Metabolomics @ Bruker
All the pieces to solve the Metabolomics Puzzle

Poster Hall Edition 2

Innovation with Integrity

Mass Spectrometry, NMR
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Metabolomics has emerged as an exciting new area of biological research and gained substantial attention in recent years. Previously, significant efforts have been made to probe and understand the genomes, transcriptomes and proteomes of a variety of organisms. Despite massive investments to sequence and identify cellular DNA, RNA and proteins, our understanding of many key biological functions remains rudimentary and incomplete. Many researchers believe that perhaps more complete answers can be gleaned from studying small molecules that are utilized for a variety of metabolic and cellular functions. Because of this belief, in many laboratories, Metabolomics is catching up with traditional Proteomics and Transcriptomics efforts and has been christened “Biochemistry’s new look” [1].

Typically, during a Metabolomics study, a large number of cell or patient samples are analyzed and compared to identify potential biomarker compounds which correlate to a disease, drug toxicity, or genetic or environmental variation. Validated biomarkers can form the basis for new diagnostic assays, and lead the way to help establish truly personalized medicine, since changes in small molecule concentrations are closely related to the observed phenotype.

Answering the Challenges of Metabolomics

Metabolomics researchers are regularly confronted with the daunting tasks of acquiring large sets of data and then analyzing them in minute detail. Due to the chemical diversity of small molecule metabolites, it is impossible to study the entire metabolome using a single analytical technique or technology. Bruker’s comprehensive solution for metabolomics is based on putting together “the pieces of the puzzle” to give a complete picture: The Metabolome’s chemical space can be fully explored by utilizing Bruker’s high performance LC-MS, GC-MS, CE-MS and NMR systems in conjunction with dedicated software for data evaluation.

One of the most challenging tasks in the Metabolomics studies is the identification of unknown compounds. Exact mass and isotopic pattern information in MS and MS-MS spectra acquired by the solariX (FT-MS) or micrOTOF and maXis series (Q)-TOF-MS instruments can be used to definitively identify unknown compounds. The intrinsic capabilities of these instruments in conjunction with the SmartFormula3D software enables accurate and reliable molecular formula generation for a wide variety of unknowns. Subsequent database queries identify likely compound structures, which can be confirmed by complementary information derived from NMR spectra.

In order to enhance separation and increase throughput, many laboratories have turned to utilization of UHPLC for Metabolomics studies. Since the mass accuracy, isotopic fidelity and resolution of maXis and micrOTOF-series ESI-(Q)-TOF instruments are independent of the acquisition rate, they are perfectly suited for a coupling to UHPLC separations.

By utilizing a Bruker solution, their high-resolution and accurate mass LC-MS capabilities enable both targeted and untargeted Metabolomics workflows. In addition, the broad dynamic range characteristics of these systems allows both, high- and low-abundance compounds to be studied within the same dataset – even within the same mass spectrum.

Finally, Bruker offers the capability to integrate both LC-MS and NMR techniques into a single analytical system for maximum metabolite coverage and molecular identification. The MetabolicProfiler™ is a powerful combination of techniques and is exclusively designed to address all analytical needs for metabolic profiling and structure elucidation.

This poster hall is a “snapshot” of some of the current work utilizing a variety of Bruker solutions for Metabolomics studies. It nicely illustrates Bruker’s aim of providing and putting together the “puzzle pieces” to give a complete picture of the metabolome. We would like to thank all of our partners and customers for their constant input and feedback. This information has served as an invaluable guide for us in our solution development efforts. A special thanks are directed to everyone who has worked to generate the outstanding posters which we have assembled in this poster hall for your benefit.

Sincerely yours,

Dr. Aiko Barsch
Market Manager Metabolomics
Metabolomics Posterbook 2012

Food Metabolomics

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Hibiscus sabdariffa (HS) is a leafy vegetable used as a soft drink. This plant contains organic and phenolic acids, anthocyanins, and flavonoids as main compounds (1, 2). Traditionally, the aqueous extracts of HS have been used in folk medicine to treat hypertension, fever, inflammation, liver disorders, and obesity.

In recent years, this plant has been studied in depth because it exerts a great number of pharmacological activities. Previous studies have shown that HS possesses antitumor and antioxidant properties (3, 4). Recent data also indicate that aqueous extracts of HS might ameliorate metabolic disturbances (5, 6). Furthermore, the hypolipemic properties of PEHS have been recently reported in a cell model for adipogenesis and insulin resistance (8). However, to establish conclusive evidence for the effectiveness of phenolic compounds from this plant on health, it is very important to define the bioavailability and the excretion of these compounds in humans. The objective of this study was to identify the urinary excretion of phenolic compounds in humans after the ingestion of a phenolic-enriched Hibiscus sabdariffa extract (PEHS).

In this study a phenolic-enriched Hibiscus sabdariffa extract (PEHS) has been obtained and encapsulated. Urine samples from a healthy volunteer were collected before and 2, 4 and 6 hours after intake of 500 mg of the PEHS for the evaluation of the urinary excretion of the phenolic compounds present in this plant extract.

This powerful analytical technique revealed the presence of several phenolic compounds from the PEHS in the urine samples. The main compounds detected previously described in the extract were hibiscus acid, hibiscus acid dimethylester, hyperosidic acid, chlorogenic acid isomers, methyl digallate, and N-feruloyltyramine. The maximum excretions of these compounds were found at 2 and 4 hours after the intake of PEHS.

**REFERENCES**


**ACKNOWLEDGMENTS**

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In this study, 3T3-L1 pre-adipocytes were propagated and differentiated according to conventional procedures. Quercetin was added at 100 μM to the media at the beginning of the study. Subsequently, samples of cytoplasm were taken at different time (3, 6, 12 and 18 hours) after quercetin addition.

**RESULTS**

The compound quercetin was quantified by HPLC-ESI-TOF analysis by integrating the area of the signals associated with the retention time of quercetin.

**CONCLUSIONS**

Quercetin was detected in all analyzed samples, except in the control. From the obtained data, the maximum concentration found in cytoplasm was at 3 hour after the quercetin addition. Nevertheless, it is important to note that at 6 hours time point the concentration of quercetin is still high. At the end of the assay, 18 hours after the addition, the level of quercetin is minimum. In all samples except in the control, quercetin-3-O-glucoside has been detected.

