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Principal component analysis of HPLC–MS/MS patterns of wheat (*Triticum aestivum*) varieties

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Abstract. Untargeted metabolomic strategy was chosen to investigate as many small metabolites as possible in a collection of 13 varieties of conventionally grown spring and winter wheat and organic wheat (*Triticum aestivum*). Metabolites were separated by high-performance liquid chromatography on a reversed-phase column (RP–HPLC) coupled with electrospray ionization tandem mass spectrometry (ESI–MS/MS). The procedure includes extraction of metabolites followed by chromatographic separation using the linear gradient of aqueous formic acid and acetonitrile with subsequent identification of compounds by MS/MS. Discrimination of the metabolomic patterns of different wheat varieties was achieved by principal component analysis (PCA). Results of PCA indicated clear differences in the patterns of wheat varieties.

The winter wheat grown in conventional conditions and the spring wheat grown in organic conditions differed from the spring wheat grown in conventional conditions by the higher content of carbohydrates. It could be explained by osmotic stress resistance. Varieties grown under organic conditions could be well distinguished from others by the results of PCA, which points to the existence of an impact of different farming systems.

Key words: HPLC–MS/MS, PCA, spring wheat, winter wheat, organic wheat, plant phenolics.

INTRODUCTION

Common wheat (*Triticum aestivum* L.) is a very diverse and widely adaptable cereal crop. Respective breeding programmes have been primarily targeted at the selection of new cultivars with higher grain yields and better end-use quality. During the last decade, the need for increasing sustainability and environmental protection have become more and more relevant in the agricultural sector. For that reason, special cultivars of wheat for organic farming are becoming a challenge for breeders [1–3].

In wheat, as in the other plants, antioxidant compounds are naturally synthesized as part of multifunctional defence systems against the detrimental effects of (per)oxidation. It is apparent that selecting and breeding wheat genotypes that are rich in antioxidants will improve agronomical traits of wheat plants, enhance the keeping quality, stability, and safety of wheat products and improve the health benefits associated with wheat consumption [2]. It is reported [4–5] that variation in the antioxidant activity in terms of 2,2-diphenyl-1-picryhydrazyl scavenging capacity, total phenolic content, and concentrations of phenolic acids most likely indicates the significance of the genotype effects on the antioxidant properties of whole wheat and wheat fractions, including bran. The patterns of variation of phenolic acid concentrations are dissimilar among genotypes [4,6]. Significant variation in the ferulic acid (a predominant phenolic acid in the grains of all of the tested soft wheat varieties or lines) concentration of wheat genotypes has been found to be correlated with disease resistance [2].

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Organic agriculture is gaining popularity and needs a variety of improvements for further optimization of the respective farming system. Organic agriculture is focused on varieties with ecologically better-adapted traits that yield without the use of fertilizers and pesticides, and is at the same time oriented to production of good quality. Several reports deal with outcomes of organic field trials, organic plant breeding, and crop production [1,3,7].

Metabolomic approaches have emerged as a valuable tool for the plant sciences, including the study of the development, phenotyping of genetically altered plants, qualitative trait analysis, and improvement of breeding strategies. In addition, metabolomic technologies can be utilized for the discovery and identification of markers of diseased and stressed plants, as well as changes following genetic modifications and characterization of different genotypes/phenotypes. In the past few years, rapid development of high-throughput tools for metabolic profiling, such as detection of the levels of multiple metabolites in a single extract, has facilitated the analysis of a broad range of metabolites. This contributes to an improved understanding of the metabolism network and the mechanism of its interaction with developmental phenotypes [8,9].

The most common separation and detection techniques for the profiling of metabolites are liquid chromatography (LC) in its high performance (HPLC) or ultra performance (UPLC) forms, gas chromatography (GC), capillary electrophoresis (CE), as well as the coupling of these instruments with detection techniques such as mass spectrometry (MS) [10–15] and nuclear magnetic resonance (NMR) [16,17]. Different techniques used in metabolomic analysis are described in several reviews [8,9,18–20].

The objective of this research was to investigate the composition of wheat extracts that contributes to the characterization of wheat varieties through the comparative profiling of metabolites found in different wheat varieties. A reversed-phase (RP)–LC–MS/MS method for the separation and identification of metabolites in the whole grains, bran, and flour of wheat was developed for that purpose.

