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Metabolomics Reveals Drastic Compositional Changes during Overwintering of Jerusalem Artichoke (*Helianthus tuberosus* L.) Tubers

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ABSTRACT: Metabolic changes were investigated in overwintering Jerusalem artichoke (*Helianthus tuberosus* L.) tubers using proton nuclear magnetic resonance (^{1}H NMR) metabolomics. Three varieties were studied; as a result of overwintering, the amount of inulin was found to decrease in Jerusalem artichoke tubers. This was mainly due to its conversion to sucrose and, at the same time, formation of inulin with a lower degree of polymerization. Major effects on the concentration of citric acid, malic acid, γ-aminobutyric acid (GABA), and adenosine were also found. Intriguingly, malic acid concentration increased and citric acid concentration decreased. These changes, together with an increase in sucrose and GABA concentrations, were ascribed to mobilization of nutrients prior to sprouting, suggesting that malic acid and GABA serve as carbon and nitrogen sources during sprouting of Jerusalem artichokes.

KEYWORDS: *Jerusalem artichokes, citric acid, malic acid, γ-aminobutyric acid, overwintering, ^{1}H NMR*

INTRODUCTION

Root and tuber vegetables, such as carrot (*Daucus carota*), potato (*Solanum tuberosum*), and Jerusalem artichoke, are important food sources. After maturation, crops are subjected to cold storage or overwintering in the soil, that is, left in the soil and covered with straw or uncovered until the following season. In northern countries carrots and Jerusalem artichokes may overwinter in the soil, which make them suitable for consumption during wintertime, whereas most other vegetable crops can only be imported or produced in greenhouses with huge energy input. For example, carrots typically mature in the late fall, when aroma and flavor compounds accumulate,[1] and during storage, either in a cool room or in the field under straw; roots retain their eating quality for several months.[2] Jerusalem artichokes can overwinter in the soil as well and are usually suitable for consumption from September until March or April the following year. During overwintering the tubers are metabolically active, and therefore tuber composition and sensory characteristics change as a function of harvest time and temperature.[3] The sensory attributes of raw Jerusalem artichoke tubers are described as nutty, earthy, apple-like, and sweet, and as a result, Jerusalem artichokes are suitable for different culinary applications.[3,4]

Jerusalem artichokes produce the fructan inulin, which is stored in the tubers as an energy source. Inulin imparts a creamy mouthfeel to low-fat foods[5] and as low-calorie substitute for sucrose in bread and yogurt.[6,7] However, the relatively low sweetness of inulin (10−30% that of sucrose, depending on the degree of polymerization) limits its usefulness as sweetener.[8,9] Inulin consists of fructose units linked through β2,1 linkages with a terminal β2-fructofuranosyl, α1-glucopyranoside linkage. These β-linkages cannot be digested in the human intestinal tract,[10−12] but are fermented selectively by beneficial bacteria in the gut, which is known as a prebiotic effect.[13,14] Jerusalem artichokes are suitable in a diabetic diet because inulin does not increase blood glucose,[15] and the content of mono- and disaccharides is relatively low in tubers.[16]

Presently no study has addressed the metabolic changes of Jerusalem artichokes during overwintering in the soil and here we investigate this crop during postharvest storage (September−March) using ^{1}H NMR spectroscopy based metabolomics. Enzymatic activities are low during storage at northern latitudes with low soil temperatures, but during overwintering it is well-known that inulin is enzymatically converted to sucrose and inulin with lower degrees of polymerization.[16,17] Although the regulation and metabolism of inulin in Jerusalem artichokes are well-studied,[18−22] other enzymatic changes are unknown. Hence, there is a need for a better understanding of the metabolism in Jerusalem artichoke tubers. In the present study we demonstrate that during overwintering, significant metabolic changes occur in Jerusalem artichoke tubers, and we discuss these responses in relation to low temperature stress response and mobilization of energy prior to sprouting.

