# AN EXACT MASS GC-MS METABOLOMIC STUDY OF NITROGEN STARVATION IN *ARABIDOPSIS THALIANA*

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## OVERVIEW

**We established a model of N starvation using Arabidopsis thaliana plants cultivated under hydroponic conditions. Plants were grown for 5 weeks on 6mM nitrate in a hydroponic device and total nitrogen starvation was applied for either two days or 10 days, allowing the analysis of intermediate and long term responses to N starvation. Roots and leaves were sampled separately and extracted for metabolite profiling. The plant extracts were derivatised with methoxyamine hydrochloride and MSTFA and their metabolic patterns analysed in both EI and CI mode on a GCT Premier™ (Waters). The exact mass data was deconvoluted, aligned, normalised and subjected to PCA analysis using the MarkerLynx Applications Manger™ (Waters). Clear separation was observed between the starved and non-starved root and leaf metabolite profiles. The sample clustering showed a more rapid depletion of the nitrogen pool in roots than in the leaves. The main discriminating compounds were found to be amino acids, sugars and organic acids.**

**The data show the wide-ranging effects of plant metabolism to moderate and severe N starvation in roots and leaves.**

## INTRODUCTION

Nitrogen (N) is an essential element for plant growth and development. In natural soils N is often a significant factor limiting plant growth. Nitrogen stress triggers various responses at the level of metabolism, gene expression and development allowing the sessile plant to adapt by short and long term mechanisms. N starvation induces important changes in metabolism N assimilated in biomolecules can be released back to inorganic nitrogen (NH4) which can re-enter metabolism in various physiological processes such as photorespiration and biosynthesis of phenylpropanoids. Due to the co-ordination of N metabolism with other pathways, e.g. C metabolism, major changes in C and N metabolism have been described (Scheible et al 1997 [1]), but more detailed analysis of metabolite profiles are needed to understand the dynamic response of the metabolic network to N stress. Recent studies on potato plants (Urbanczyk-Wochniak and Fernie2005 [2]) and Chlamydomonas (Bolling and Fiehn 2005 [3]) have demonstrated the immense changes in metabolite profiles after N starvation.

## METHODS

### Sample Preparation

Arabidopsis thaliana plants were cultivated under hydroponic conditions using the method of Orsel et al[4]. The plants were grown for 5 weeks on 6mM nitrate in a hydroponic device using short days. Total nitrogen starvation was applied for either two days or 10 days and roots and leaves were sampled separately.

40mg of Arabidopsis leaves and roots (fresh material) were extracted with 1mL extraction buffer (Gulberg et al [5]). The extraction buffer (chloroform/ methanol/ water(1:3:1 V/V/V AT –20°C) allows the extraction of lipophilic and hydrophilic metabolites in one phase. All samples were vortex shaken for 3 minutes and then centrifuged for 10 minutes at 3000rpm and 4°C (glass vials). 300µL of supernatant was dried by speed vac concentration and the dried pellets were conserved under argon and stored at –80°C.

The dried extracts were derivatised using a 2-stage process based on the method of Fiehn et al [6]. 20µL of 40mg/mL methoxyamine hydrochloride in pyridine was added to the dried extracts and held at 28°C for 90minutes. This was followed by the addition of 180µL of MSTFA for 30minutes at 37°C.

## INSTRUMENTATION

*Figure 1. Waters GCT Premier™*

## GC CONDITIONS

| | | | |
|---|---|---|---|
| GC | Agilent 6890N | | |
| Column | J&W Scientific DB-5MS<br>30m x 0.25mm i.d. x 0.25µm film | | |
| Flow Rate | 1.0mL/min Helium | | |
| Injection Volume | 1µL split 20:1 | | |
| Temperature Gradient | Temperature (°C) | Time (min) | Rate (°C/min) |
| | 85 | 2 | 15 |
| | 320 | 5 | |
| Transfer Line | 280°C | | |
| Solvent Delay | 4.3 min | | |

## MS CONDITIONS

| Parameter | Value |
|---|---|
| Ionisation Mode | EI and CI+ |
| Source Temp | 200°C |
| Electron Energy | 70eV |
| Trap Current (EI+)<br>Emission Current (CI+) | 200mA<br>200mA |
| Mass Range | m/z 50-1000 |
| Scan Time | 0.19 secs |
| Inter-scan delay | 0.01 secs |
| Data | Centroid (DRE) |
| CI Reagent Gas | 90:10 Methane: Ammonia |
| Lock Reference EI Exact Mass | Chloropentafluorobenzene 201.9609 |
| Lock Reference CI+ Exact Mass | 2,4,6-Tris(trifluoromethyl)-1,3,5-triazine 286.0027 |

## RESULTS

The data acquired was processed through the Waters MarkerLynx Application Manager and the resulting PCA scores plot from all of the EI data is shown in figure 2. This shows clear separation of leaves and roots and the samples from each stage of the nitrogen starvation experiment. The CI data showed similar clustering in PCA (not shown) but was principally used for molecular weight confirmation.

