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Metabolomics Strategies Using GC-MS/MS Technology

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Key Words

- Arabidopsis Thaliana
- Biological Systems
- GC-MS/MS
- Metabolomics
- MRM
- Quantitation
- Targeted Phase

Introduction

Biological systems are highly complex systems which can be described and analyzed at all levels of complexity and where we still are at the beginning of understanding. Over millions of years, organisms developed incredible ways to survive and interact with their often hostile environments.

Different molecular levels of proteins and metabolites as well as their interactions, including gene expression, and underlying hierarchy of biochemistry and physiology can be summarized as the "genotype-phenotype relationship".[1]

This link between genotype and phenotype is an issue of great interest and leads to the need for analyses at different molecular levels, the so called "omics" approaches: transcriptomics (gene expression), proteomics (protein translation) and metabolomics (metabolic network).[2]

The "metabolome" can be defined as the quantitative complement of all the low molecular weight molecules present in cells at a particular physiological or developmental stage enlarged by the concept of metabolomics introduced as the global analysis of all metabolites in a sample.[3]

Metabolites represent all native small molecules (non-polymeric compounds) that are participants in general metabolic reactions and are substrates, intermediates or products of biochemical pathways which are involved in plant development, growth, reproduction as well as stress response mechanisms.[4] The levels of metabolites can be considered as the response of biological systems to genetic or environmental changes.[1]

The physico-chemical diversity of biological small molecules makes a metabolomic analysis very difficult. Therefore, different analytical strategies are necessary and are ideally combined with each other.[4] Analyses focusing on a group of metabolites as well as watching as many metabolites as possible at specified environmental or developmental stages are called metabolite profiling. On the other hand metabolite target analyses are restricted to specific metabolites of interest, which can be selectively monitored and quantified. However, there is a discrepancy of detectable peaks in a typical metabolomics sample and the number of assignable chemical identifications due to a restricted availability of non-complete reference libraries and technical problems such as ultracomplex coelution of compounds and non-optimal mass spectral deconvolution

in gas chromatography-mass spectrometry. Therefore, in metabolomics we propose a workflow to increase the number of identifiable and selectively quantifiable chemical structures.

In Figure 1 this central strategy in metabolomics is described. First, a semi-quantitative discovery phase identifies as many metabolites as possible from biological samples in a complete unbiased manner. Reference libraries are developed based on existing or novel tools including biochemical knowledge and qualitative data from discovery phases. At the same time, existing reference libraries are continuously updated with novel compounds from natural product libraries or chemical synthesis.

Based on these reference libraries very accurate deconvolution, identification and quantification of single compounds can be performed using multiple reaction monitoring (MRM) with GC-MS/MS-technology. Both the discovery phase as well as the targeted phase are performable with one instrument guaranteeing reproducibility in fragmentation pattern, peak intensities, mass accuracies etc. Intriguingly, this strategy emerges also in proteomics labs building integrative platforms of untargeted (discovery) and targeted (MRM profiling) protein profiling as well as combined metabolomics/proteomics platforms.[5, 6, 7, 8, 9, 10, 11]

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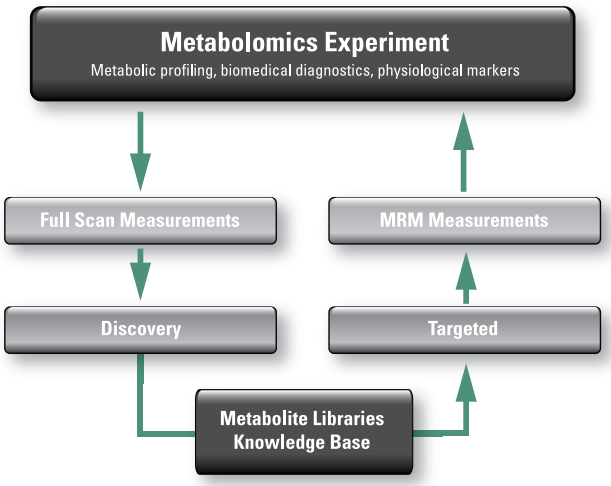