MATERIALS AND METHODS

Plant Material. Growth conditions, harvest, and preparation of Jerusalem artichokes have been described elsewhere.[3] In short, three different varieties were chosen for the experiments, representing an early variety (Mari), a medium to late variety (Rema), and a late variety (Draga). The Jerusalem artichokes were grown in sandy loam soil in a complete block design with three replicates, and for each block a representative sample was analyzed. Jerusalem artichokes for analysis were taken from the soil 30, 38, and 46 weeks after planting of tubers in the middle of April 2009. These are referred to as first, second, and third harvests. The corresponding cumulative average daily temperatures above 0 °C were 2716, 2898 and 2916, respectively

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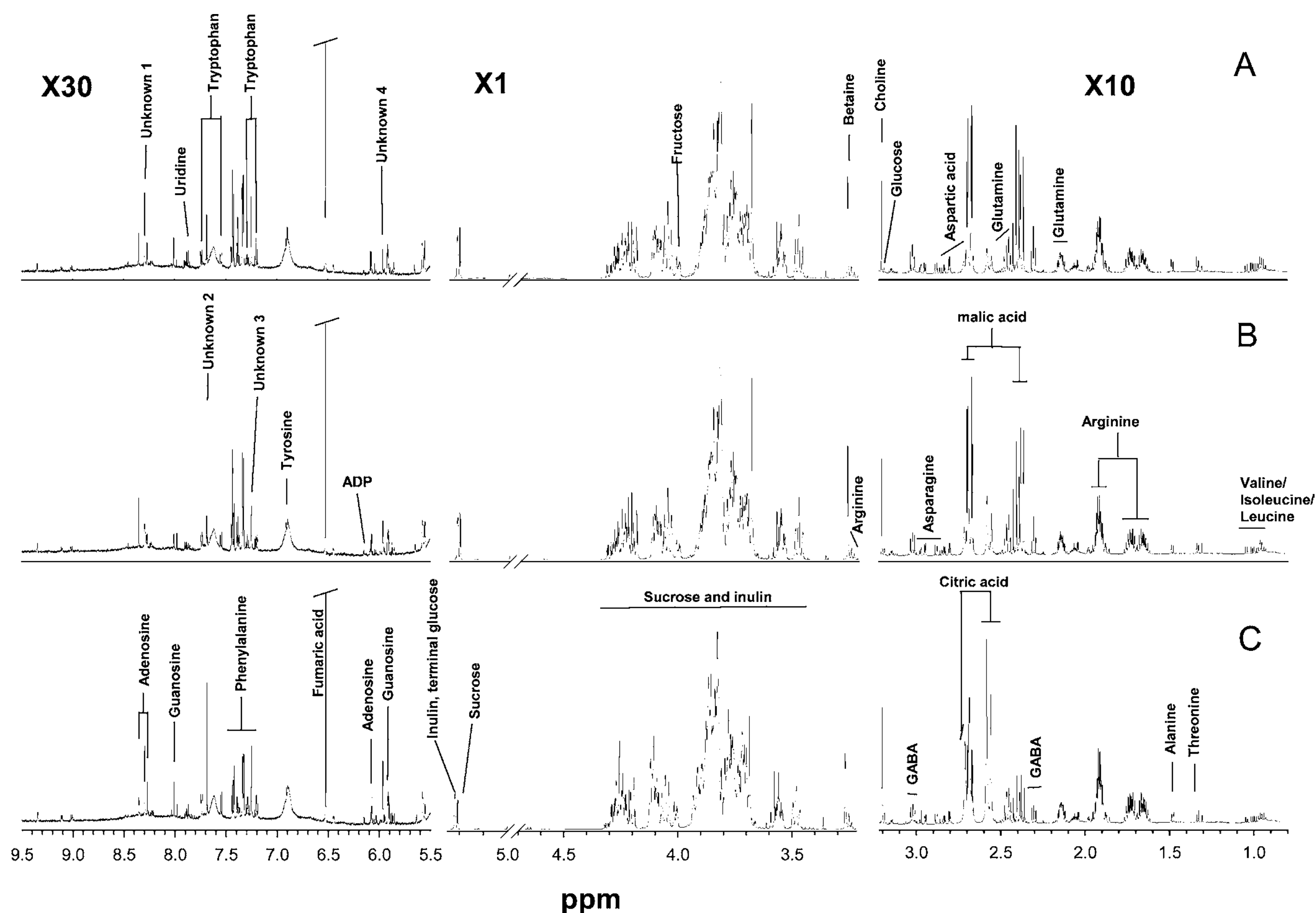

Figure 1. Representative spectra of Jerusalem artichokes, variety Draga, at first (A), second (B), and third (C) harvests. The region from 0.5 to 3.2 ppm has been expanded by a factor of 10 and the region from 5.5 to 9.5 ppm by a factor of 30. Furthermore, signals from residual HDO have been removed, and the signal from fumaric acid (6.53 ppm) has been truncated.