**REFERENCES**


**ACKNOWLEDGEMENTS**

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**SUPPLEMENTARY MATERIAL**

See supplementary material for additional information.
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Food Metabolomics

Metabolic consequences of aronia juice intake by human volunteers

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Introduction

Metabolomics is a powerful technique which focuses on high throughput characterization of metabolome (the complete set of low-MW metabolites in biological samples) in order to obtain a molecular profile. This approach has shown a considerable potential in the field of nutrition, so, there is an increasing interest in the use of metabolomics as a tool for the understanding of the interaction between diet and metabolome and to be a promising technique for biomarker discovery related to food intake [1].

Our study was conducted with the aronia fruit (Aronia melanocarpa) common name black chokeberry which belongs to the Rosaceae family. This berry stands out in high amount of flavonoids, quercetin (71.13 mg Kg⁻¹ 93.07%) and kaempferol (3.53 mg Kg⁻¹ 8.9%). Aronia berries are one of richest plant sources of anthocyanine, mainly containing cyanidin glycosides [2].

In a recent study, anthocyanines showed significant potency of antiobesity and ameliorate adipocyte function [3].

The aim of this study was to observe the single and five days changes in the metabolic profile of healthy volunteers following the consumption of aronia juice and to identify the key metabolites.

Materials and methods

Aronia melanocarpa is a berry of Rosaceae family and is rich in anthocyanins that are mainly cyanidin glycosides. This fruit is documented to be rich in flavonoids and anthocyanines and it contains 0.55% of anthocyanines and 0.3% of cyanidin-3-sophoroside. The anthocyanine content in this berry is one of the highest of any known natural source. The berry has the property that it is rich in bioflavonoids and anthocyanines.

The study was conducted on healthy volunteers following the consumption of aronia juice and to identify the key metabolites.

Biomarker discovery

Future identification of metabolites: MS/MS analysis and Databases (HMDB, KEGG, Massbank)

Assay design

Number of patients: 8 (4 men and 4 women)
Age: 28-47
Race: Caucasian
BMI means: 24.5 Kg/m²
Wash out
Wash out
Wash out
Wash out
Day 1
Day 2
Day 3
Day 4

- Workflow for metabolic analysis:

- Assays development

- Data pre-processing

- Multivariate statistical analysis

- Future identification of metabolites: MS/MS analysis and Databases (HMDB, KEGG, Massbank)

Results

Data LC-MS from treatment samples (after aronia juice intake) and control samples (before aronia juice consumption) were analyzed with multivariate statistical analysis, PCA (Principal Component Analysis) and Student’s t-test (Figure 1). In this analysis six hundred masses were approximately generated of which six were selected as the most significative ones (three in positive mode and three in negative mode) (P-value <0.05) (Table 1 and 2). We have chosen the size at m/z 561.0086 as an example to show the score and loading plot. In these graphs, the separation between treatment and control groups can be displayed showing an interesting dispersion in the loading plot.

After statistical analysis, the significative ions were processed by SmartFormula (Figure 3). These molecular formula of the largest features were generated based on the MS data.

Conclusions

- Metabolomics approach with HPLC-q-TOF analysis is a powerful tool for the discovery of metabolic changes in nutritional interventions with humans.
- Four days of aronia juice intake alters the urinary metabolic profile.
- New markers of aronia juice consumption were isolated.

Acknowledgments

This work has been cofunded by financial support of the Spanish CICYT (AGL2007-61694) and CONSOLIDER-INGENIO 2010 (CSD2007-00063) projects. We wish to thank also Spanish EEC for PhD fellowship grant, partially funded by the European Social Fund. Authors are grateful to Maria Bague for help with the aronia juice.

References

Bacterial Metabolomics

COMPREHENSIVE WORKFLOW FOR METABOLIC PROFILING AND IDENTIFICATION OF SECONDARY METABOLITES FROM MYXOBACTERIA

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Introduction

Extracting the relevant information from complex data sets is an important bottleneck in (microbial) metabolomics research. Compared to a focused targeted approach, this objective becomes even more challenging in untargeted metabolomics, aiming at the comprehensive analysis of a particular bacterial strain. Besides the identification of all known compounds it is important to assign the subset of interesting compounds that are “worth” further examination.

Here, we present the combination of targeted and untargeted metabolomics utilizing statistical methods of data mining. ESI-UHR-Q-TOF based analysis of myxobacterial extracts allows for fast generation of high resolution MS as well as MS² spectra which support our screening workflow for the discovery of novel secondary metabolites.

Sample Set

The sample set consisted of 6 biological replicates of a Sorangium cellulosum strain and 6 replicates of the growth medium blank. Myxobacterial extracts were prepared using a methanol extraction of an adsorber resin which was added to the cultivation broth (Ambiflex VAD-X). Samples were diluted 5x with methanol prior to injection. For each sample two technical replicates were measured.

Methods

Sorangium cellulosum extracts were separated using a Hichrom 110C column(150 x 2.1 mm, 1.7 µm particle size) in an isocratic manner using 40 % methanol and 60 % H2O + 0.1 % FA(A) and 100 % methanol for B. For all samples 1500 µl/min and 45 °C using H2O + 0.1 % FA(A) and 100 % methanol for B. For all samples were performed at 600 µl/min and 45 °C using H2O + 0.1 % FA (A) and ACN + 0.1% FA (A). The gradient started at 6 % B for 6.5 min before increasing to 60 % B in another 19 min.

MS measurements were performed using positive ionization mode at a resolution of 100,000. The fragmentation voltage was set at 2000 V. Data were measured in the range 150-1500 m/z, repetition code: 2 y for MS, 3 y for MS².

Pre-processing of data and statistical interpretation using t-test and Benjamini-Hochberg adjustment was performed on all fields. MS/MS experiments were performed at 29.4eV and 11.87 min #1401.

Feature Finding

Myxobacterial extracts represent a complex mixture of small molecules derived from the medium and bacterial secondary metabolites covering several magnitudes in dynamic range. The feature finding algorithm (FMF) assigned approximately 3500 features for each measurement (Fig. 1).

