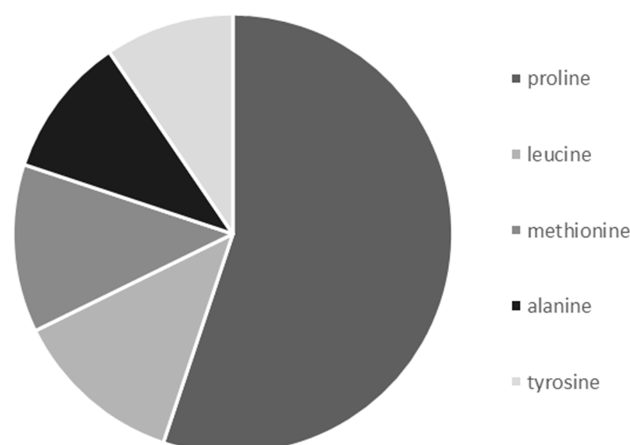

Figure 24. Frequency of the occurrence of the most represented selected amino acids in nectar samples of *H. niger*.

The results show that the average quantity of selected amino in nectar samples of H. niger (Figure 24) is proline (1.71 µg/mL) > leucine (0.39 µg/mL) > methionine (0.38 µg/mL) > alanine (0.32 µg/mL) > tyrosine (0.29 µg/mL).

4. Discussion

Nectar provides pollinators with different nutrients for energy and life processes [4]. Pollinators have trouble finding food, particularly in early spring when snow can still be present and temperature is not favourable. In our study, we focused on the nectar of two late winter and early spring species, *G. nivalis* and *H. niger*. Both flowers have different shapes and also sizes of the nectary, which is supposedly connected to the flower size and its effect on nectar secretion [38]. We wanted to study whether these two species are comparable—if they react the same way to abiotic factors, if they have the same nectar composition, and if smaller flowers really have less nectar. Nectaries in *H. niger* are more visible [39] than in *G. nivalis*. In our study, we investigated the nectar secretion and composition of sugars, phenolic compounds, and amino acids in the nectar of *G. nivalis* and *H. niger*.

Environmental factors have an impact on nectar secretion [8,14–16,18,40]. Based on other research, humidity and temperature [16] have the greatest impact on sugars and

secretion, but we noticed that *H. niger* does need a higher temperature for good secretion, while *G. nivalis* does not. When the weather is sunny and warm, *Helleborus* flowers are more open [31]. Because there was still a lot of humidity in the air on the day of our sampling, it is possible that we obtained such a large nectar quantity because some water could have fallen into the nectaries—the flowers were exposed. Based on our results, we noticed that the amount of nectar in *G. nivalis* and *H. niger* is highest in the morning. The reason could be the more closed flowers for *G. nivalis*; evaporation is lower in closed flowers [11]. We noticed that every time the flowers were more open, we had trouble finding any nectar. Other researchers [8,36] also noticed that there is less nectar when *G. nivalis* flowers are open. It was also mentioned that the *Galanthus* genus secretes nectar just once per day [8], but our results show some nectar secretion later in the day, too. It could also be possible that we did not collect all the nectar in the morning. Based on our other research [41] on the *Salvia* genus and other species, it was expected that the nectar productivity would be higher between 9 a.m. and 12 noon, which is also important for pollinators because this is the time when they usually pick nectar and pollen [42]. Nectar productivity in *G. nivalis* was also higher when the temperatures were lower and there was no sun. It is possible that the reason is that flowers were closed because of the lack of sun, which did not allow evaporation from flowers. Usually, when humidity is higher, the flowers of *G. nivalis* are closed, and when temperature increases and humidity drops, flowers start opening. The lack of sun also affects *G. nivalis* flowers. When the sun is present, flowers are open, and when it is not, flowers close [35]. Weryszko-Chmielewska and Chwil [36] also determined that the nectar secretion is higher in closed flowers than in more open flowers. The reason can be evaporation [11]. Even if UVB radiation sometimes has an effect [43], we cannot confirm that. It was surprising that we did not find any connection between air humidity and nectar productivity, since it is known that it has an effect [8,14–16,18,40,41]. We also noticed that on some days, both species lacked nectar or they had a really low amount of nectar, which was unexpected. The reason could be the high temperatures for winter/early spring because on some days we measured air temperatures above 20 °C. It is known that plant species that more commonly grow in warmer environments tolerate higher temperatures more easily [14,44], which is not common for our researched plant species. For *H. niger* nectar secretion, soil humidity has an effect, which matches other studies and suggests that nectar secretion is greater with higher soil humidity [19], but it does not seem to affect *G. nivalis*.