After harvest, Jerusalem artichokes were peeled and shredded into 4 × 4 mm sticks using a food processor (Robot Coupe, CL50, Vincennes, France), snap frozen in liquid nitrogen, and freeze-dried (Gamma 1−20, Martin Christ, Osterode, Germany). Dried plant material was milled in a micro hammer mill equipped with a 1.0 mm sieve (Culatti AG, Zurich, Switzerland) and stored at −24 °C for no more than 2 months prior to analysis.

Metabolite Extraction. Fifty milligram dry samples were weighed into 2 mL Eppendorf tubes. Extraction was performed through the addition of 1 mL of 90 mM phosphate buffer, pH 6.0, in deionized water (Elga, Marlow, UK). Then samples were sonicated for 15 min at room temperature. After centrifugation (10000*g*, 10 min, 4 °C), 500 μL extracts were transferred to NMR tubes and mixed with 100 μL of D_2O, including 0.005% sodium-3-trimethylsilyl-[2,2,3,3-d_4]-propionate (TMSP) (Aldrich Chemicals, Steinheim, Germany). Preliminary studies showed that aqueous samples could be left in the autosampler for 5 h at room temperature without any visible changes in the spectra (data not shown).

^{1}H NMR Spectroscopy. ^{1}H NMR spectra of extracts were obtained on a Bruker Avance III 600 equipped with a TXI probe (Bruker Biospin, Rheinstetten, Germany) operating at a ^{1}H frequency of 600.13 MHz and 298 K. All spectra were referenced to TMSP. One-dimensional ^{1}H NMR spectra of aqueous extracts were acquired with a single 90° pulse, a relaxation delay of 5 s, and 64 scans sweeping 12.7 ppm (offset 4.7 ppm) with 32K data points. Comparison of relaxation delays of 5 and 20 s showed that 5 s was sufficient to quantitate compounds of interest relative to TMSP. The water peak was reduced by irradiation of the water signal with a weak presaturation pulse. Prior to Fourier transformation, the data were multiplied by a 0.3 Hz line-broadening function. The ^{1}H NMR spectra were phased and baseline-corrected manually.

Metabolites were identified using pure standards and two-dimensional (2-D) ^{1}H−^{1}H correlation spectroscopy (COSY) and ^{1}H−^{13}C heteronuclear single-quantum correlation (HSQC). The COSY spectra were acquired with a spectral width of 6130 Hz in both dimensions, 8K data points, and 512 increments with 32 transients per increment. The HSQC spectra were acquired with spectral widths of 8000 Hz in the F2 dimension and 25000 Hz in the F1 dimension, a data matrix with a size of 1K × 256 data points, and 64 transients per increment. All spectra were manually phased and baseline corrected.

Data Analysis and Visualization. NMR data points were bucketed into spectral bins, 0.006 ppm wide. Further correction of NMR spectra was not necessary. Spectral regions from 9.5 to 0.5 ppm were used for multivariate data analysis, leaving out signals from residual nondeuterated water (5.0−4.7 ppm). No normalization was carried out because freeze-dried samples were used for extraction, removing any effects of differences in dry matter content between samples. Furthermore, due to the dominating carbohydrate peaks in the spectra, normalization would lead to values highly dependent on the content of carbohydrates.

Multivariate data analysis was performed using SIMCA-P+ (ver. 13.0, Umetrics AB, Umeå, Sweden). The data set was analyzed using scaling to unit variance. The unsupervised method principal component analysis (PCA) was chosen for the initial analysis, and orthogonal projection to latent structures discriminant analysis (O-PLS-DA)[23] was used for further investigation of differences between harvest times. For validation, seven cross-validation groups were