Resulting LC–MS/MS chromatograms were statistically evaluated by a variety-based comparison of peak heights using principal component analysis (PCA) of the whole data set.

EXPERIMENTAL

Materials

Wheat grains, flour, and bran were obtained from Jõgeva Plant Breeding Institute (Estonia). The varieties investigated were Manu, Meri, Triso, Vinjett, (items 1–4), and Spelta (*Trticum spelta*, item 5) as spring varieties; Anthus, Björk, Olivin, Portal, and Tarso (items 6–10) as winter varieties; and Manu, Meri, and Vinjett (items 11–13) as spring organic varieties (all harvested in 2009).

All chemicals were of analytical grade and used as received. Methanol and formic acid from Fluka (Buchs, Switzerland) were used for extraction and separation, respectively. Acetonitrile and methanol of ultra gradient grade used in the chromatographic experiments were from Romil (Cambridge, UK). Deionized water was prepared by a Milli-Q system from Millipore (Bedford, MA, USA).

Extraction procedure

It is supposed that the extraction method is of fundamental importance for any analysis. Ordinarily, the extraction conditions must be adapted to the type of compounds finally identified. While this study was aimed as untargeted, methanol was used as the extraction solvent according to the results of our previous work [10], which allowed a better extraction performance in a reproducible way and guaranteed a high stability of extracts compared with the methanol/water mixture in a ratio 50 : 50. As the primary goal of this work was to provide qualitative information about metabolites in wheat, the extraction was performed only once.

The extraction procedure was as follows: the wheat grains were ground to a fine powder using an ordinary grinder; the bran layer and flour were used as received. About 2.0 g of finely ground wheat was weighed and extracted with 10 mL of methanol in an ultrasonic bath at 36 °C for 30 min. After sonication, samples were centrifuged for 5 min (3000 rpm), and liquid phases were filtered through a 0.45 μm filter. Liquid phases were taken to dryness in a rotary evaporator, re-dissolved in 0.5 mL of methanol, and injected directly into the LC system.

To obtain more hydrophilic extraction conditions, a methanol/water mixture in a ratio 50 : 50 was used as the solvent in a similar extraction procedure. The analytical samples obtained were stored at –18 °C.

LC–MS/MS analysis

Samples were analysed using LC/ESI–MS/MS in the negative ion mode on a 1100 Series LC/MSD Trap-XCT (Agilent Technologies, Santa Cruz, CA, USA). The ion trap was connected to an Agilent 1100 Series HPLC instrument consisting of an autosampler, solvent membrane degasser, binary pump, and column thermostat. The HPLC 2D ChemStation software with a ChemStation Spectral SW module was used both for the process guidance and for the processing of the results.

The sample components were separated on a Zorbax 300SB-C18 column (2.1 mm × 150 mm; 5 μm particle size; Agilent, Santa Cruz, CA, USA) with a guard column filled with the same type of sorbent. The column was eluted at 0.3 mL/min with a linear gradient from 0.1% aqueous formic acid (solvent A) and 5% of acetonitrile (solvent B) to 30% B in 40 min followed by 90% B for 15 min. The column temperature was maintained at 35 °C. The sample injection volume was 15 μL. All experiments were performed in duplicate.

The conditions of MS/MS detection in the auto MS(n) regime with the scan mode standard enhanced were as follows: m/z linear spectra interval 100–1000 amu; target mass 400 amu; number of precursor ions 2; maximal collection time 100 ms with 15 averages; compound stability 100%; drying gas (N_2 from generator) speed 10 L/min, temperature 350 °C, pressure 30 psi; collision gas (He) pressure 6×10^{-6} mbar.

Principal component analysis

PCA is a powerful tool for data analysis, identification of data patterns, and expressing data, which enables the highlighting of a group's similarities and differences [21]. It can be assumed that the content and/or diversity of metabolites in the collection of varieties under investigation should be different. Winter and spring varieties are classified in terms of the growing season and are therefore considered as different phenotypes.

For PCA, the chromatograms of wheat varieties were transformed to a table (a matrix) of peak intensities of metabolites. In this table, a row corresponds to a certain variety and a column to a metabolite (represented via a corresponding extracted ion peak intensity). If we denote this matrix as D, the PCA procedure decomposes the matrix D as follows: $D = ST^T$ (here the superscript means transpose). Assuming that the dimension of D is nxm, where n is the number of varieties under study and m is the number of measured peaks, the dimension of a scores matrix, S, is nxp and the dimension of the loadings matrix, T, is mxp where $p << n$. Plotting the first row of S versus its second row, a PCA plot is obtained where each point represents a variety. Moreover, if the first two components of T are overlaid onto the scores plot as vectors, the directions of these vectors explain the scatter and clustering of the varieties that are plotted on the scores plot.

