ORIGINAL RESEARCH PAPER

Discriminant Analysis of *Gentiana* Headspace Gas Chromatography – Mass Spectrometry Extract Data from *In Vitro* Cultures

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Abstract

A set of *Gentiana* L. species was successfully grown in vitro under the same conditions, and 72 samples from various cultures of these species (root, shoot, cotyledon callus, hypocotyl callus, and root callus) were obtained. The investigated species were *G. affinis*, *G. andrewsii*, *G. bhutanica*, *G. burseri*, *G. cachemirica*, *G. capitata*, *G. crassicaulis*, *G. dahurica*, *G. decumbens*, *G. freyniana*, *G. frigida*, *G. gelida*, *G. grossheimii*, *G. kurroo*, *G. macrophylla*, *G. paradoxa*, *G. robusta*, *G. scabra*, *G. septemfida*, *G. siphonantha*, and *G. tianschanica*. The obtained samples were extracted with a methanol-acetone-water (3:1:1) mixture, evaporated to dryness, and subjected to thin layer chromatography (TLC) on silica gel in sandwich mode with ethyl acetate-methanol-water (8:2:2) as the mobile phase. The resulting dry extracts were subjected to gas chromatography – mass spectrometry (GC-MS) fingerprinting of the headspace volatile fraction. Total ion count and average mass spectrum vectors were collected as two blocks and scaled independently to form a complex dataset. The major direction separating root or shoot samples from callus samples was found not to be fully associated with the highest variance as this information was placed in the first and fourth principal components of the principal component analysis (PCA). Therefore, linear discriminant analysis was performed on the first four (only the informative) components to reveal features responsible for the separation of culture types in the multivariate space.

Keywords

in vitro; linear discriminant analysis; principal component analysis; fingerprinting; *Gentiana*; gas chromatography

1. Introduction

Gentiana L. (gentian) contains almost 400 species worldwide, whereas approximately 30 species grow in Europe (Xu et al., 2017). Because of their bitter content, they are used in folk medicine to treat the lack of appetite. In addition to the bitter compounds, many xanthones, flavonoids, alkaloids, iridoids and triterpenoids have been reported as active substances in gentian plants (Pan et al., 2016). These compounds can act biologically in various ways, including as hepatoprotective, choleretic, anti-inflammatory, antinociceptive, antiamnesic, antiplatelet, and antiproliferative agents (Mirzaee et al., 2017; Pan et al., 2016).

Literature on gas chromatography (GC) of gentians is scarce. Koshioka et al. (1998) identified endogenous gibberellins in *Gentiana triflora* Pall. using GC with mass

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[3] The paper presents a part of PhD thesis research of Sebastian Gadowski.

spectrometry (MS) and the Kovats retention indices. Mihailović et al. (2011) analyzed Serbian samples of *Gentiana asclepiadea* L., highlighting the importance of essential oils and identifying major compounds. Mustafa et al. (2016) compared the volatile profiles of *Gentiana lutea* L. root materials from wild and cultivated sources. Gas chromatography was also used to identify bitter substances in *Gentiana olivieri* Griseb. (Azadbakht et al., 2020) as well as for profiling beverages containing the root of *G. lutea* L. as the bitter ingredient (Biehlmann et al., 2020; Gibitz-Eisath et al., 2022).

No studies have performed comparative analysis of many gentians using GC. The lack of such studies can be attributed to the limitation associated with reliability of such comparisons. The phytochemical composition of plant material can depend on various vegetation factors, such as soil, weather, and light conditions. For instance, a significant correlation between γ-pyrone content and cultivation altitude was reported in Montenegro for *G. lutea* L. (Balijagić et al., 2012). Gentiopicrin and swertiamarin also varied significantly according to the altitude of *Gentiana macrophylla* Pall. cultivated in China (Sadia et al., 2020), whereas many active compounds of *Gentiana straminea* Maxim. varied significantly according to cultivation location in the Tibetan Plateau (Zhou et al., 2021).