Table 1. Chemical Shift Assignments in Aqueous Extracts of Jerusalem Artichoke Tubers

compound	chemical shift	assignment type[a]
β-adenosine	6.08 (d, *J* = 6.3 Hz, Rib, C-1, αCH), 8.35 (s, adenine C-8, ring CH), 8.46 (s, adenine C-2, ring CH)	b
alanine	1.48 (d, *J* = 7.1 Hz, C-3, CH_3)	d
arginine	1.69 (m, C-4, CH_2), 1.91 (m, C-3, CH_2)	d
asparagine	2.86/37.5 (m, C-2, CH_2), 2.96/37.4 (m, C-3, CH)	d
aspartic acid	2.66/39.3, 2.81/39.3 (dd, *J* = 3.7, 17.2 Hz, C-3, CH_2)	c
betaine	3.26/56.4 (s, N−$(CH_3)_3$)	c
choline	3.20/56.7 (s, N−$(CH_3)_3$)	c
citric acid	2.57/47.4, 2.70/47.4 (d, *J* = 15.0 Hz, C-1/C-3, CH_2)	d
formic acid	8.46 (s, C-1, CHOOH)	a
fructose	4.00/72.1 (m, C-5, CH), 4.03/66.4 (dd, C-6, CH)	d
fumaric acid	6.52/137.8 (s, C-2/3, −CH=)	c
GABA	1.90 (m, C-3, CH_2), 2.30 (t, *J* = 7.2 Hz, C-2, CH_2), 3.02 (t, *J* = 7.3 Hz, C-4, CH_2)	d
glucose	3.25/77.1 (dd, C-2, βCH), 4.65/98.8 (d, *J* = 7.7 Hz, C-1, βCH), 5.24/94.9 (d,*J* = 3.9 Hz, C-1, αCH)	c
glutamine	2.14/29.1 (m, C-3, CH_2), 2.46/33.9 (m, C-4, CH_2)	d
guanosine	8.01 (s, guanine, C-8, =CH−), 5.92 (d, *J* = 5.4 Hz, rib, C-1, CH)	a
inulin	3.52 (t, *J* = 9.0 Hz, glc, C-2, CH), 4.11/77.0 (t, fru, C-4, CH), 4.27/79.6 (d, fru, C-3, CH), 5.44/95.0 (d, glc, C1, CH)	d
isoleucine	0.94 (t, *J* = 7.2 Hz, C-5, CH_3), 1.01 (d, *J* = 7.2 Hz, C-3′, CH_3)	d
leucine	0.96 (t, *J* = 6.1 Hz, C-5,5′, CH_3)	d
malic acid	2.36/45.2 (dd, *J* = 10.1, 15.1 Hz, C-3, CH), 2.68/45.4 (dd, *J* = 3.1, 15.1 Hz, C-3, CH′), 4.31/73.1 (dd, *J* = 3.1, 9.9 Hz, C-2, CH)	d
phenylalanine	7.33 (d, *J* = 6.9 Hz, C5/C9-ring CH), 7.39 (m, C7-ring CH), 7.43 (m, C6/C8-ring CH)	b
sucrose	4.06/77.0 (t, fru, C-3, CH), 4.22/79.2 (d, fru, C-4, CH), 5.42/95.0 (d, *J* = 4.0 Hz, glc, C-1, CH)	d
threonine	1.33/22.9 (d, *J* = 6.6 Hz, C-4, CH_3)	d
tryptophan	7.21 (m, C-10-ring, =CH−), 7.29 (s/m, C5/C11-ring, =CH−), 7.55 (d, *J* = 8.1 Hz, C-8-ring, =CH−), 7.74 (d, *J* = 8.0 Hz, C-9-ring, =CH−)	d
tyrosine	6.90 (m, C-5/9, ring CH), 7.20 (m, C6/8, ring CH)	b
unknown 1	8.30 (s)	
unknown 2	7.69 (s)	
unknown 3	7.25 (s)	
unknown 4	5.96 (s)	
unknown 5	3.22	
uridine	5.91 (d, rib, C-1, αCH), 7.87 (d, *J* = 7.8 Hz, uracil, ring CH)	b
valine	0.99 (d, *J* = 7.0 Hz, C-4, CH_3), 1.04 (d, *J* = 6.8 Hz, C-4′, CH_3), 2.26 (m, C-3, CH)	d

[a]Assignment types: a, tentative assignment through comparison with published data; b, comparison with pure standard; c, 2-D experiments, d, both b and c.

chosen randomly. Cross-validated analysis of variance (CV-ANOVA)[24] and cross-validated scores were used to test the robustness of the models. Initial analyses showed that no robust models that discriminate between second and third harvest times could be obtained. Hence, modeling focused on the first versus second/third harvest times. To improve interpretation, the approach of Cloarec et al. was used;[25] that is, loadings were multiplied by the variable standard deviations and plotted on a parts per million scale. Subsequently, back-scaled loadings were colored according to the loading weights.