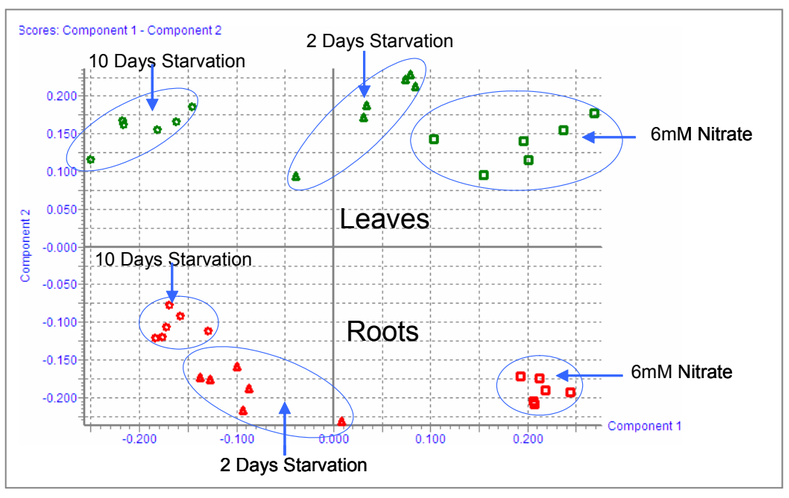


*Figure 2. MarkerLynx PCA scores plot of EI data.*

Representative TIC chromatograms from the EI analysis of the derivatised leaf extracts are shown in figure 3 and from the root extracts in figure 4.

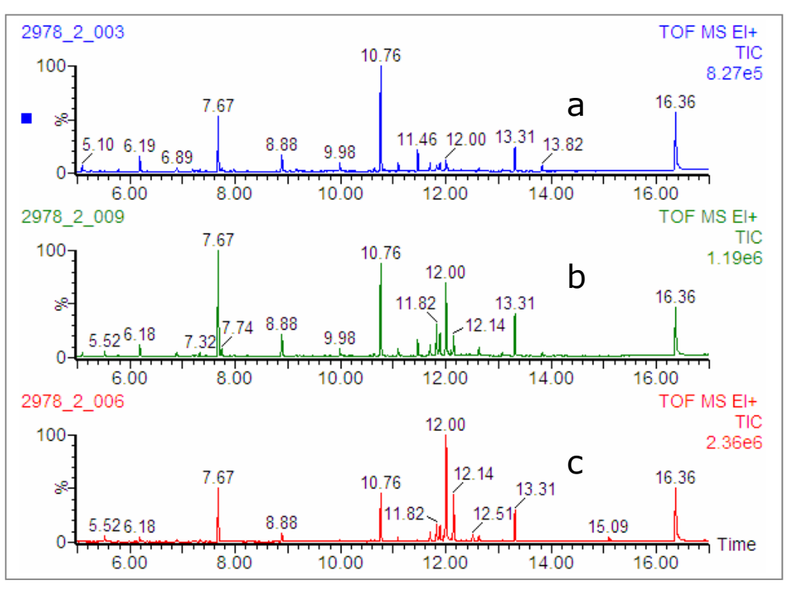


*Figure 3. TIC chromatograms for EI data from leaf extracts from a) unstarved, b) after 2 days and c) after 10 days nitrogen starvation.*

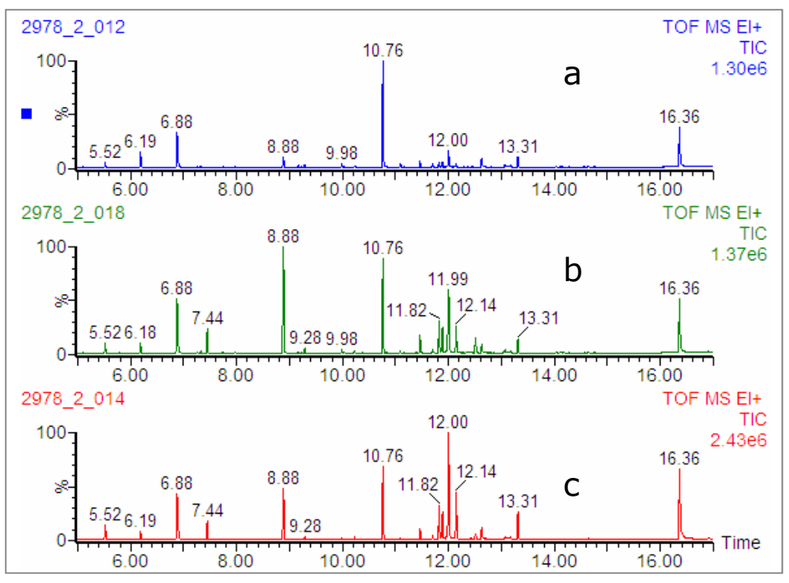


*Figure 4. TIC chromatograms for EI data from root extracts from a) unstarved, b) after 2 days and c) after 10 days nitrogen starvation.*

Visual examination of the chromatograms shows distinct differences between the metabolic profiles of the leaves and roots with the major peak at 7.67 min. in the leaf samples, assigned as fumaric acid, only being present at trace levels in the root samples. Conversely the silanol trimethyl-phosphate peak observed at 6.88 min. in the TIC of the root samples was only present at low level in the leaf samples.