These differences can be suppressed by the cultivation of various gentians in the same location, such as in botanical gardens. However, this approach is very difficult in practice, as gentians occur mainly at high altitudes, and their cultivation in the lowlands is simply unsuccessful. A promising alternative is to grow such plants in vitro under optimized conditions (Thiem et al., 2008) and such an approach can provide a high content of the active ingredients (Drobyk et al., 2015a, 2015b). When all plants are grown in the same medium in a conditioned room, the differences in phytochemical composition arise only from differences between species.

These facts encouraged us to analyze a representative set of in vitro cultured gentian species. Our previous research (Gadowski et al., 2022) focused on thin layer chromatography (TLC). This study is a continuation of this research using headspace GC, which is a broad and common technique used to analyze various types of samples (Wang et al., 2008). In the case of plant material, fresh or dried plants can be placed directly in the sample vial, and the gas phase above the sample can be analyzed. Owing to the possibility of sample heating, a large part of biologically active phytochemical constituents can be detected with this method, as they become volatile at high temperatures.

2. Material and Methods

2.1. Plant Material

Plant material was obtained from the seeds supplied by various botanical gardens. We obtained whole plants for the separate analysis of roots (K) and shoots (P), as well as calli obtained from the cotyledon (L), hypocotyl (H), and root (R). Previously described methods were followed for the various *in vitro* procedures used in these experiments (Mikuła et al., 2011; Tomiczak et al., 2015, 2019). Table 1 lists the 72 samples of the 21 species used in this study.

Approximately 1.25 g of fresh plant material was weighed accurately for the extraction. Extraction was performed in an ultrasonic bath at a temperature of 35–40 °C for 30 min. For the extraction mixture, we used 25 mL of methanol, acetone, and water in a ratio of 3:1:1. Extraction was performed three times for each sample. The combined extracts were evaporated to dryness by using a vacuum evaporator. The dry residue was dissolved in methanol (5 mL).

2.2. Chromatography

The samples were analyzed using a 7890A gas chromatograph coupled to a triple quadrupole mass spectrometer combined with a 7697A headspace sampler (Agilent Technologies, Palo Alto, CA, USA). Separation was carried out using a chromatographic capillary column HP-5MS ((5%-phenyl)-methylpolysiloxane), 30 m × 0.25 mm i.d., 0.25 µm film thickness) with pure helium (99.9999%; Messer, Chorzów,

Table 1 List of analyzed samples with their abbreviations. The last letter of the abbreviation indicates the analyzed part or culture: roots (K), shoots (P), and callus obtained from cotyledon (L), hypocotyl (H), and root (R).

Abbr.	Species	Material source
AffiK AffiP	*G. affinis* Griseb.	Perm Botanical Garden, Russia
AndrH AndrK AndrL AndrP AndrR	*G. andrewsii* Grieseb.	Biologisches Institut Der Universität Stuttgart, Stuttgart, Germany
BhutH BhutK BhutL BhutP BhutR	*G. bhutanica* Grubov (*G. vernayi* C.Marquand)	Lithuania
BursH BursK BursL BursP BursR	*G. burseri* Lapeyr.	Botanical Garden Teplice, Teplice, Czech Republic
CachK CachP	*G. cachemirica* Decne.	Lithuania
CapiH CapiK CapiL CapiP CapiR	*G. capitata* Buch.-Ham ex D.Don	Botanical Garden Teplice, Teplice, Czech Republic
CrasH CrasK CrasL CrasP CrasR	*G. crassicaulis* Duthie ex Burkill	University of Tartu, Botanical Garden, Tartu, Estonia
DahuH DahuK DahuL DahuP DahuR	*G. dahurica* Fisch.	Botanischer Garten, Friedrich-Schiller-Universität Jena, Germany
DecuH DecuK DecuL DecuP DecuR	*G. decumbens* L.f.	Berlin-Dahlem Botanical Garden, Berlin, Germany
FreyK FreyP	*G. freyniana* Bornm. ex Freyn (*G. septemfida* Pall.)	Lithuania
FrigH FrigK FrigL FrigP FrigR	*G. frigida* Haenke	Perm Botanical Garden, Russia