Quantification was performed by signal integration relative to the internal standard, TMSP.

RESULTS AND DISCUSSION

In the present study the ^{1}H NMR metabolome of Jerusalem artichoke tubers was followed during overwintering in soil. Our data demonstrate the usefulness of metabolomic analysis for classification according to variety and harvest time. In previous studies, metabolomics was used to classify potato varieties,[26,27] to describe the life cycle of potatoes,[28] and to map the metabolic changes occurring in soybeans during storage[29] and in grass and legume cultivars during the season.[30]

Assignment. ^{1}H NMR spectra were assigned on the basis of pure standards, 2-D experiments, and comparison with published data.[31−33] Jerusalem artichoke extracts contained signals from inulin. This is evident in Figure 1 as a broad peak at 5.44 ppm, which was assigned to the glucose unit in inulin. Furthermore, from COSY spectra it was found that the peak shape at 5.44 ppm probably could be attributed to inulin molecules with different degrees of polymerization. Other assignments are described in Figure 1 and Table 1.

Metabolite Variation during Storage. PCA modeling of ^{1}H NMR spectra revealed a classification of Jerusalem artichoke tubers according to both variety (along PC2, Figure 2A) and harvest time (along PC1 and PC3, Figure 2B). There was a slight interaction effect between variety and harvest time; that is, in Figure 2A, PC2 described some of the variation associated with harvest time of Draga and Rema tubers (for clarity, this is not shown in the score plot). To avoid such interactions during analysis and interpretation of the data, an O-PLS-DA model using harvest time as class variable was then constructed. In this model two groups were defined; one group corresponded to the first harvest and the second group corresponded to the second and third harvests. The rationale for this choice was based on (i) the fact that the PCA model did not reveal differences between second and third harvest times; (ii) cross-validated predictive ability (Q2) was low in O-PLS-DA models, where the second and third harvest times were treated as

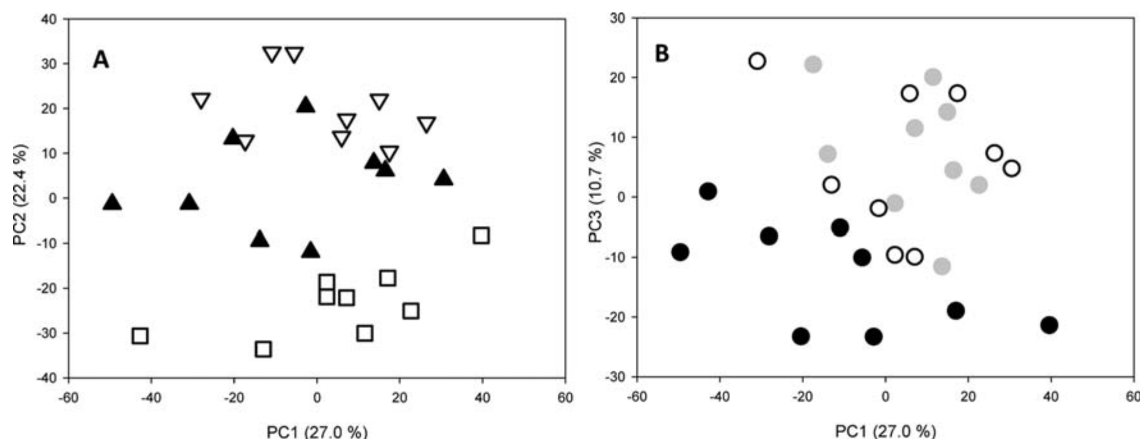

Figure 2. PCA score plots showing the grouping of Jerusalem artichokes according to variety (A) and harvest time (B). Symbols in panel A correspond to 'Mari' (□), 'Draga' (▲), and 'Rema' (▽) and in panel B to first (black), second (gray), and third (white) harvests.

separate groups (for example, Q2 was only 0.4 in a model with all three groups and 0.0 in a model discriminating between the second and third harvest times); and (iii) cross-validated score plots of these two models revealed no classification between groups, indicating overfitting.