Un-targeted Analysis

The result was evaluated by a t-test comparing extracts and blanks and value-count filtering for features which were present in at least 11 out of 12 measurements in the extract.

A resulting subset of 176 features was exported as a scheduled precursor list (SPL) for directed precursor selection in a subsequent MS/MS experiment (Fig. 2).

Fig. 1: Overall 24 runs were analyzed. The feature finding algorithm assigned ~3500 features per run.

Fig. 2: Features that are only present within bacterial extracts are assigned by means of a t-test. An SPL of 176 features was exported automatically for further CID analysis.

Fig. 3: A result of target compound identification as presented in TargetAnalysis. Example: Assignment of several adducts for the same compound gives more reliable hits.

Targeted Analysis

An in-house database (Myxobase) covering ~700 myxobacterial secondary metabolites representing more than 100 distinct compound classes with retention time and sum formula information was used to identify the known compounds (Fig. 3A).

The 176 filtered features obtained by the untargeted workflow were then compared to the TargetAnalysis result to assign known metabolites. 22 features are related to TA hits or fragments thereof.

To increase the confidence in compound identification by targeted analysis common adduct information was additionally taken into account. The Myxobase system enables the export of a “screening file” for TargetAnalysis which automatically creates common adduct sites for a given sum formula (Fig. 3B).

Fig. 4: Un-targeted MS/MS evaluation of unknown features by MS/MS analysis. Fragment Explorer allows for the annotation of highly accurate MS² peaks, supporting the identification.

Fig. 5: MS/MS spectra of Myxobacter nov. assignments from two different MS² experiments. Un-targeted analysis: MS² spectra evaluation using Fragment Explorer.

Parent ion selection for MS²

Automatic precursor selection during acquisition routinely allows for the fragmentation of dominant species. However, compounds of interest may be low abundant and coeluting with matrix compounds from cultivation media. In such cases, the preselection of parent ions can increase the number of MS² spectra for compounds of interest.

Manual definition of hundreds of precursors is time consuming. Therefore, the subset of 176 features was used for directed precursor selection via an SPL (Fig. 2).

37 % of the precursors of interest were not fragmented precisely during an auto-MS² run. Using SPL lists generated based on t-test results could improve the MS² coverage to 100 % for the target compounds.

Unknown identification

The number of features representing potentially novel metabolites was reduced further by matching specific neutral losses within coeluting features.

Guided MS/MS acquisition allows for the assignment of new metabolites and facilitates their identification (Fig. 4).

Searching in publicly accessible databases did not yield any hits.

113 features remained for further evaluation by MS and NMR experiments.

Conclusions

High resolution accurate mass LC-MS data sets allow for both targeted and untargeted analysis.

Complexity is reduced by statistical data evaluation.

Parent ion selection is significantly improved by a scheduled precursor list (SPL).

ESI-UHR-Q-TOF-MS data from 43 out of 176 filtered features from myxobacterial extracts could be identified based on MS² data, thereby effectively supporting de-replication during screening.

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References


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The impact of high-resolution MS and NMR techniques on the discovery of novel natural products from myxobacteria

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Introduction

Microbial natural products represent a rich and still largely untapped resource for novel chemical scaffolds. Their discovery enables characterization of the underlying biosynthetic pathways and at the same time chemists are good at finding compounds with potent biological activity. UHPLC-coupled UHR-QTOF-MS and database-mining approach is exemplified by the recent discovery of myxosprinomides, an intriguing family of novel secondary metabolites from Myxococcus xanthus (2, 3).

Methods

- **Bacterial strains**: Various myxobacterial strains were cultivated in complex media and extracts were separated by RP chromatography.
- **In vitro experiments**: Myxochelin A, Chondromyces crocatus Sorangium cellulosum and M. xanthus wildtype and knockout strains were used.
- **LC-SPE-NMR** was used for dereplication.
- **Accurate-mass MS2 fragmentation** for the rapid identification of known and novel compound classes due to its ability to generate a partial or even complete structure based on MS/MS data.
- **LC-SPE-NMR** is an important key tool in the discovery workflow (Fig. 4).

Targeted Profiling

- Full scan spectra enable the targeted screening for known myxobacterial compounds based on highly selective EIC traces, using Myxoprin as an analyte database (Fig. 1, 2).
- Profiling reports are parsed into M YXOBASE with all analyte database (Fig. 1, 2).

Metabolome Mining

- The number of known compounds identified to date from many myxobacterial strains is often significantly lower than expected from genome sequence information [1, 2].
- The comparison of feature-extracted secondary metabolites using statistical tools thus has the potential to uncover previously “hidden” natural products (Fig. 3).
- This comprehensive genome- and metabolome-mining approach is exemplified by the recent discovery of myxosprinomides, an intriguing family of novel secondary metabolites from Myxococcus xanthus (2, 3).

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References

[1] “The biosynthetic potential of myxobacteria and their impact on medicine and agriculture”.

Fig. 3 Statistical treatment of extracts derived from wild-type and gene-engineered strains of Myxococcus xanthus. Feature-extracted OM discover the discovery of a novel compound from the myxosprinomide (c506) by using a combination of LC-SPE-NMR/MS and M YXOBASE for myxosprinomide-c506 (right).

Identification

- Accurate-mass MS2 fragmentation is used for the identification of compounds differentiating bacterial strains, for evaluation of chemical novelty and as starting point for structure elucidation.
- LC-SPE-NMR is an important key tool in the discovery workflow (Fig. 4).