Figure 1: Overall strategy combining full scan MS-analyses of metabolites and targeted analysis. Full scan MS-analysis provides a completely unbiased identification of metabolites and metabolite dynamics. This information is used for sample classification and biological interpretation and the set up of metabolite libraries as well as knowledge bases. Physiological markers are selectively identified and quantified from complex metabolomics samples in a high sample throughput manner with the GC-MS/MS-MRM-technology.

We used this strategy for the investigation of plant metabolism and phytohormone profiling. What makes plants unique is the fact that they "cannot run away", meaning that they are sessile organisms and cannot escape environmental pressures. A long evolutionary history has led to a molecular flexibility that is unique and diverse to cope with the current environmental conditions as well as inhabiting specialized ecological niches. The tremendous phenotypic plasticity is mainly caused by the vast number of metabolites – up to 5 million – occurring in the plant kingdom. This represents one of the richest sources of novel bioactive compounds. The huge amount of metabolites and natural products is generated by modified substrate specificity of enzymes or varying isoforms, occurring in different cell compartments or having altered kinetic characteristics.[12]

In this work we exemplify the overall strategy depicted in Figure 1 and develop a highly sensitive and selective method for the detection as well as absolute quantification of metabolites using the triple stage quadrupole GC-MS/MS-MRM-technology.

Experimental Conditions

Sample Preparation

Leaf material of *Arabidopsis thaliana* Col-0, *Medicago truncatula* grown under different climates, *Clusia minor* as well as a microbial sample were homogenized under liquid nitrogen and about 50 mg fresh material was applied to extraction procedure. A slightly modified water/chloroform/methanol mixture was used to extract water soluble metabolites.[13] After phase separation the polar phase was dried out in a vacuum centrifuge and derivatized in two steps: methoxyamination (methoxyaminhydrochlorid dissolved in pyridine) to suppress keto-enol tautomerism, followed by trimethylsilylation using MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) to derivatize polar functional groups. Total derivatization volume was 100 µL.

Standards were desolved in methanol or water, diluted into various concentrations, dried out and derivatized according to plant material.

GC-MS/MS Conditions

GC: Thermo Scientific TRACE GC Ultra and TriPlus Autosampler

Oven:	
Initial:	70 °C hold 1 min
Ramp 1:	1 °C/min to 76 °C
Ramp 2:	6 °C/min to 330 °C, hold for 5 min
Post-run Conditions:	10 min at 325 °C
Column:	5% Phenyl (equiv) Polysilphenylene-siloxane Thermo TR 5MS SQC 15 m × 0.25 mm ID × 0.25 µm
Injection:	1 µL at 230 °C constant temperature splitless mode (Splitless time 2 min), split flow 10 mL/min
Carrier:	He, constant flow at 1 mL/min
Transferline:	340 °C

Mass Spectrometer: Thermo Scientific TSQ Quantum GC

Ionization Mode:	EI positive ion
Emission Current:	100 µA
Ion Source Temperature:	250 °C
Scan Mode 1:	Full Scan (*m/z* 40-600) Scan time 250 ms
Scan Mode 2:	SRM (Selected Reaction Monitoring)
Indole-3-acetic Acid:	*m/z* 319.15 (202.24)
Glucose:	*m/z* 319.21 (129.00)
Salicylic Acid:	*m/z* 266.90 (249.00; 73.08)
Scan Time:	10 ms
Peak Width:	Q1 0.7 Da (FWHM)
Collision:	CID (Collision Induced Dissociation)
Collision Gas:	Argon
Pressure:	1 mTorr
Collision Energy:	
Indole-3-acetic Acid:	20 V
Glucose:	20 V
Salicylic Acid:	15 V