The major changes observed as a result of the nitrogen starvation however were an increase in intensity of the peaks in the retention time range 11.75 to 12.25 min. corresponding to an increase in sugars.

Some of the minor compounds showing a significant change as a result of nitrogen starvation are tabulated below. The table shows their identity based on the best fit from the NIST library. The molecular masses were confirmed by exact mass CI analysis with the calculated masses of the protonated molecular ions and the masses reported by MarkerLynx being shown.

| RT (min) | Identity from NIST Library | Elemental Composition | Calculated $[M+H]^+$ | Measured $[M+H]^+$ | Error ppm | Comment |
|---|---|---|---|---|---|---|
| 5.09 | alanine | $C_9H_{23}NO_2Si_2$ | 234.1346 | 234.1345 | -0.4 | ↓ roots & leaves |
| 7.18 | proline | $C_{11}H_{25}NO_2Si_2$ | 260.1502 | 260.1509 | 2.3 | ↓ roots & leaves |
| 7.26 | glycine | $C_{11}H_{29}NO_2Si_3$ | 292.1584 | 292.1587 | 1.0 | ↓ roots & leaves |
| 7.44 | glycerate | $C_{12}H_{30}O_4Si_3$ | 323.1530 | 323.1520 | -3.1 | ↑ roots |
| 9.22 | pyrogluta-mate | $C_{11}H_{23}NO_3Si_2$ | 274.1295 | 274.1295 | 0.0 | ↓ roots & leaves |
| 9.28 | GABA | $C_{13}H_{33}NO_2Si_3$ | 320.1897 | 320.1911 | 4.4 | ↓ roots |

*Table 1. Calculated and reported masses for some minor components showing a significant change on nitrogen starvation.*

The pyroglutamate identified is in fact the degradation product of glutamine. An example of the CI data obtained for glycerate with elemental compositions within a 5ppm window are shown in figure 5.

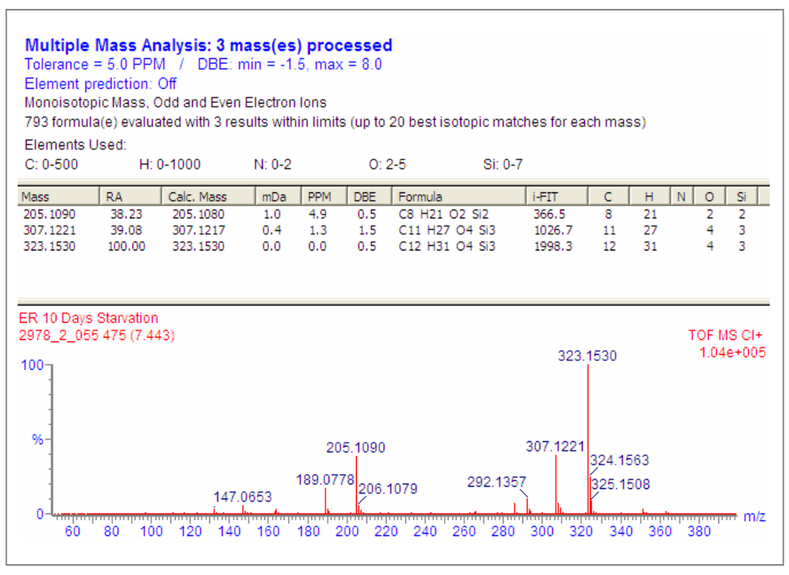


*Figure 5. CI spectrum and elemental composition report for glycerate at 7.44 min which showed an increase in roots after nitrogen starvation.*

## CONCLUSIONS

- **We have studied N starvation in Arabidopsis thaliana focussing on root and leaf metabolic profiles after moderate and severe N starvation.**
- **PCA analysis of the data showed that the 2 day root samples clustered more closely with the 10 day samples than the corresponding leaf samples indicating a more rapid depletion of the nitrogen pool in the roots.**
- **The compounds showing a change were identified by searching the NIST EI library and confirmed by exact mass CI with measurements of <5ppm being routinely obtained.**
- **The principle changes observed were a decrease in amino acids and an increase in sugars.**
- **A detailed comparison of these two organs over the time course of starvation will further the understanding of the plant adaptive behaviour upon N stress.**

## REFERENCES

1. Scheible et al. Plant Cell. 1997, 9, 783-798.
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6. Fiehn et al Anal. Chem., 2000, 72, 3573-3580.

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