Poland) as the carrier gas, with a constant flow of 1.0 mL min^{-1}. The mass spectrometer was tuned using perfluorotributylamine to m/z values of 69.0, 264.0, and 502.0. The GC column was operated in the temperature-programmed mode with an initial oven temperature of 40 °C (held for 2 min), ramped to 250 °C at a rate

Table 1 *Continued.*

Abbr.	Species	Material source
GeliH GeliK GeliL GeliP GeliR	*G. gelida* M.Bieb	Botanical Garden Teplice, Teplice, Czech Republic
GrosK GrosP	*G. grossheimii* Doluch. (=*G. septemfida* subsp. *grossheimii* (Doluch.) Halda	Lithuania
KurrK KurrP	*G. kurroo* Royle	Lithuania
MacrK MacrP	*G. macrophylla* Pall.	Botanical Garden of Johannes Gutenberg University, Mainz, Germany
ParaK ParaP	*G. paradoxa* Albov.	Lithuania
RobuH RobuK RobuL RobuP RobuR	*G. robusta* King ex Hook.f.	Botanical Garden Teplice, Teplice, Czech Republic
ScabK ScabP	*G. scabra* Bunge.	Botanical Garden Teplice, Teplice, Czech Republic
SeptK SeptP	*G. septemfida* Pall.	Lithuania
SiphK SiphP	*G. siphonantha* Maxim. ex Kusn.	Botanical Garden Teplice, Teplice, Czech Republic
TianK TianP	*G. tianschanica* Rupr. ex Kusn.	Botanical Garden Teplice, Teplice, Czech Republic

of 15 °C min^{-1}, and held at this temperature for 4 min. The temperatures of the injector, ion source, MS transfer line, and quadrupoles were 250 °C, 230 °C, 300 °C, and 150 °C, respectively. The injections (1 μL) were performed in the split mode with a split ratio of 10:1. The mass detector was operated in the scan mode with standard electron impact conditions (70 eV). To eliminate metastable helium species, helium gas (2.25 mL min^{-1}) was used as the quench gas. The data were collected over a mass-to-charge range of 30 to 500 m/z at a rate of 4 scans s^{-1}. The headspace sampler was connected to the GC system front inlet via a heated fused-silica transfer line. A 1 mL sample loop was employed. The temperatures of the headspace oven, loop, and transfer line were set to 90 °C, 100 °C, and 115 °C, respectively. The injections (sampling time, 0.8 min) were performed in the flow-to-pressure mode (15 psi). The system was operated using the software Agilent MassHunter B.07 (build 7, service pack 2). The extraction of organic compounds from the examined herbal samples was performed using 20 mL headspace vials containing 2 g of dried and ground material. The vials were sealed with a polytetrafluoroethylene-lined septum and an aluminium crimp cap and then conditioned for 20 min at 90 °C. Once equilibrium was reached, the vials were pressurized to 15 psi for 1 min. Chromatograms were recorded for 17 min, starting from the third minute of operation (3–20 min).