Results

In Figure 2, a full scan mode analysis of a complex sample is shown with assignments of identified and quantified metabolites. Supervised and unsupervised multivariate statistics are used to validate these multiple analyses of biological samples and replicates. This information is used for sample classification, biological interpretation and screening of physiologically interesting compounds or metabolic markers (Figure 3, PCA plot).[14]

One of the great challenges in metabolomics is to cope with the high dynamic range from very low abundance metabolites like phytohormones to highly concentrated compounds, like energy-related carbohydrates. For low concentrated metabolites and especially the analysis of chromatographic regions with ultra complex coelution of different compounds, MRM-strategies are developed. Very low detection limits and a dynamic range of 4 orders of magnitude can be presented for phytohormones. Compared to previous studies using single quadrupole and ion trap MS instruments the analysis of indole-3-acetic acid (IAA) shows up to 10 folds more sensitivity, salicylic acid (SA) up to 20 times.[15, 16]

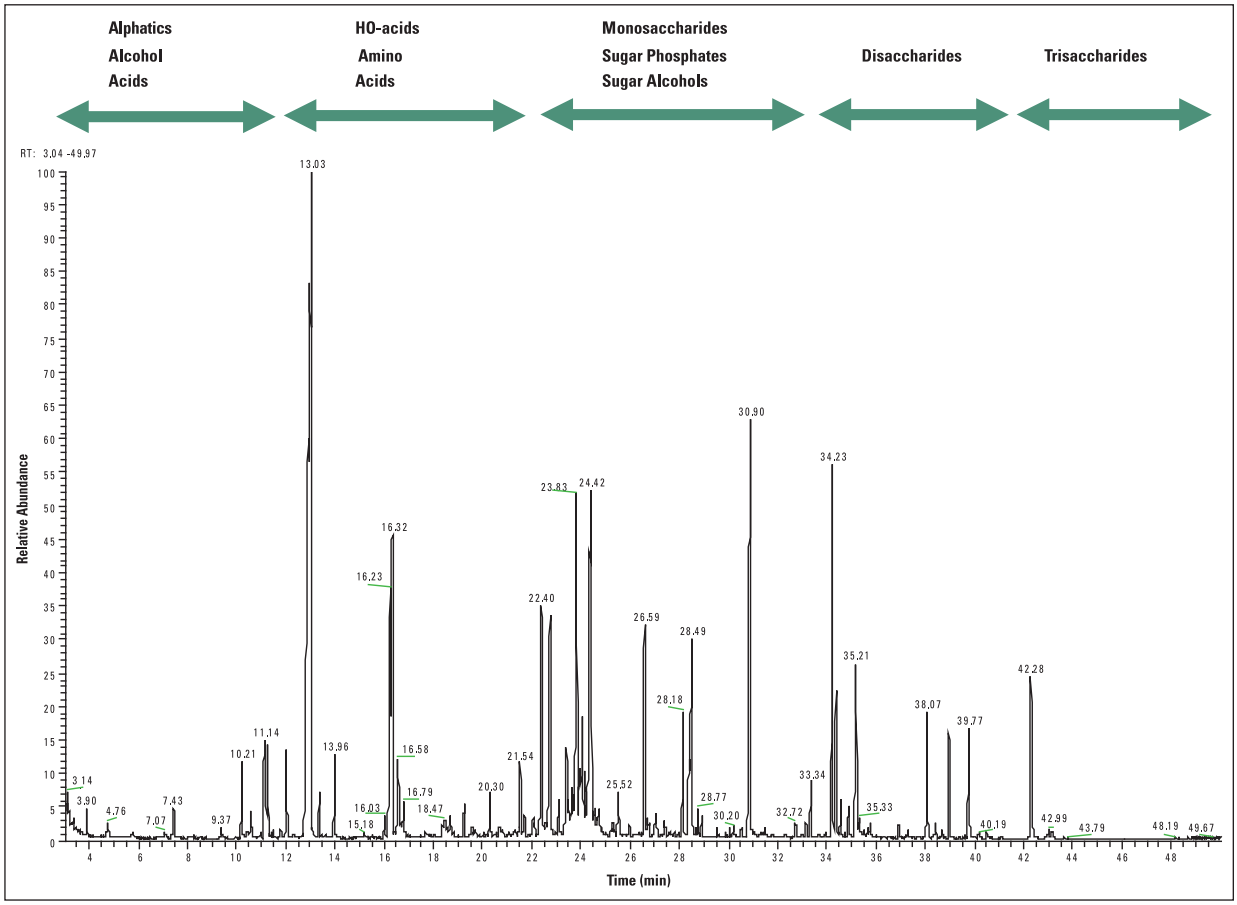


Figure 2: A complex metabolite profile measured in the full scan mode of the GC-MS/MS instrument. Chemical classes are assigned to the corresponding chromatographic regions.

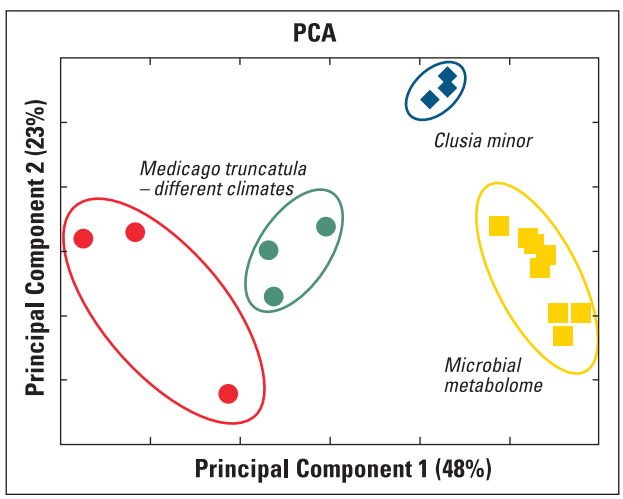


Figure 3: Principal components analysis of different biological samples. To classify the samples quantitative full scan metabolite profiles were measured with the GC-MS. Biological replicates are shown in the same color. Each biological sample can be unambiguously distinguished from the others. Individual metabolites are identified by this multivariate analysis, which discriminate the biological samples.[17]

Figure 4a–c shows an 18-level calibration curve for glucose in the range from 1 fmol to 1 nmol injected amount on column, representing 4 orders of magnitude with an excellent linearity of 0.9985. In Figure 4b and Figure 4c linear calibration curves for SA and IAA are shown.

A representative example of excellent selectivity is presented in Figure 5, demonstrating the ability of separating coeluting analytes, using different SRM transitions. Indole-3-acetic acid and glucose have similar retention times and the same parent ion; however, they are well separated by the GC-MS/MS-MRM analysis.

The calibration curves are used for the quantification of low-abundance compounds, like the phytohormone IAA as well as highly concentrated metabolites in plants, like glucose (Figure 6a-b). The calculated amount of IAA in *Arabidopsis thaliana* leaves is 213 fmol per injection, equivalent to 358 pmol/g fresh weight. As IAA is typically found in *A. thaliana* in the range of 100-1000 pmol per g fresh weight, the results correspond to expected values.[15]