PCA was carried out in a Matlab (Mathworks, Natick MA, USA) environment using a standard singular value decomposition procedure. For data processing, the peak intensities were replaced by their logarithms to reduce the influence of large and mean-centred peaks.

RESULTS AND DISCUSSION

Comparison of different wheat varieties

LC/ESI–MS/MS analysis of wheat extracts revealed the presence of different features. Twenty-three major features were detected in the wheat grain extracts and almost fifty in the bran extracts.

To compare different varieties of wheat, the data as a whole were subjected to PCA after LC–MS/MS analysis. In Fig. 1, the LC–MS/MS data are represented in two first principal component coordinates. Each point represents a peak height of a particular m/z value. As can be seen, this approach enabled good separation and grouping of the investigated varieties. To find out which feature (m/z) is responsible for the separation of samples (i.e. sorting), loading vectors located columns of the p matrix are also presented in Fig. 1. It is evident that the vectors corresponding to the m/z values 341 and 452 are those mainly responsible for the separation (Fig. 1). Also, organic, spring, and winter varieties as three standalone groups can be distinguished in Fig. 1. Those varieties that belong to the spring, winter, or organic groups have more common features and are bunching together, with two exceptions: Anthus (item 6) and Björk (item 7). According to our data, Anthus and Björk have a lower gluten content than the other investigated varieties. Anthus was also characterized as an exception in our previous work [22]. Still, more grain samples of different varieties have to be analysed to confirm this point.

Both the winter wheat grown in conventional conditions and the spring wheat grown in organic conditions differed from the spring wheat grown in conventional conditions (Fig. 1): the first two groups had a higher content of various oligosaccharides. This can be explained with osmotic stress resistance [23].

Flour and bran samples and the whole grains

The flour and bran samples of the grain and the whole grains of the same varieties were separately analysed. Spring Vinjett (item 4), organic Vinjett (item 13), and winter Björk (item 7) were used for variety comparison.

In Fig. 2, chromatograms of different fractions of these varieties are represented in PCA coordinates. It can be seen that the vectors corresponding to the m/z values 341 and 563 are those mainly responsible for the separation. It can also be observed from Fig. 2 that only the bran fractions of the investigated varieties are significantly distinct from each other. It may be assumed that differences between the bran fractions are strongly dependent on variety. It is widely accepted that wheat antioxidants and other beneficial phytochemicals (including phenolic compounds) are concentrated in the bran fraction of wheat grain [2].