Summary

- LC-UHR-QTOF-MS and database-assisted analysis enhance the natural products identification workflow
- Metabolome mining using statistical tools enable novel metabolite discovery
- LC-SPE-NMR/MS for rapid dereplication and structure elucidation

For research use only. Not for use in diagnostic procedures.
**Discovery of Novel Myxobacterial Secondary Metabolites by Mass Spectrometry-based Metabolome Mining**

**INTRODUCTION**

Bacterial Metabolomics for Infection Research, Braunschweig, Germany

**Myxoprincomide biosynthesis: a molecular assembly line "under construction"**

**Discovery of myxoprincomides by comprehensive analysis of the Myxococcus xanthus DK1622 secondary metabolome**

1. **High-resolution LC-MS measurements of samples from knockout mutant strains (replicates) and Principal Component Analysis (PCA) of **
   - **EIC 506.2713 m/z**
   - **• Mxp897 module 6 (Val, Leu)**
   - **• Mxp1622 module 2 (Leu, Met)**
   - **Val – Ser – Leu**

2. **Structural elucidation of**
   - **Myxoprincomide c506**
   - **NMe-Ser – OHVal Phe – – OHVal – Ser – Leu**
   - **(811 Da)**
   - **• Mxp1622 secondary metabolome**
   - **DNM at 489 Da**
   - **• Myxobase**

3. **Summary**

- Metabolome mining led to the discovery of myxoprincomides from M. xanthus, which are biosynthesized in a modular manner by NRPS/PKS modules.
- A number of myxoprincomides which could be isolated and structurally elucidated are actually biosynthetic intermediates, drawing the picture of a NRPS/PKS assembly line "under evolutionary construction".

**REFERENCES & ACKNOWLEDGEMENT**


**Funding from DAAD is gratefully acknowledged (NSC).**

We wish to thank Bruker Daltonik for their continued support and for providing their LC-MS software tools.
Metabolomics studies based on Gas chromatography – Mass spectrometry (GC-MS) are well established and typically employ electron impact (EI) ionisation. Currently, many possible biomarkers remain "unknowns" in these studies. This is due to the lack of reference spectra for a majority of biologically relevant compounds. Hyphenating GC with ES(I)-Q-TOF technology by atmospheric pressure chemical ionisation (APCI) permits the characterization of compounds which can not easily be identified by classical GC-EI-MS.

Corynebacterium glutamicum, a gram positive bacterium, is used in the industrial production of amino acids and can be grown on different carbon sources. Glucose is metabolized via glycolysis and tricarboxylic acid cycle (TCA) whereas propionate is catabolized through the methylcitric acid pathway (see Fig. 1). An involvement of the prpD gene, encoding 2-methylcitrate dehydratase, in the degradation of propionate has previously been shown by Plassmeier et al. [1].

Here, we used a GC-APCI-MS based metabolic profiling approach to analyse metabolite extracts of a prpD mutant C. glutamicum strain grown on glucose, with a propionate pulse in the exponential growth phase. The benefits of high resolution MS data in combination with GC separations to facilitate structure elucidation will be demonstrated.

**References**


**Methods**

C. glutamicum ΔprpD2 strains were cultivated and harvested as described in [1].

- Two biological replicates were grown on glucose, with a propionate pulse in the exponential growth phase.
- Two technical replicates of each culture were harvested by centrifugation before and 1 h after the propionate pulse.
- Dried methanolic metabolite extracts and reference standards were derivatized by methoxymation and trimethylsilylation.
- GC chromatography conditions: Inj. volume: 1µl; Column: HP-5000 (30 m x 0.25 mm id., 0.25 µm); Inj. temperature: 250°C; Helium carrier gas: 1 ml/min; Temp. Gradient: 3 min @ BPC, 3°C/min to 325°C.
- MS: Bruker Daltonics microTOF-Q LT interfaced via a Bruker GC-APCI source. Data was acquired in positive ionization mode from 85 – 750 m/z at 4 spectra per second.

- Relevant features were extracted using the mPristine Mass Profiling software. Feature intensities were normalized to the intensity of the internal standard ribitol.
- Principal Component Analysis (PCA) as well as sum formula generation by SmartFormula® were performed using ProfilingCare® 2.0. MS/MS data was correlated with structural hypothesis using the FragmentExplorer® in DataAnalysis® 4.1.

**Results**

High resolution MS data acquisition by coupling of gas chromatography via an APCI source to a microTOF-Q LT instrument enabled us to use the same data (pre-)processing methods established for LC-TOF-MS data.

- A PCA based on all extracted features revealed a clear separation of samples according to the carbon sources used for cultivating the C. glutamicum ΔprpD2 cells (Fig. 2.A scores plot).
- Peak intensities for all samples of two selected compounds are displayed as bucket statistics in Fig. 2.B. Both show a higher abundance in cells metabolizing propionate.
- Utilizing exact mass and isotopic pattern information SmartFormula® generated 18 possible elemental formulae for compound 27.2min: 144.0839/140.0839/116.0890 234.1340/220.1400/196.1350 in Public Databases using the Compound Crawler®
- The molecular formula of Compound 9.3min: 234.13 m/z was Rank 1 (Fig. 3 A).
- FragmentExplorer® 3D spectrum with annotated sum formulae for compound 27.2min: 144.0839/140.0839/116.0890 234.1340/220.1400/196.1350 (Fig. 2 B). The spectrum revealed a clustering of samples according to the carbon sources used for cultivating the C. glutamicum ΔprpD2 cells (Fig. 2.B).
- Combining accurate mass and isotopic pattern information of MS and MS/MS spectra, sum formulae suggestions for this compound could be reduced to a single hit (Fig. 3.B). The formula C9H24O7Si4 by SmartFormula® is in accordance to derivatized 2-methylitric acid (Fig. 3 inset) and could be confirmed in comparison to the reference standard.

**Conclusions**

- PCA analysis of GC-APCI-QTOF-MS data revealed several compounds elevated in C. glutamicum extracts following an addition of propionate.
- 2-methylitric acid and alanine were identified using accurate mass and isotopic pattern information in MS and MS/MS spectra – proof of concept for the identification of target compounds using high resolution MS technology.
- TOF instrument performance (mass accuracy, resolution and isotopic fidelity) are independent of the chromatographic separation. Therefore these systems are perfectly suited for coupling to gas chromatography.

**Fig. 1:** Metabolic pathways of the glycolysis, TCA cycle and propionate metabolism highlighting the step catalyzed by PrpD2, which is not present in the ΔprpD2 mutant. Adapted from [1].