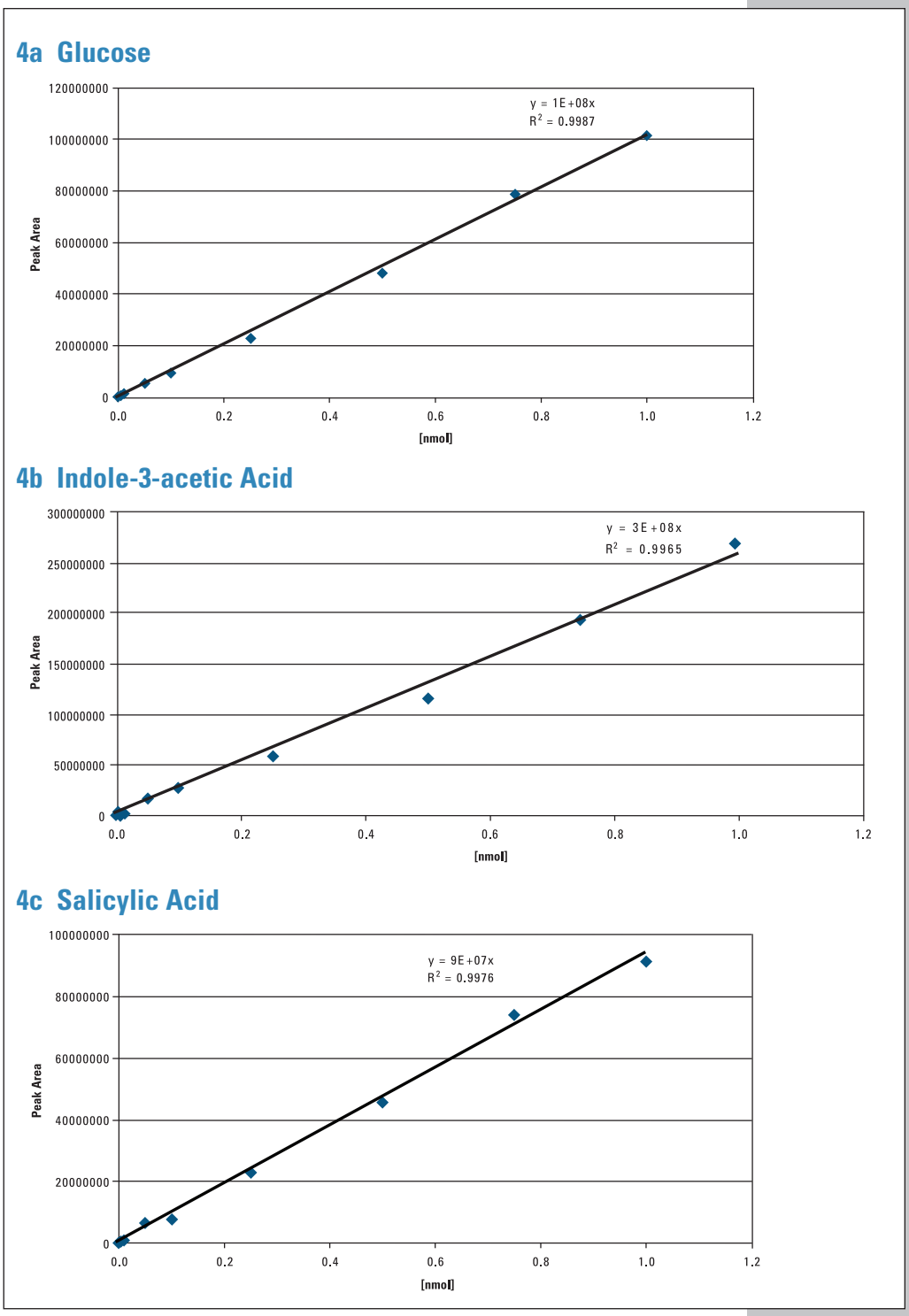


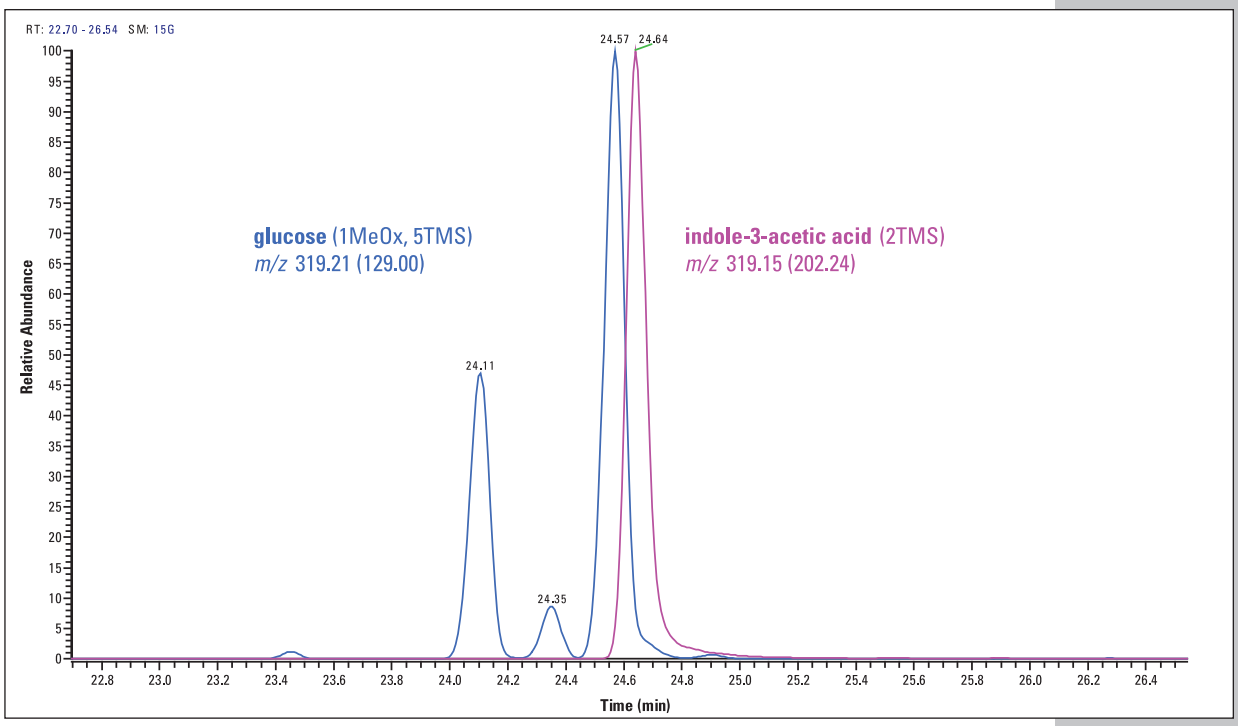
Figure 4: Calibration curves using GC-MS/MS-MRM analysis

4a: Linear calibration curve for glucose from 1 fmol to 1 nmol on column

4b: Calibration curve for indole-3-acetic acid in the range of 10 fmol to 1 nmol on column

4c: Calibration curve for salicylic acid (7.5 fmol to 1 nmol per injection)

Figure 5: SRM chromatogram of IAA and glucose reference compounds at the level of 50 pmol injected amount