In the final model, classification persisted in the cross-validated score plot (Figure 3) and Q2 was 0.91. Significance test of residuals (CV-ANOVA) showed that model residuals were significantly lower (p = 2.0 × 10^{-6}) than the residuals calculated for the global mean, also indicating that the model was not overfitted. The cross-validated score plot confirmed that the projection of second and third harvests was similar but differed from that of the first harvest. Furthermore, the projection of 'Mari' samples after the first harvest should be noted. These samples had slightly higher cross-validated scores on PC1 than the other samples taken after the first harvest, indicating a higher degree of maturity and a metabolite composition more closly resembling that of the samples from the second and third harvests. This was consistent with the fact that 'Mari' matured earlier than 'Draga' and 'Rema', and it was the only variety that flowered during the growing season (unpublished observation).

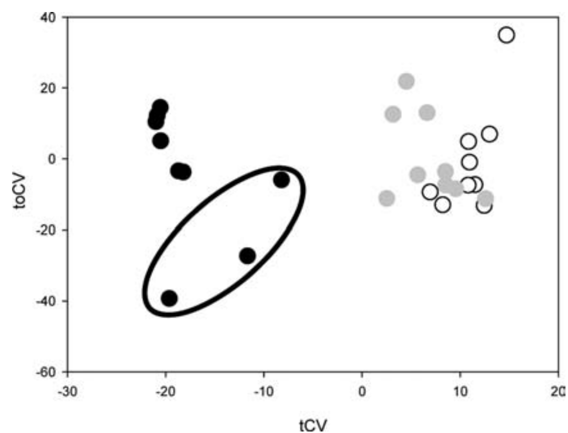

Figure 3. O-PLS-DA cross-validated score plot. Encircled samples correspond to early-harvested 'Mari' samples. Symbols as in Figure 2B.

Carbohydrates. In Figure 4, back-scaled loadings from the O-PLS-DA model are plotted on the parts per million scale, which allows us to compare this plot directly with the identifications obtained in Table 1 and Figure 1. Furthermore, the loading weights are shown as color codes, which enable us to identify significant contributions from less abundant metabolites.

The most prominent contributors to the discrimination between first and second/third harvest times were signals from sucrose and inulin (Figure 4). The results clearly showed that

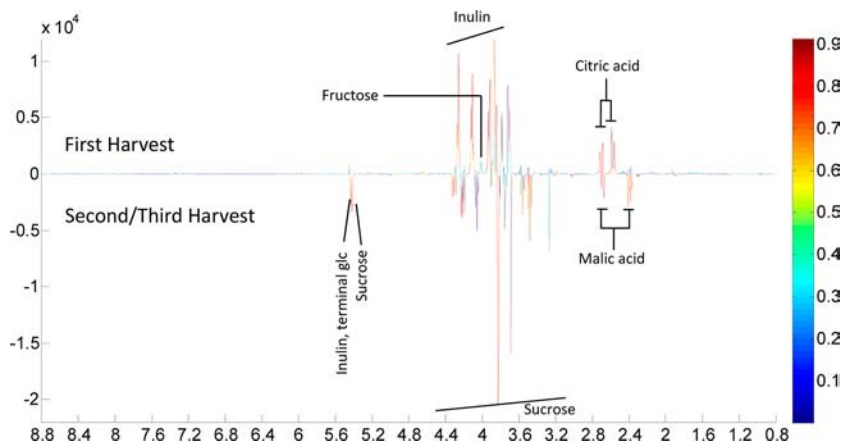

Figure 4. Plot of O-PLS-DA back-scaled loadings contributing to the discrimination between Jerusalem artichokes at first and second/third harvests, as shown in Figure 3. Loadings are colored according to loading weights.

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the sucrose content of tubers increased during storage. Signals from inulin had both negative (5.44 ppm) and positive (4.4–3.6 ppm) loadings. This result seems inconsistent but can probably be explained by two concurrent effects: The absolute amount of inulin was higher after the first harvest, as reflected by the signals at 4.4–3.6 ppm, whereas the signal at 5.44 ppm, which was assigned to the terminal glucose unit, indicated that the concentration (in mol L^{-1}) increased during storage. In other words, these results reflect a decrease in the degree of polymerization of inulin during overwintering.

No effect of overwintering on fructose content was observed (Figure 4), but glucose levels decreased to a very low level (Figure 5D), probably due to degradation of inulin to a lower degree of polymerization, for which glucose units are needed.