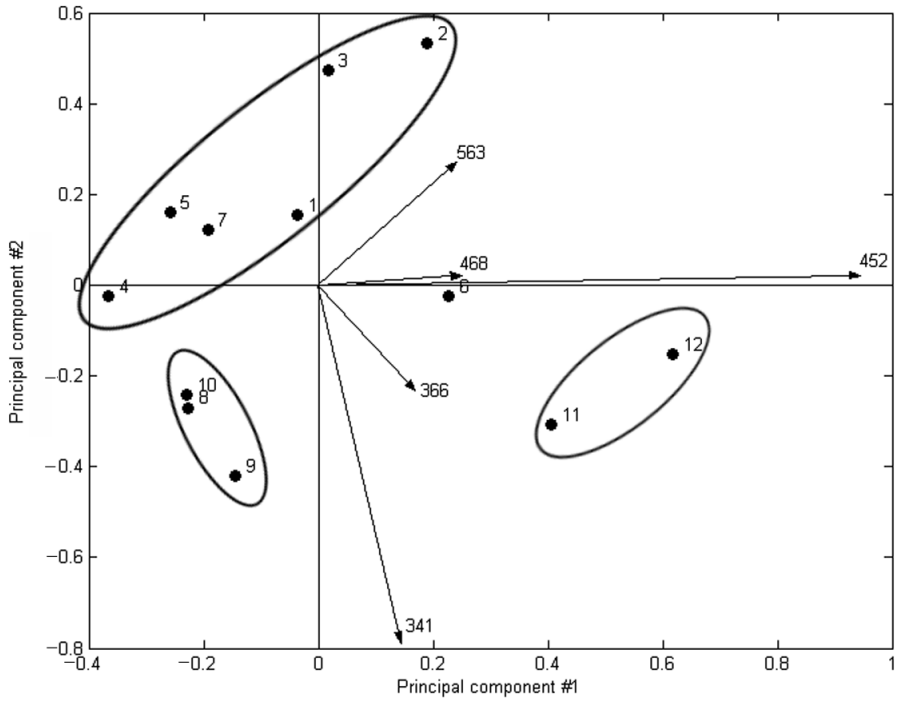

Fig. 1. Representation of the LC–MS/MS base peak chromatograms of the methanol extracts of 12 wheat varieties in the first principal component coordinates (accounting for 71% of the variability). Each point represents one particular chromatogram. Vectors are loadings and the numbers 341, 366, 563, 468, and 452 refer to the m/z values primarily responsible for the scattering. 1–5 conventionally grown spring wheat, 7–10 winter wheat, 11 and 12 organic spring wheat.

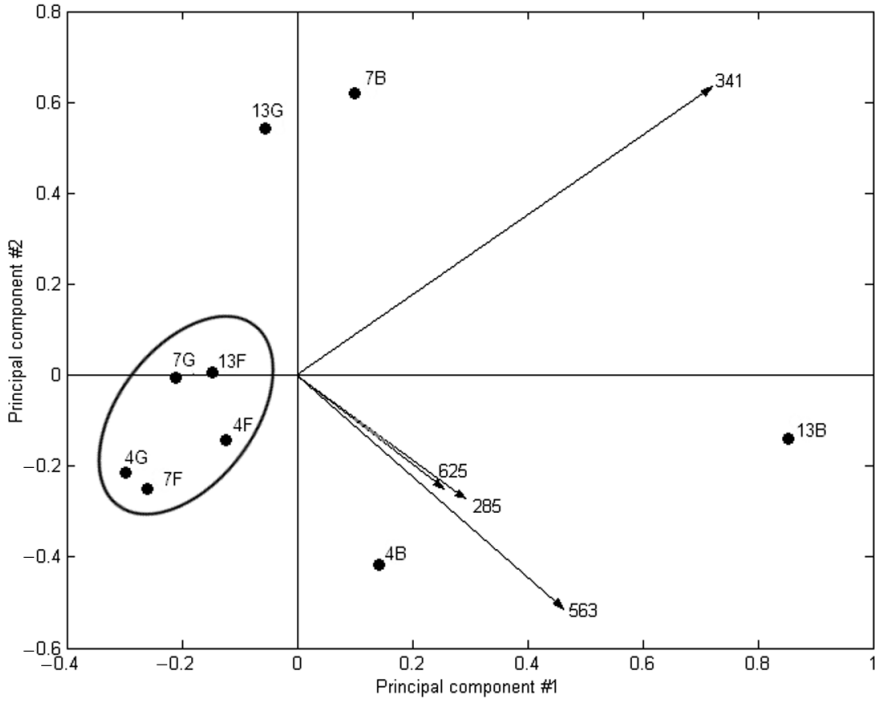

Fig. 2. Representation of LC–MS/MS base peak chromatograms of the methanol extracts of grains (G), bran (B), and flour (F) of three varieties (spring Vinjett (item 4), winter Björk (item 7), and organic Vinjett (item 13)) in the first principal component coordinates (accounting for 67% of the variability). Each point represents one particular chromatogram. Vectors are loadings and numbers 341, 563, 285, and 625 refer to the m/z values primarily responsible for the scattering.

Another observation that can be made in Fig. 2 is that the organic variety may show a characteristic behaviour. This could be explained by the organic growing conditions. Nevertheless, substantially more samples of different varieties have to be analysed to confirm the last finding.

Identification of selected key metabolites

Figure 3 shows the sample base peak chromatogram of the grains of winter wheat variety Anthus. Compounds that were responsible for scattering in PCA results formed the interest group for the identification (Figs 1 and 2). The characteristics of the selected peaks obtained from LC–MS/MS analysis are presented in Table 1. Identification was performed by the interpretation of the MS/MS fragmentation patterns of corresponding analytes, their accordance with literature data, and chromatographic behaviour.