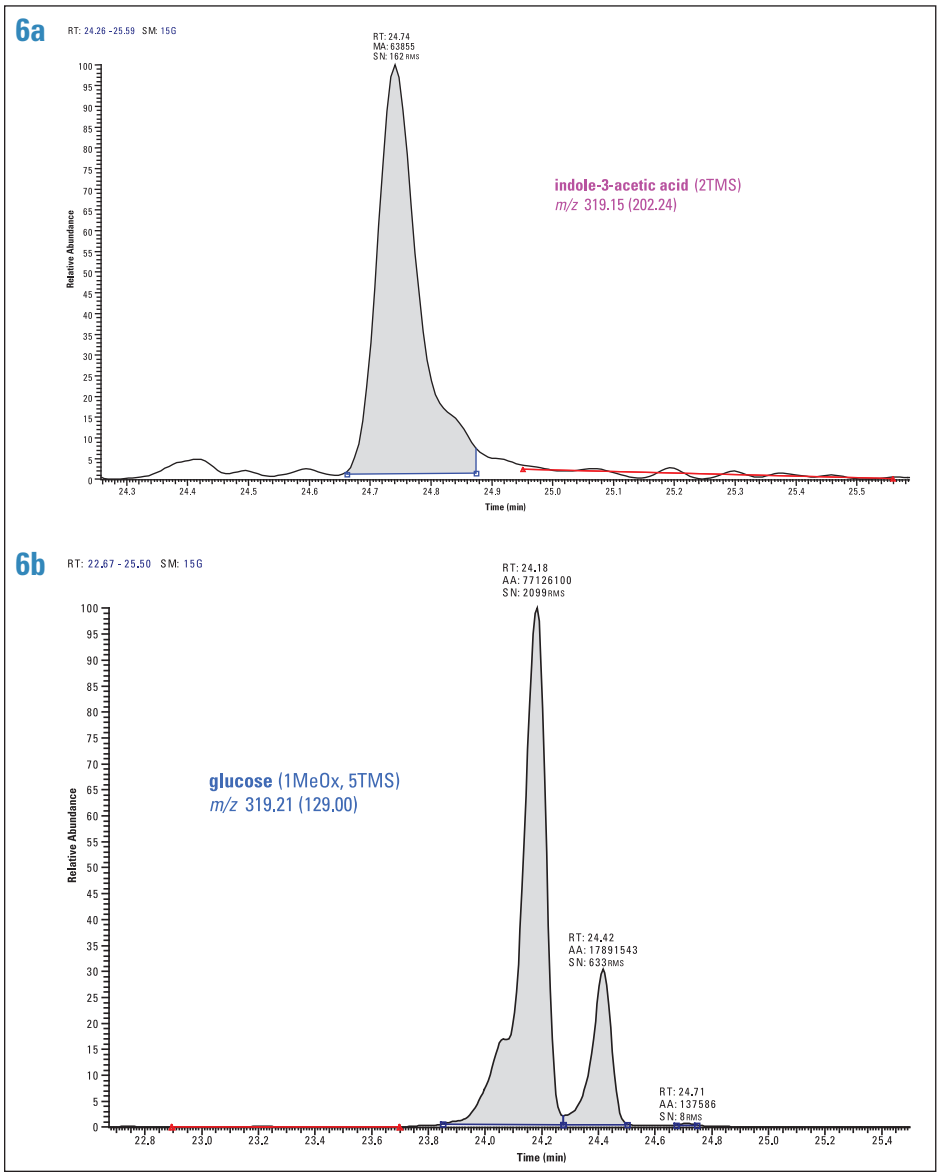


Figure 6: GC-MS/MS-MRM analysis of real complex metabolite samples from Arabidopsis thaliana leaves.

6a: SRM chromatogram of an Arabidopsis thaliana leaf sample containing 358 pmol/g fresh weight indole-3-acetic acid (equivalent to 213 fmol on column).

6b: SRM chromatogram of an Arabidopsis thaliana leaf sample containing 1.6 µmol glucose per g fresh mass (equivalent to 0.9 nmol on column).

Conclusions

The TSQ Quantum GC™ GC-MS/MS instrument provides both the discovery phase analysis and very accurate and selective identification and quantification over a high dynamic range of interesting metabolite markers from complex mixtures using the MRM mode. Compounds, which cannot be separated through gas chromatography, can be separated using compound-specific MRM-transitions in one run.

The TSQ Quantum GC is an optimal instrument for use in metabolomics to perform the cycle of metabolite screening on the one hand, and targeted metabolite analysis in complex samples, on the other (see Figure 1). Aims of ongoing work are to extend the current approach to the analysis of hundreds of target metabolites in one GC-MS/MS-MRM run.

By this approach, metabolome coverage will increase continuously while, at the same time, accurate and selective identification and quantification is performed in a high sample throughput manner.

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