**Fig. 2:** A: PCA scores and loading plot (Explained Variance FC1 vs. FC2, 44.4% vs 29.9%). B: Isotopic pattern of PrpD2 mutant strain grown on glucose before and after a propionate pulse. The scores plot revealed a clustering of samples according to the carbon sources used for cultivating the C. glutamicum ΔprpD2 mutant strain mainly contributing to the grouping of samples observed in A.
**Bacterial Metabolomics**

**GC-APCI-MS/MS based identification of metabolites in bacterial cell extracts**

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**Introduction**

In order to analyze and understand the metabolism of microorganisms, it is important to know all the components and their interaction. The goal of metabolomic research is to measure concentrations of as many metabolites as possible. It is estimated that about 800 different chemical entities, covering a wide range of polarities, reactive behavior and sizes, are involved in a microbial metabolism.

Conventionally used ionization techniques in gas chromatography – mass spectrometry, like Electron ionization, generate reliable and unique fragment patterns. Commercial and in-house libraries can be used to identify the separated compounds. For identification of unknown compounds additional information is needed.

**Identification strategy**

We screened GC-MS chromatograms of bacteria especially for unknown substances with biological relevance. The microorganisms were cultivated on glucose and stable isotope-labeled [U-13C]-glucose to allow unambiguous identification of biologically related compounds. Sum formulae were calculated with high resolution - mass spectrometry and structural information were gained by MS/MS fragmentation. We present the evaluation of GC-APCI-MS chromatograms using the example of Pseudomonas putida and the structural analysis of a compound from the polar cell extract of Yersinia pseudotuberculosis. This compound does not match to already known derivatives from our in-house library, the Golm Metabolome Database (GMD) and the NIST08 database.

**Metabolome analysis**

**Metabolomic profiling with GC-APCI-MS**

Normally, 250-300 targets were detected in GC-MS chromatograms of *P. putida*. Only 100 derivatives could be identified. With GC-APCI-MS measurements it is possible to detect up to 350 chemical species with accurate mass. We used the same chromatographic methods on every GC-MS system for a fast compound recovery.

The presence/absence of a mass shift between the 12C- and the co-eluting 13C-labeled metabolite derivative allows for a fast compound recovery.

**Mass spectrometry based structure elucidation**

The analysis of 70 eV fragmentation pattern of (derivatized) unknown compounds is difficult without specific information. For instance, the molecular ion is low abundance or not detectable.

**References**


**GC-APCI-MS system**

Agilent 7890A gas chromatograph equipped with a 28-MMS column (Phenomenex), 29:1 split ratio, 5 m×0.15 mm×0.25 μm GC-column, Agilent 6890N, 5975C, Agilent OpenLAB core 3.5.0.0.
Untargeted metabolic profiling of yeast extracts by UHR-Q-TOF analysis to study arginine biosynthesis mutants

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  * Bruker Daltonik GmbH, Bremen, Germany  ** Bruker Daltonics, Fremont, CA

Introduction

Many primary polar metabolites, such as amino acids, are poorly retained by reversed phase LC column chemistries which are widely used for untargeted metabolic profiling studies. Densitometry of primary amino and aromatic amino acids is performed in these studies relying on the reaction of primary amino groups with dansylation reagents, thus enabling a standard RPLC separation with simultaneous enhancement of the signal intensities in electrospray ionization [1].

We analyzed dansylated metabolite extracts from yeast wild type and deletion mutants in the arginine biosynthetic pathway using a novel UHR-Q-TOF instrument. To prove the hypothesis that upstream or downstream metabolites are altered in abundance in mutants we applied an untargeted metabolomics workflow. Target compounds could be identified based on accurate mass and isotopic pattern information as well as based on fragment ions of the dansylated target compounds.

Methods

**UHR-LC-MS:**
- **Column:** IonPac CS22 (50 mm x 4 mm i.d.)
- **Flow rate:** 0.5 µl/min.
- **Injection volume:** 5 µl
- **Mobile phase:** A: H₂O, B: AcOH (99.9%)
- **Gradient:** 0-100% B in 10 min
- **Acquisition rate:** 2.5 Hz

**HRMS**
- **MS:** hrESI, 7.3-7.4 min
- **Maxis:** 0.75-0.80 min
- **Impact:** drift time
- **Data acquired on the maXis Impact UHR-QqTOF, Bruker Daltonik GmbH**

Sample: Yeast wild type as well as arg4 and arg7 gene deletion mutant strains were grown on a synthetic growth medium. Six biological replicates were harvested by centrifugation at the indicated time points. Metabolites were extracted using the Folch method of standard extraction. Densylation was performed by dansylation (see Fig. 1) and separated by HPLC by dansylation (see Fig. 1) and separated by HPLC

Fig. 1. Dansylation reaction schema

**Table 1: Selected differences comparing arg4 and wild type samples.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass [m/z]</th>
<th>Error [ppm]</th>
<th>Atomic formula</th>
<th>-log10 of the p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dns-Isoleucine</td>
<td>284.11 m/z (1+)</td>
<td>0.3 ppm</td>
<td>C11H17N3O2</td>
<td>5.10</td>
</tr>
<tr>
<td>Dns-Leucine</td>
<td>285.11 m/z (1+)</td>
<td>0.3 ppm</td>
<td>C11H17N3O2</td>
<td>5.10</td>
</tr>
<tr>
<td>Dns-Proline</td>
<td>285.11 m/z (1+)</td>
<td>0.3 ppm</td>
<td>C11H17N3O2</td>
<td>5.10</td>
</tr>
<tr>
<td>Dns-Tryptophan</td>
<td>286.11 m/z (1+)</td>
<td>0.3 ppm</td>
<td>C11H17N3O2</td>
<td>5.10</td>
</tr>
</tbody>
</table>

Results

Densitometry of purified amino acids method development

A mix of pure amino acids was derivatized by dansylation (see Fig. 1) and separated by C18 RP chromatography. The high mass accuracy of the acquired full scan HS data enabled to create 2D trace width high resolution Extracted Ion Chromatograms for the target compounds, providing a very high selectivity (Fig. 2).

As shown for the example of Dns-methionine (Fig. 3) the correct sum formula could be calculated with a mass error of 1 ppm and a very good m/z value which provided a measure for the measured and theoretical isotopic fit. The HS/MS spectrum of dansylmethionine contained a characteristic fragment ion near m/z 171. The SmartFormula tool generated the fragment ion distribution and annotated this information which included structural information including the FragmentExplorer, which also enabled a quick annotation of the HS/MS spectrum.