Nectar contains water and different compounds. The concentration and chemistry can vary between families, species, and even populations and flowers on the same plant [3–5,11–13,18,19]. Sugars as a source of energy are the most important compounds in nectar [3,5,11]. The three main sugars are sucrose, fructose, and glucose. Based on their ratio, we can divide nectar into different types: sucrose-dominant, sucrose-rich, hexose-rich, and hexose-dominant [23]. There is a big difference between the sugars in the samples of *G. nivalis* and *H. niger*. *H. niger* has sucrose-dominant nectar and *G. nivalis* has hexose-dominant nectar. It has been established that sucrose is the main sugar in *H. niger* [7]. Other studies [23,26] show that bees prefer high-sucrose nectar, so based on our results, *H. niger* has better nectar for bees. In a previous study in Slovenia [9], it was proven that bees are the most important *H. niger* pollinators. Percival [45] studied sugars in the nectar of many species and found that the shape of the flower could also influence nectar composition. Deeper flowers or nectaries produce sucrose-dominant nectar, while shallow flowers and nectaries produce either glucose- or sucrose-dominant nectar, which, based on our results, we can confirm for *G. nivalis* flowers. Sometimes, the presence of microbes affects nectar composition. Microbes can be transferred from flower to flower by pollinators and this can also affect nectar composition with yeast, which increases the total sugar concentration and percentage of fructose, while the percentage of sucrose is decreased [46]. Another reason why sucrose levels can be lower is injured nectaries. Usually, such injury is caused by frost [47]. Otherwise, *G. nivalis* is a species that can tolerate cold temperatures. The *Galanthus* genus has a special adaptation called psychrokliny so that frost cannot damage

the plants [48]. *H. niger*'s sucrose-dominant nectar could also be good food for other pollinators, such as ants, which also prefer higher sucrose concentrations in nectar [49]. In early spring, bees are usually not so active because of cold temperatures, and ants can be really important pollinators for *H. niger*.

Nectar also contains phenolic compounds, which are quite common. Some make nectar better and attract pollinators, whereas some can repel them [27,30]. The presence of phenolic compounds can also enhance worker bees' resistance to the queen's signals. Isoquercetin and quercetin were also present in our samples. Particularly, quercetin can affect bee colonies by making them more resistant to the queen bee's hormones, which can actually cause more queen bees [50]. Quercetin is also preferred by bees [51]. The most abundant phenolic compound was rutin, which is important because it has a protective effect against some insecticides [52]. We also decided to check for the presence of hyperoside and chlorogenic acid, which are also common in nectar [53–55]—the levels of both were lower than those of rutin and isoquercetin. Since both nectars consist of important phenolic compounds for the life of bees as protection against insecticides, both species are important bee forage in early spring.

The amount of amino acids in nectar is lower, but it is also important for pollinators [5]. Some ants, for example, prefer nectar with a mix of different amino acids and sugars [49]. Our study showed that proline is the most abundant amino acid. Proline is important for early-stage insects because it plays an important role in maintaining the good condition of muscles necessary for locomotion. It is also the dominant amino acid in the hemolymph of insects, including bees [56]. High concentrations of proline have also been found in other research [57]. They state that proline is present in nectar in quite high percentages, representing 45–60% of all amino acids. In our study, we also examined the presence of tyrosine and alanine, which were also found in other studies in smaller amounts [28], which we confirmed with our own study. High proline levels could also be the result of pollen contamination in nectar [51]. It was surprising that *G. nivalis* has a lot of methionine, which is known for its pest toxicity [29]. Leucine was also present in both samples, since it is also important for bees [56].

We assume that the quantity of sugars in nectar is not connected to abiotic factors but to nectar quantity. When the temperature was lower, the amount of sugars in *G. nivalis* was higher, which could be connected to the reason for higher nectar productivity. The difference between species was that when the temperature increases, the amount of sugars in *H. niger* also increases but decreases in *G. nivalis*; however, the reason is probably that the flowers of *G. nivalis* are open at higher temperatures and evaporation is higher [11]. Our results also show that air temperature, air humidity, soil temperature, and even UVB radiation have an effect on phenolic compounds. However, we found only a low correlation for chlorogenic acid and tyrosine, so it is hard to confirm the effect of abiotic factors, since there is no other research that we could check our results against. There was no effect of abiotic factors on amino acids.

5. Conclusions

In early spring, the range of nectar for pollinators is limited. There are fewer flowers and it is even more important to obtain rich bee forage. It is important to obtain more information about early spring species. Since bees forage nectar in the field, it is important to know how abiotic factors affect the nectar quantity and quality of those species. It has been established that environmental factors, such as soil temperature and humidity and air temperature and humidity, influence nectar secretion. There are no reliable data on UVB radiation and whether it affects the nectar or not. The effects of those factors are not the same for our investigated species. Soil and air temperature affect *G. nivalis* nectar secretion, while soil humidity and temperature affect *H. niger* nectar secretion. It is important to know that increasing temperature could cause lower nectar secretion because this means shorter bee foraging on this species. UVB radiation does not have any effect on nectar secretion. Based on our results, both species are important, as pollinators forage in late winter and

early spring. Carbohydrates (sugars) are the most important compound in nectar, since they are a source of energy for pollinators—especially in early spring, when this energy is even more important. The main sugar in the nectar of *H. niger* is sucrose, while the main sugars of *G. nivalis* are hexoses—glucose and fructose. Bees prefer sucrose-rich nectar, which *H. niger* has, but *G. nivalis* also gives bees a lot of pollen. Rutin, with its protective role for bees against insecticides, is the most predominant phenolic compound in both species' nectar, whereas proline, which is important for bee muscles, is the most abundant amino acid in both species' nectar. Abiotic factors do not seem to have any effect on amino acids, but they seem to affect sugar and phenolic compound levels. Since bees and other pollinators need good food early in the spring, this study can contribute to a better understanding of the importance of keeping large populations of these two important plant species for pollinators.

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