2.3. Chemometric Analysis

All data were computed in the GNU R 4.1 computational environment, operated under R Studio (www.r-project.org, www.rstudio.com). The obtained total ion count (TIC) chromatograms were imported from the CSV file, which can be found in each
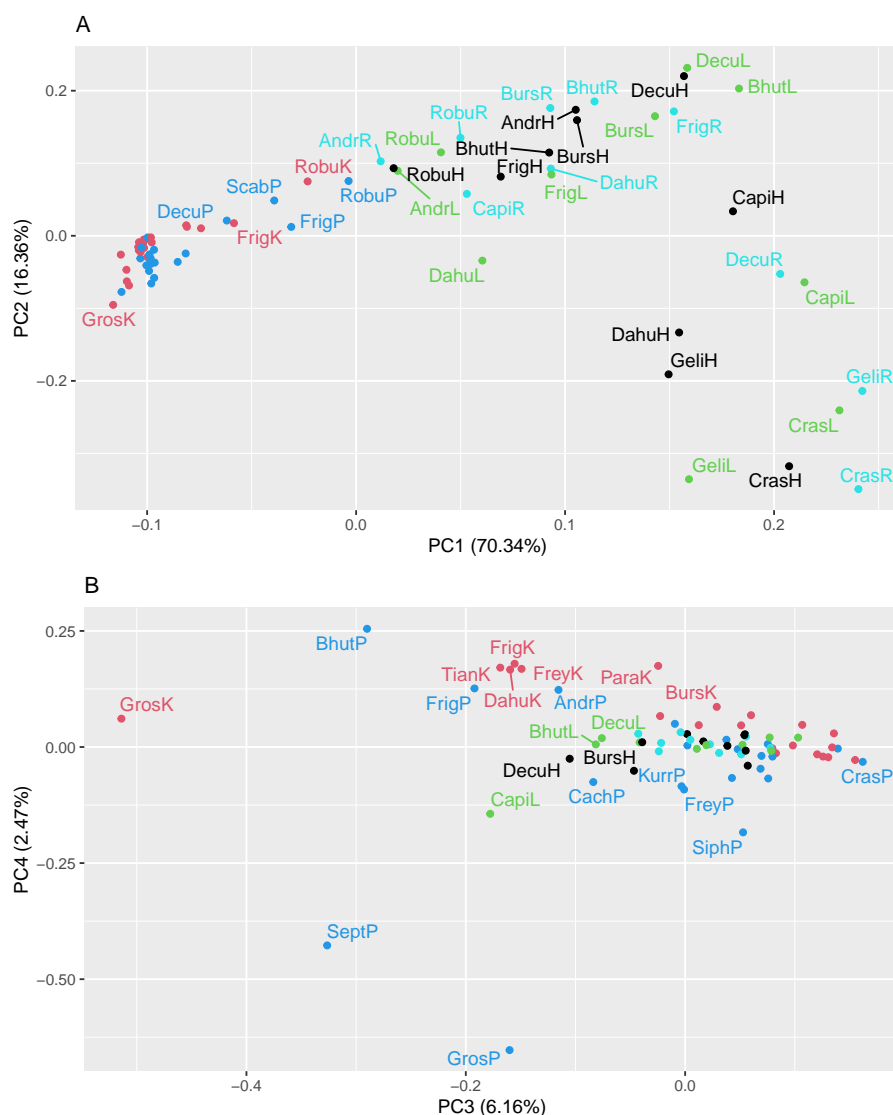
Figure 1 Scores of principal component analysis: (A) PC1 vs. PC2, (B) PC3 vs. PC4. The points and labels are colored according to the tissue type. For an explanation of the labels, see Table 1.

data folder. Average mass spectra of the samples were obtained by converting the entire dataset to MZXML with the ProteoWizard "mzconvert" tool, and subsequently importing to R with the "readMzXmlData" package with a resolution of 0.1 m/z.

3. Results

Two matrices with 72 rows (samples) were obtained. The m/z matrix had 4201 columns (mass ranging from 30.0 to 450.0, with steps of 0.1), whereas the TIC matrix had 1201 columns (time points from 4 to 14 min, with steps of 5 s). The principal component analysis results of the concatenated matrix are presented in Figure 1. Figure 2 presents the linear discriminant analysis performed on the first four principal components. The coefficients of the discriminant functions are also illustrated. The features along the mass dimension are presented in Figure 3, and those along the time dimension are presented in Figure 4.