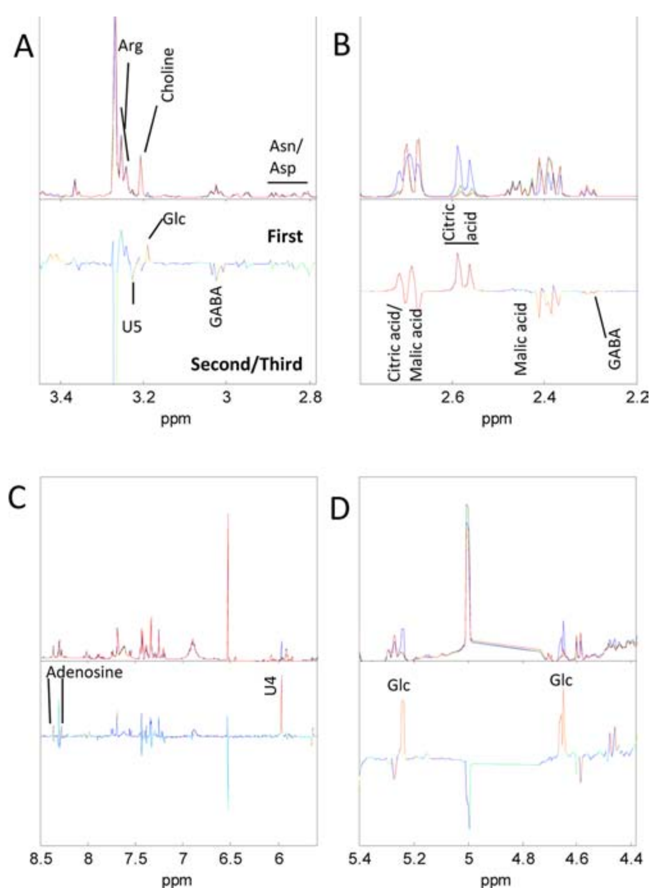

Figure 5. Pairwise depiction of spectra (upper plots) and O-PLS-DA coefficients (lower plots) in selected regions: 2.8–3.5 ppm (A); 2.2–2.8 ppm (B); 5.6–8.5 ppm (C); 4.4–5.4 ppm (D). Spectra are shown in blue (first harvest), green (second harvest), and red (third harvest). Compounds that contributed to discrimination are assigned in the coefficient plots, whereas other compounds are assigned in the spectra.

These observations are generally in accordance with the literature[16,17,19,22] and probably reflect that depolymerization of inulin and formation of sucrose are important postharvest responses when Jerusalem artichokes are stored at temperatures below 0 °C.[34] In fact, in the present study temperatures between the second and third harvests generally were below 0 °C, and the cumulative average daily temperature above 0 °C was only 18 °C between these two harvest points, whereas it accumulated to 182 °C between the first and second harvest points.

Sucrose probably plays a dual role during overwintering. In addition to its protective effect against crystallization of water at low temperatures, mobilization of sucrose during overwintering is needed for tuber respiration and later sprouting. However, sprouting in Jerusalem artichoke tubers is associated with a dramatic increase in the fructose content,[35] which was not observed in the present study. Hence, the tubers were probably still dormant during the three harvest points studied here.

Other Metabolites. The changes in carbohydrate content were accompanied by changes in the content of a number of other metabolites: citric acid, malic acid, adenosine, and GABA. In Figure 5B the contribution of citric acid and malic acid to the classification in the O-PLS-DA is shown; whereas the content of citric acid was highest at the first harvest, the reverse was observed for malic acid. As shown in Table 2 the ratios between citric acid and malic acid concentrations were 1–2 in tubers of 'Rema' and 'Draga' at the first harvest. During overwintering, citric acid concentrations decreased and malic acid concentrations increased, and hence ratios of 0.2–0.02 were obtained. The early maturing variety, 'Mari', had a citric acid/malic acid ratio of 0.3 already at the first harvest, and it further decreased to 0.001, mainly because citric acid was almost depleted during overwintering. Hence, the citric acid/malic acid ratio seems to be an important indicator of developmental stage of the tubers.