Peak **1**. A molecular ion at m/z 341 had a constant neutral loss (CNL) of 162 Da, which corresponds to the loss of a hexose moiety (glucose, galactose, or fructose) linked to the rest of the molecule by an O-glycosidic bond. The ions with m/z 179 and 161 indicate scission of the glycoside bond to form the complementary monohexose molecule. The other product ions, with m/z 143, 119, 131, and 101, which were present in the product ion mass spectrum from each hexose, are formed by CNLs of H_2O and CH_2O-group. The identified disaccharide could be sucrose or maltose. The fragmentation behaviour was consistent with the literature data [24].

Under peak 1, a lower extracted ion peak of a molecular ion with m/z 503 was found. Its MS/MS fragmentation ions at m/z 377, 323, 341, and 179 refer to tri-hexoside, but its peak intensity was too low for further PCA. The detected tri-glycoside can be raffinose, which is also found in wheat.

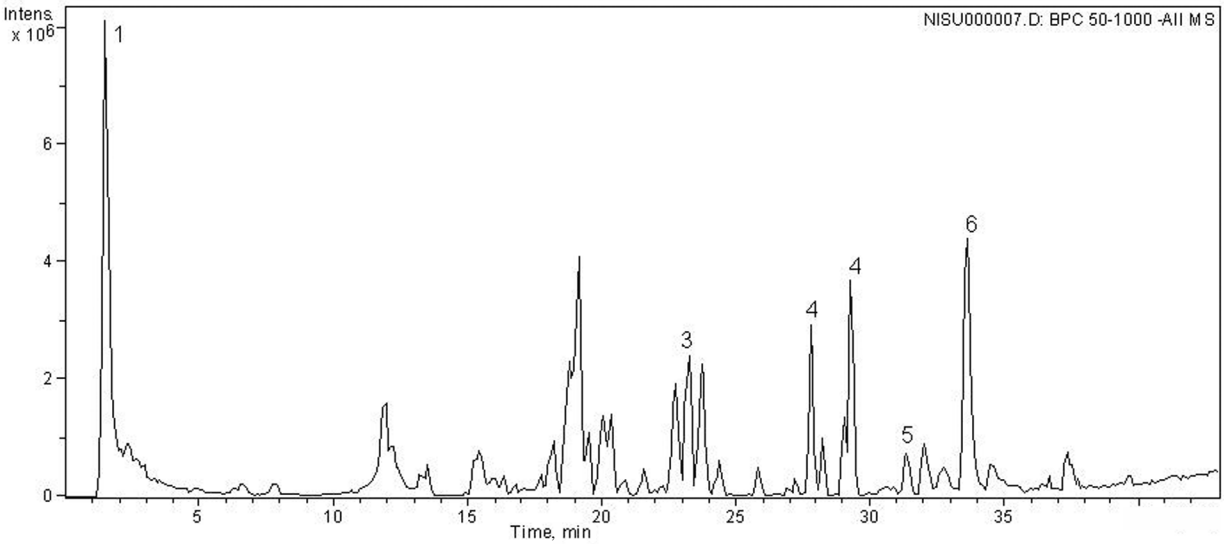

Fig. 3. LC/ESI–MS/MS base peak chromatogram of the methanol extract of the winter wheat variety Anthus (whole grains). Peak numbers indicate tentative compounds primarily responsible for the scattering in PCA: 1 – unspecified dihexoside; 3 – hexose-hexose-N-acetyl; 4 – apigenin-6-C-pentoside-8-C-hexoside; 5 – unknown; 6 – putatively rhamnoside.

Table 1. Retention times (t_r), deprotonated molecular ions, and fragment ions obtained from LC/ESI–MS/MS analysis of metabolites in the variety Anthus. In the column 'Fragment ions' base peaks are shown in bold

Peak No.	Tentative compound	$[M-H]^-$, m/z	t_r, min	Fragment ions, m/z
1	Dihexoside (unspecified)	341	1.5	**179**/161/143/119/131/101
2	Apigenin-6/8-C-pentoside-8/6-C-hexoside	625	11.9	**485**/179/221/383/323/341
3	Hexose-hexose-N-acetyl	366	23.3	**186**/204/142/246
4	Apigenin-6-C-pentoside-8-C-hexoside	563	27.8 29.3	**353**/383/443/473/503
5	Unknown	468	31.3	**332**/306/161/289
6	Rhamnoside	452	33.6	**306**/316/135/145/332
7	Luteolin	285	41.7	**241**/285/175/199/151

Oligosaccharides are indicators of osmotic stress resistance in plants; they tend to accumulate in a stress situation. In Fig. 2, organic and winter wheat samples are clustered and separated from spring samples due to m/z 341 and 366, m/z 341 refers to saccharides. The content of m/z 341 in the organic and winter samples was almost twofold in comparison with the spring varieties (contrary to [24]). This could be due to stress conditions such as an insufficient supply of nutrients in the case of organic samples.