4. Discussion

Initially, we attempted to place fresh plant material directly into the headspace sample vial. However, this method results in very weak chromatograms with unacceptable
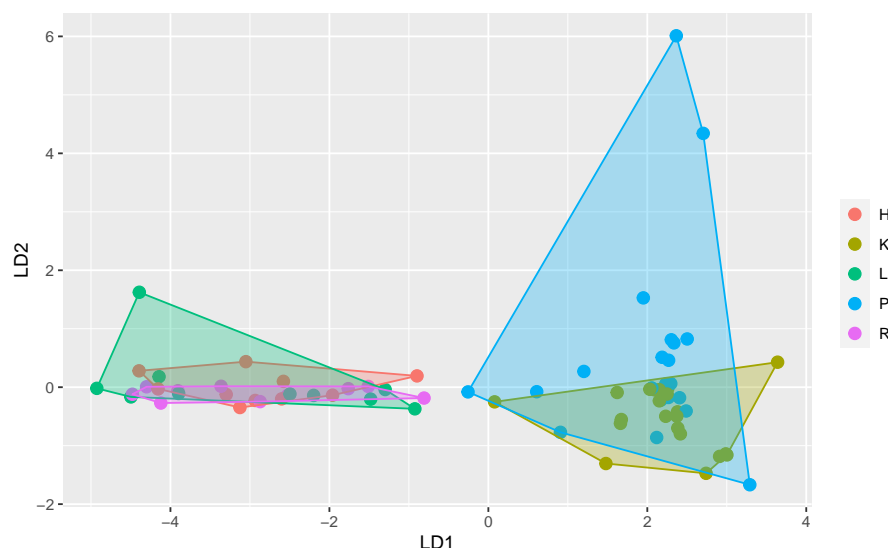
Figure 2 Scores of the principal component–linear discriminant analysis with convex hull polygons grouping tissue types. For an explanation of letters indicating tissues, see the "Plant Material" section.

sensitivity. Therefore, we decided to perform liquid extraction, evaporate the extract, and dissolve it in methanol, similar to the method followed in our previous TLC study (Gadowski et al., 2022). Although there was a risk of loss of volatile compounds, the resulting chromatogram had many more peaks.

We decided to reject mass values below 50.0 before the preprocessing step, as these values caused large variance disturbances in the data analysis. The matrices were concatenated, and each block was scaled separately to the unit variance.

The use of two vectors (the TIC and average MS spectrum vectors) instead of a large matrix was proposed and discussed in our previous paper (Wróbel-Szkolak et al., 2022). Briefly, this approach allows a substantial reduction in the computer resources needed for the computation, as each sample, being a large matrix, can be converted to two small vectors, those of TIC and average mass spectrum. Although it is a lossy compression, this method was demonstrated to separate the main sources of variance regardless of its nature (such as one peak, tailing baseline, and column bleeding with several peaks).

Preliminary analysis of this dataset by principal component analysis (Figure 1) revealed that the four first principal components (PC1–PC4) explained 70.3%, 16.4%, 6.2%, and 2.5% of the variance, and the subsequent components contained only noise or irrelevant information. As shown in Figure 1A, PC1 modelled the difference between the shoot or root cultures (negative values) and callus cultures (positive values). There was no clustering trend along PC2 or PC3 (Figure 1B). A slight tendency to separate the roots (positive values) and shoots (negative values) was also observed along PC4 (Figure 1B).

Because of the discriminative tendency along the PC1 and PC4 axes, one can conclude that the features responsible for cultural differences can be highly complex, and that they are separated from the directions modelling the largest variance. To investigate this phenomenon, we used linear discriminant analysis on the first four principal components. As shown in Figure 2, this method identified the combinations of the first four PCs that best discriminated the culture analyzed: the first discriminant fully separated root or shoot and callus cultures, and the second discriminant represented the difference between shoot (positive) and root (negative) cultures; however, there were several samples that were not separated. The remaining discriminants did not exhibit any discriminative power.