Citric acid and malic acid are both intermediates in the citric acid cycle, and their differential formation and depletion are therefore intriguing. Many of the citric acid cycle intermediates serve other functions in plant cells, for example, as precursors of both ascorbate and isoprenoid synthesis and in photorespiration. Furthermore, the steps in the citric acid cycle are differentially regulated and should not just be seen as a series of reactions.[36]

In *Arabidopsis thaliana*, malic acid functions as a carbon source during the night and is thus used as an energy resource when carbohydrates are depleted.[37] This role of malic acid is only transient and seems to be under diurnal control in *Arabidopsis*, as is the case for sucrose and starch.[38] In heterotrophic plant tissue (such as tubers) malic acid acts as a mobile energy source,[39] and in barley seeds, malic acid, isocitric acid, and succinic acid were stored in the vacuole. The same was true, to some degree, for citric acid.[40]

Knowledge about the metabolism in Jerusalem artichoke tubers is sparse. However, our results indicate that malic acid

Table 2. Citric Acid and Malic Acid Concentrations (Millimoles per Liter) in Jerusalem Artichoke Extracts

	first harvest[a]			second harvest[b]			third harvest[c]		
variety	citric acid	malic acid	ratio[d]	citric acid	malic acid	ratio[d]	citric acid	malic acid	ratio[d]
Mari	2.81 ± 0.06	9.4 ± 0.2	0.3	0.07 ± 0.02	13.9 ± 0.7	0.005	0.01 ± 0.06	13.0 ± 0.4	0.001
Rema	5.1 ± 0.7	4.6 ± 0.4	1.1	0.68 ± 0.06	10.1 ± 0.2	0.07	0.2 ± 0.1	11.2 ± 0.5	0.02
Draga	6.53 ± 0.04	3.48 ± 0.07	1.9	2.1 ± 0.1	9.4 ± 0.7	0.2	1.1 ± 0.2	10 ± 1.1	0.1

[a]Harvest 30 weeks after planting. [b]Harvest 38 weeks after planting. [c]Harvest 46 weeks after planting. [d][Citric acid]/[malic acid].

serves a role as an energy resource which is built up during the winter. Malic acid is hence ready for mobilization as temperature increases and tubers begin to sprout after a period of vernalization. As shown above, the accumulation of malic acid is more pronounced in the early-maturing variety, Mari, and could be an indicator for the tubers ability to start sprouting.

Adenosine and one unknown metabolite (unknown 4, 5.9 ppm) were more abundant after the first harvest (Figure 5D), whereas GABA concentrations and an additional unknown (unknown 5, 3.22 ppm) were higher in Jerusalem artichokes after the second/third harvest (Figure 5A).

Adenosine serves as precursor for DNA synthesis and participates in the synthesis of other biomolecules.[41] These processes are important during cell division, and the decrease in adenosine content during overwintering could be a result of recruitment of adenosine for DNA synthesis.

GABA is synthesized from glutamate through the action of glutamate decarboxylase in the cytosol. After transport to the mitochondria, GABA is converted to succinic acid anhydride and subsequently succinic acid, and thus GABA serves as a fuel for the citric acid cycle. This series of events is often referred to as the GABA shunt.[42] The ^{1}H NMR spectra were very crowded around the chemical shift of succinic acid (2.30 ppm), and it was not possible to determine if succinic acid content also changed during overwintering. Thus, it remains unknown if malic acid and GABA serve a unique function. However, information about fumaric acid, glutamine, aspartic acid, asparagine, and arginine was obtained from the ^{1}H NMR metabolomic data, and none of these compounds changed concentrations to the same extent as GABA during overwintering (Figures 4 and 5A,B). We therefore assume that GABA and malic acid accumulate prior to sprouting to serve as carbon and nitrogen reserves during sprouting.[43] In line with this assumption, it has been shown that the GABA shunt provides building blocks during the germination of *Castanea sativa* L. seeds and seedlings.[44]

This study provides a metabolomic investigation of Jerusalem artichoke tubers. The connection between overwintering and transformation of metabolites indicates that malic acid and GABA are key metabolites in Jerusalem artichoke sprouting after overwintering in the soil at low temperatures.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

2-D, two-dimensional; COSY, ^{1}H−^{1}H correlation spectroscopy; CV-ANOVA, cross-validated analysis of variance; GABA, γ-aminobutyric acid; ^{1}H NMR, proton nuclear magnetic resonance; HSQC, ^{1}H−^{13}C heteronuclear single-quantum correlation; O-PLS-DA, orthogonal projection of latent structures discriminant analysis; PCA, principal component analysis; TMSP, trimethylsilylpropionate.

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