Untargeted Metabolomics of Yeast Mutants in the Arginine Synthesis Pathway

The established method was applied to study yeast mutants blocked in the arginine biosynthetic pathway. Fig. 4 shows the biochemical pathway of arginine with the reactions catalysed by the proteins encoded by arg4 and arg7 highlighted.

Fig. 2. 2D trace width high resolution EICs for dansylated standards reveal a significant retention for derivatized amino acids on a reversed phase column.

Comparison of arg7 vs. Wild Type

In order to determine statistically significant differences between sample groups, a student’s-t test was performed comparing wild type vs. arg4 and wild type vs. arg7 samples. Fig. 5 presents the volcano plot of the t-test result, plotting the log2 of fold changes vs. -log10 of the p-value, for the arg 7 vs. wild type comparison. The calculated sum formula fits to the data set of 156 features with a 60 fold increase in arg7 samples and a p-value of 0.0000026 had an accurate mass of 436.1568 m/z. Fig. 6 shows the overlaid measured spectrum and the simulated spectrum for C₁₉H₂₆N₃O₅S which was ranked #1 with the best isotopic fit by Smartformula. This sum formula fits to Dansylated N-acetylornithine with the calculated sum formula as the metabolite downstream of the reaction catalysed by the arg7 gene product. EIC traces for the target compound were plotted in Proficurve, which could validate the accumulation of Dansyl N-acetylornithine in arg7 mutant samples (Fig. 6 B inset).

Comparison of arg4 vs. Wild Type

Table 1 presents several selected compounds which were significantly different (p-value <0.05) comparing arg4 and wild type samples. Using accurate mass and isotopic pattern information elemental composition as well as standard reporter ions for indicating a derivatized compound, the SmartFormula tool generated a quick annotation of the HS/MS spectrum.

All features were extracted from the raw data using “Find Molecular Features”. This algorithm can combine isotopic peaks, charge states and adducts which belong to the same compound into one feature. Based on these extracted features an unsupervised clustering could clearly separate the derived metabolic extracts from wild type and both mutant strains (Fig. 5).

Fig. 3. Example - Dansylmethionine A: MS spectrum measured on a m/z range of 380-400 B: HS/MS spectrum with annotated fragment ion distribution. C. SmartFormula result for the given sum formula which produced a significant reporter ion indicating a derivatized compound. D. SmartFormula2D result for the given sum formula based on precursor and fragment ion information. E. FragmentExplorer links molecular formulae from SmartFormula2D result with structural suggestions for fragment ions and enables a quick annotation of the HS/MS spectrum.

Fig. 4. Arginine biosynthesis highlighting steps blocked in yeast arg7 and arg4 deletion mutants.

Fig. 5. Unsupervised clustering: Dendrogram reveals a class separation according to the analysed yeast wild type and mutants.

Conclusions

- Densitometry of metabolites enables a RP-LC separation of primary metabolites like amino acids
- Data acquired on the maXis Impact instrument permits an unambiguous molecular formula generation for known and unknown compounds
- An untargeted metabolomics workflow, analysing arginine biosynthesis yeast mutant strains, uncovered unexpected and unmetabolic changes in the arginine pathway.

UHR-QqTOF
A workflow for metabolic profiling and determination of the elemental compositions using MALDI-FT-ICR MS

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Introduction
Metabolomic studies can lead to the enhanced understanding of mechanisms for drug or xenobiotic effects and an increased ability to predict individual variations in drug response phenotypes. Tandem mass spectrometry (MS) has been extensively used for this purpose, however, an identification of metabolites in this approach is dependent on spectral databases or comparisons with authentic reference standards. This is hampered by the fact that only about 20% of all existing metabolites are currently available. Since Fourier transform ion cyclotron resonance (FT-ICR) MS provides an ultrahigh mass resolution and accuracy which enables isotopic fine structure analysis it allows for highly confident chemical annotations. In addition, due to this high resolving power a separation of complex samples by liquid chromatography is often not essential. Here we present a metabolic profiling workflow based on matrix assisted laser desorption ionization (MALDI)-FT-ICR MS which offers rapid profiling capabilities for metabolic analyses.

Methods
HepG2 cells, a human liver carcinoma cell, were treated for 24 h with the anticancer drug 5-fluorouracil (5FU) at IC50 (10 µM; 5FU-Low) and excess (50 µM; 5FU-High) concentrations. Intracellular metabolites of five biological replicates (n=5) were extracted by a toluene extraction method using methanol/water/chloroform=2/2/1. A solvent FT-ICR MS (Bruker Daltonik GmbH, Bremen, Germany) has been used for MS measurements. The MS system was equipped with a 9.4 tesla superconducting magnet and an ESI/MALDI dual ion source.

Measurements were carried out in MALDI mode, 3-kynurenine (3Kyn) was applied as matrix in negative ion mode. Data collection was performed using the omniPC algorithms (Bruker Daltonik GmbH). The acquired data were evaluated using multivariate statistical analysis. Principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were carried out using SIMCA P+ version 12.0.1. Elemental compositions were calculated using CompoundCrawler software tool (Bruker Daltonik GmbH). The derived formula candidates were queried in the public database Chemspider using the CompoundCrawler software tool (Bruker Daltonik GmbH).

Results and Discussion
5FU acts as a pyrimidine analogue and is transformed inside the cell into different cytotoxic metabolites which can be incorporated into DNA and RNA, finally inducing cell cycle arrest and apoptosis by inhibition of cellular DNA synthesis. Although these drugs are well-known and well-established anticancer drugs that inhibit nucleotide biosynthesis, there is little information concerning the metabolic response of cancer cells against 5FU. Metabolic responses of HepG2 cells to this anticancer drug were studied by comparing extracts of treated and untreated cells by metabolic profiling.

For comprehensive identification of metabolites, FT-ICR MS was selected as analytical platform. MALDI-FT-ICR MS analysis revealed thousands of peaks in the spectra. We evaluated the complex FT-ICR MS data by PCA and OPLS-DA to differentiate metabolic states of HepG2 cell treated with different concentrations of 5FU. As shown in Fig. 1A, metabolic profiles of 5FU-High and control groups could be separated. The OPLS-S-plot comparing 5FU-High and control samples is shown in Fig. 1B. Several loadings were responsible for this separation. Elemental compositions for these compounds were calculated and queried in public databases to retrieve possible structures. As shown in Table 1, those could be nucleotides and amino acids.