To identify the chromatographic features modelled by these two discriminants, their coefficients (each had four coefficients, assigned to each principal component) were
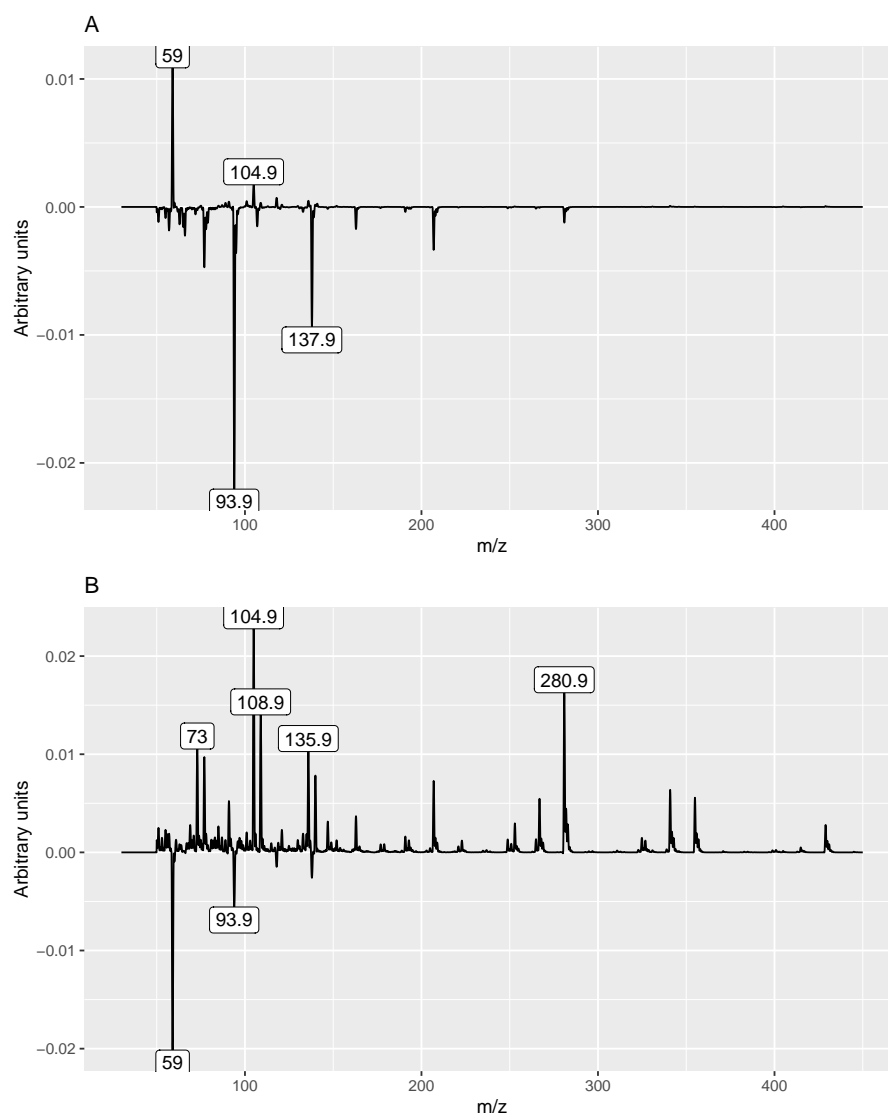
Figure 3 Linear discriminant analysis loadings of the first (A) and second (B) principal component–linear discriminant along the mass spectra dimension.

converted to the original multivariate space with matrix multiplication by the loadings of principal component analysis. The resulting matrix can be perceived as the linear discriminant analysis loadings (discriminant coefficients) in the original space.

Analysis of the loadings (Figures 3 and 4) revealed that the main feature responsible for the difference between callus and shoot or root cultures (first discriminant) was the presence of a peak with a retention time of 9.275 min (Figure 4A) and mass values of 93.9 and 137.9 (Figure 3A). The peak was present in callus cultures but absent in root or shoot cultures and contained mass and relative abundance values as follows: 93.9, 100%; 137.9, 48.7%; 77, 24.6%; 66, 9.8%; 106.9, 7.6%; and 51, 6.2%. Unfortunately, the identity of the peak could not be determined using the NIST library.

The second discriminant was connected to several peaks (6.8, 7.99, 8.49, and 10.16 min) and mass values: 73, 104.9, 108.9, 135.9, and 280.9. They were more intense in shoot samples than in root samples.