Peak **2** showed a molecular ion at m/z 625. Compound 2 was tentatively identified as apigenin-6/8-C-pentoside-8/6-C-hexoside in accordance with the literature data [12,25].

Peak **3**. MS analysis showed a molecular ion at m/z 366 and a fragmentation pattern similar to those of di-C-glycosides. The MS/MS fragmentation gave CNLs of 180 (galactose or glucose), 162 (hexose), 120 (characteristic for a C-hexoside), and 42 (loss of CH_2CO group). On the basis of these results, a hexose-hexose-N-acetyl structure was proposed for compound 3.

Peaks **4** with t_r = 27.8 and 29.3 min contained the same molecular ion as $[M–H]^–$ = 563 and were characterized by the same complex of daughter ions indicating isomers. MS/MS data showed fragments at m/z 473 and 443, indicating the presence of a C-hexosyl unit. The fragment at m/z 503 corresponds to the fragmentation of pentose. The ions at m/z 353 (aglycone+83) and 383 (aglycone+113) supported the conclusion that apigenin was the aglycone for compound 4. Therefore, its general structure could be apigenin-6-C-pentoside-8-C-hexoside, putatively shaftoside/isoschaftoside [12,25–28].

Peak **5** corresponded to a major molecular ion at m/z 468. The fragment ions included ions at m/z 332, 306, 161, and 289. We were not able to identify the compound. A similar (in molecular ion and fragmentation) compound was found in globrous canaryseed groats [29].

Peak **6** contained a molecular ion at m/z 452. The MS/MS data showed fragments at m/z 306, 316, 135, 145, and 332. A fragment ion at m/z 306 refers to the deprotonated glutathione (GSH) moiety and CNLs of 120 and 146, indicating the presence of a rhamnoside group.

Peak **7** showed an intense molecular ion at m/z 285. The respective compound was putatively identified as luteolin [27].

CONCLUSIONS

Differences in the metabolomics patterns of wheat varieties could be individualized in the results of PCA. It was found that both the winter wheat grown in conventional conditions and the spring wheat grown in organic conditions had a high content of various oligosaccharides differing thus from the spring wheat grown in conventional conditions. This phenomenon can be explained with osmotic stress resistance. According to our present knowledge, no such result has been reported previously in the open literature for wheat.

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Nisu- (*Triticum aestivum*) sortide peakomponentide analüüs kõrgsurvevedelikkromatograafiliste mass-spektrite alusel

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On välja töötatud fikseerimata sihtmärgita metaboolne strateegia, et määrata nii palju väikese massiga metaboliite kui võimalik 13 suvi-, tali- ja mahenisu (*Triticum aestivum*) sordikogumis. Metaboliidid lahutati kõrgsurvevedelikkromatograafiliselt pöördkolonnis (RP–HPLC), mis oli ühendatud elektronpihustusionisatsioon-tandem-massispektromeetriga (ESI–MS/MS). Protseduur sisaldas metaboliitide ekstraktsiooni, kromatograafilist eraldamist sipelghappe ja atsetonitriili vesilahuste gradiendis ning lahutatud ühendite tandem-massispektromeetrilist identifitseerimist. Erinevate nisusortide metaboolsete mustrite eristamine saavutati peakomponentide analüüsi meetodit (PCA) kasutades. PCA tulemused osutavad nisusortide selgele erinevusele.

Tavapärastes tingimustes kasvanud talinisu ja mahetingimustes kasvanud suvinisu erinevad tavapärastes tingimustes kasvanud suvinisust suurema süsivesikusisalduse poolest. See on seletatav osmootse stressi parema taluvusega. Mahetingimustes kasvanud sordid on teistest sortidest eristatavad PCA tulemuste põhjal, mis osutab erinevate kasvatamisviiside mõjule.