To enhance the confidence of the formula assignments, isotopic fine structures were compared with the theoretical ones. Figure 3 shows that the ultrahigh resolution spectrum for two compounds is in accordance to the theoretical spectra of the corresponding sum formula which were suggested by elemental composition analyses. The high spectral resolution of >400,000 FWHM was required not only for separation of both monoisotopic peaks close each other but also for the isotopic fine structure separation. Since metabolomics samples are typically highly complex mixtures, this level of ‘ultrahigh resolution’ is one of the key features for non-targeted metabolomics and biomarker discovery.

Table 1. Summary of elemental composition analyses and database search.

<table>
<thead>
<tr>
<th>No.</th>
<th>m/z</th>
<th>Formula</th>
<th>Component</th>
<th>Search</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>204.071</td>
<td>C17H11N3O2</td>
<td>cyclic-ADP-ribose</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>294.054</td>
<td>C10H13N3O6PNa</td>
<td>5FU</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
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<td>C17H11N3O2Na</td>
<td>cyclic-ADP-ribose</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>329.058</td>
<td>C10H13N3O6Na2</td>
<td>5FU</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>330.071</td>
<td>C10H15N3O6SNa2</td>
<td>Glutathione</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
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<tr>
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<tr>
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<td>ADP</td>
<td>Yes</td>
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<tr>
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<td>330.04949</td>
<td>C10H11N5O6P</td>
<td>ADP</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Conclusions
High-throughput metabolic profiling was achieved by MALDI-FT-ICR MS. Ultrahigh resolution mass spectra can provide more reliable elemental composition analysis by resolving isotopic fine structures. Candidate compounds were found through database search. Metabolic profiling results indicate that 5FU seems to inhibit the biosynthesis of the nucleotides and amino acids in human liver carcinoma cell.

Fig. 1. PCA scores plot between 5FU-High, 5FU-Low, and control groups (A) and OPLS-S-plot of 5FU-High versus control (B).

FT-ICR MS

For research use only. Not for use in diagnostic procedures.

SUMMARY
MALDI-FT-ICR MS analysis was successfully employed for metabolic profiling of HepG2 cells treated with the drug 5FU. PCA and OPLS-DA of ultrahigh resolution MS data could differentiate sample groups and find several responsible loadings. Elemental composition analyses provided candidates which could be confirmed by the ultrahigh resolution data permitting a comparison of measured and theoretical isotopic fine structures. The presented method enables rapid and non-targeted metabolic profiling.
NMR-based screening of neonate urines

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2INFAI Institute for Biomedical Analysis and NMR Imaging GmbH, Köln, Germany

Introduction

NMR is a quantitative technique which can be used for identification of clinically relevant metabolites after a simple preparation of body fluids. A routine 1D proton spectrum contains hundreds to thousands of spectroscopic lines. (Fig. 1: typical 1H-NMR neonate urine spectrum). Hence from a single unspecific measurement it is possible to obtain information on a plethora of metabolites in a multi-marker approach.

NMR can deliver targeted and nontargeted results from one measurement of neonate urine in short time, thus combining quantification of a large set of compounds indicative of inborn errors with the statistical analysis of deviations from normality.

Study Design

700 neonate urine samples from 8 centers in Turkey were collected and spectra were recorded in full automation in two different laboratories (INFAI GmbH and Bruker BioSpin GmbH).

Targeted Analysis: Quantification

Typically, analysis of body fluids relies on direct quantification of preselected diagnostic biomarkers. The NMR signals of such biomarkers can be described in knowledgebases and quantified in urine spectra. Two examples of quantification are illustrated in Fig. 2. These examples based on a real spiking study and, as a result, we can compare the quantified values with the real concentration values.

Untargeted Analysis

The ability to quantify a large number of compounds in every sample allows valid concentration distributions to be accumulated. Some were found to be different from the typical value sets published in textbooks and literature about inborn error analysis, where the number of samples previously used has been typically limited to small cohorts. Fig. 3 shows distributions of two examples with the textbook ranges drawn in.

The idea of getting references ranges for relevant metabolites can be extended to the complete spectroscopic NMR-pattern of a urine. This leads to models which can even detect deviations of unknown or not yet recognized compounds, a so called “untargeted analysis”. Figure 4 illustrates this methodology on the example of 4-Hydroxyphenyllactic Acid (marker for Tyrosinemia I). The coloured model shows the natural variance of more than 700 urine samples. The spectrum with the 4-Hydroxyphenyllactic Acid does obviously not fit into this cohort. This approach allows an automated screening procedure for the detection of diseases without any previous knowledge.

References

NMR Based Metabolomics in Clinical Studies of Human Population: Quantification

D. Krings, C. Cannet, B. Schütz, H. Schäfer, M. Spraul, F. Fang
Bruker BioSpin GmbH, Rheinstetten, Germany

Introduction
NMR is a quantitative technique which can be used for identification of clinically relevant metabolites after a simple preparation of body fluids. With the resulting NMR spectra it is possible to detect and quantify selected metabolites and thus creating a snapshot of the current metabolic phenotype.

Preliminary Studies
Taking into account the natural variability in urine NMR-profiles, we perform a virtual spiking study on the basis of 700 urine spectra of Turkish neonates. Consequently, we obtain ranges for reliable quantification of the respective metabolites. In Fig. 1 we show results of the detection of Methylmalonic Acid and 3-Phenyllactic Acid produced in 50000 spiking experiments for each metabolite.

Detection and Quantification Example
Finally, real spiking experiments can be used to verify the implementation that based on the virtual spiking studies. A process of a complete quantification procedure is illustrated in Fig. 4. After a successful detection we fit the curve of the concentration and obtain a quantification result. If the result reaches or exceeds the detection limit a distribution with possible real concentration values can be generated. The distribution based again on the results of a virtual spiking study in 700 urine spectra.