The peak with a retention time of 6.8 min and principal mass of 280.9 was identified as polysiloxane column bleeding. The peak at 7.99 min contained the following mass and relative abundance values: 108.9, 100%; 139.9, 51.7%; 109, 13.4%; 52.9, 7%; 44, 6.5%; 109.1, 5.2%; 73, 5.2%, whereas that at 10.16 min these values were 340.9, 100%; 73, 62.4%; 428.9, 49.9%; 342.9, 23.3%; 341.9, 22.8%; 146.9, 22.7%; 324.9, 21.5%; 430.9, 15.2%; 429.8, 11.4%; 341.3, 9.1%; 341, 7.3%; 72.9, 6.9%; 206.9, 6%; 429.3, 5.5%; 341.2,
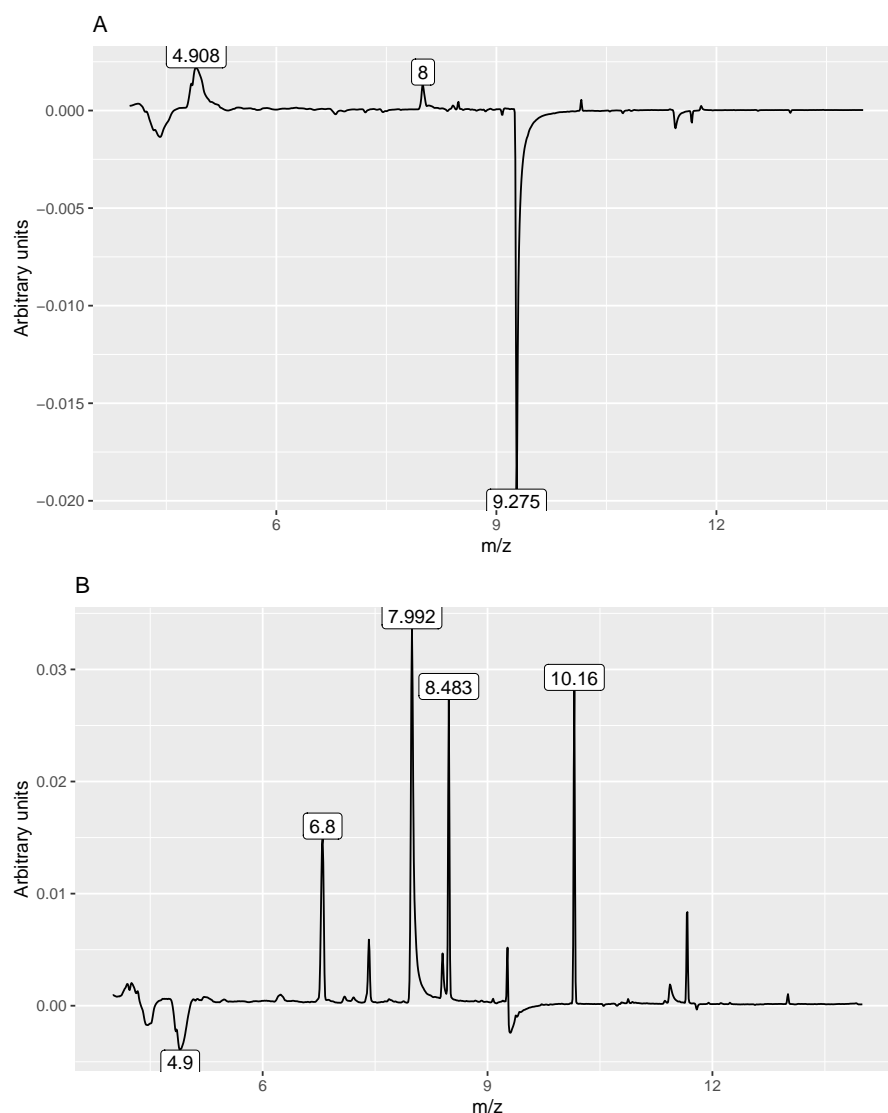
Figure 4 Linear discriminant analysis loadings of the first (A) and second (B) principal component–linear discriminants along the time dimension.

5.3%; and 340.8, 5.2%. The last peak with a retention time of 8.49 min contained mass and relative abundance values of 354.9, 100%; 108.9, 14%; 355.9, 7.4%; 355, 6.6%; 42.9, 6.5%; 94.7, 6.4%; 338.9, 6%; 355.3, 5.9%; and 31.1, 5.2%. We could not identify these two substances with the available libraries because we obtained several very different hits with comparative similarity.

5. Conclusion

This preliminary study of in vitro samples of *Gentiana* identified GC-MS features responsible for the differences between in vitro culture types. Further investigation is planned to identify these features and interpret them in the context of actual knowledge of genus phytochemistry.

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