Quantification Accuracy
After successful detection of a metabolite we start a quantification procedure. Again the variability of the chemical background in different spectra can influence the results. A statistical analysis of such experiments delivers distributions of possible true concentrations values when measured a certain concentration value. Such distributions are exemplarily shown in Fig. 3 for Methylmalonic Acid.

Detection Limits
In each spiking experiment we store the true and false detections. The curves in Fig. 1 are functions that represent the true and false detection rate in dependency on the spiked concentration value. The courses of such curves are determined by several characteristics of the searched signal like the chemical background of the search region and the complexity of the signal pattern. Fig. 2 shows a comparison of three spectra after spike experiments with Methylmalonic Acid and 3-Phenyllactic Acid.
Introduction

Presently, the key bottleneck of plant metabolomics is structural confirmation and elucidation of secondary metabolites. Nicotiana attenuata is a well-established model system to monitor plant-herbivore interactions with metabolomics being a novel approach to investigate the underlying biology [1]. 17-Hydroxyepierythronin aldehyde diterpene glycosides (HGL-DTGs) are abundant direct defense compounds with their mode of action being largely unknown [1-3]. New acyclic HGL-DTGs (attenoside, nicotianoside I, II, III) were characterized using MS and NMR [1H, 13C] after extraction of several hundred grams of raw plant material [2.3]. Such scale is not compatible to the analytical scope of metabolomics. Here, we present novel tools facilitating the identification process of natural products when mass spectral libraries are not yet available and the sample amount is limited [1-3].

Methods

Repeatedly enriched samples in HGL-DTGs from Nicotiana leaves were generated by extraction with 80% methanol, subsequent washing and preparative chromatography [2.3]. Samples were subjected to LC-MS analysis and detailed fragmentation studies by means of direct infusion measurements (3µl/min; dilution 1:10). Standard extracts of various Nicotiana species were prepared as described in [1].

Chromatographic separation was carried out using an RSLC system (Dionex) with a 100x2mm Acclaim RSLC 120 CL8 column, flow rate 0.3ml/min, Solvent A: water + 0.1% HCOOH, Solvent B: acetonitrile = 0.1% HCOOH, linear gradient from 5%B to 70%B in 10min.

Identification

Nicotiana attenuata was stored in the LibraryEditor. Information using SmartFormula3D and combined evaluation of mass accuracy, FindMolecularFeatures (FMF) and Dissect 4.1 software (Bruker Daltonik) using the AutoMS/MS experiment. As the glycosidic pattern of adduct formation in ESI positive mode, only the [M-H]- and the formic acid adduct ([M+HCOO]-) in ESI positive mode, BPC and ESI positive and negative mode in a scan range from 50-1500m/z (MS fullscan & +MS, MolFeature, 6.40-6.65min #377-396) are observed. In general MS spectra observed. Adduct information can be recognized by the fragment ion ratios are easily changed by the presence of a HGL-DTG. This fragment in combination with the neutral losses of sugars and malonyl groups is a very good indicator for the identification results of different HGL-DTGs in extracts (no special enrichment) from Nicotiana using the library search that was then created. The identification results of different HGL-DTGs in extracts (no special enrichment) from Nicotiana using the library search are summarized in Fig. 5.

Conclusions

HGL-DTGs in complex samples can be recognized by:
- distinct adduct pattern of [M+H]+, [M+Na]+ and [M+Na]+ in ESI positive mode
- rich in-source fragmentation with neutral losses of sugars and malonyl groups
- backbone fragment 271, 242m/z

Identification algorithms (FMF, Dissect) have been successfully applied in the early identification steps. Dedicated MS/MS interpretation incorporating structural assignments was performed leading to a well characterized MS/MS library.

All presented tools for identification of HGL-DTGs will be applied now to other Solanaceae species.

References

MS-based dereplication and classification of diterpene glycoside profiles of 23 Solanaceous plant species

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1Max-Planck Institute for Chemical Ecology, Jena, Germany
2Bruker Daltonics, Bremen, Germany

1. Introduction

HGL-DTG profiles of 23 Solanaceae species

2. Methods

Varieties and classification of HGL-DTGs produced by different groups

3. Workflow

LC/MS-Chromatogram

4. Results

4.1 Identification of diterpene glycosides

5. Conclusion

Due to the presence of multiple labile glycosidic bonds, HGL-DTGs exhibit intense fragmentation which provides information valuable for structure elucidation. The set of spectra recorded for each compound was used to develop a library containing 988 different diterpene glycosides. We identified 1035.5395 [M+H]+, 127.0411 [M+H]+ and 845.4300 [M+Na]+ as the characteristic adduct ion for diterpene glycosides.

Acknowledgment and Reference

Institutional Acknowledgements: Dr. Ilona Grabherr and Mr. Paul Pusich for technical support and use of the HRMS and DIA TOF for funding.

References:
3. Other references are given in the text.
GC-APCI-QTOF-MS: An innovative Technique for Metabolite Profiling Studies

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1. Motivation
GC-MS-based metabolite profiling of crude plant extracts reveals more than 100 compounds. Thereof, more than half can be identified using comprehensive mass spectral libraries. The rest remains unknown, in order to identify the unknown compounds we applied GC-APCI-QTOF-MS in succession to our metabolite profiling studies since this technique allows for the determination of elemental compositions of quasi-molecular and fragment ions. For the mapping of spectral features of both techniques we used the retention index (based on unknown). In order to identify the unknown compounds we applied GC-APCI-QTOF-MS in succession to our metabolite profiling studies since this technique allows for the determination of elemental compositions of quasi-molecular and fragment ions. For the mapping of spectral features of both techniques we used the retention index (based on unknown).

2. Concept

2.1 Metabolite Profiling by GC-EI-QUAD-MS

2.2 Multivariate Statistics (ICA, ANOVA, Hotelling)

2.3 MST Annotation by Library Search

3. Materials and Methods

3.1 Plant Cultivation

3.2 Extraction of Roots

3.3 Derivatization

3.4 Structure Elucidation of unknown MSTs

4. MST Annotation

Sugar Breakdown and Glycolysis

Amino Acid Biosynthesis

Miscellaneous Metabolites

5. Mapping of unknown MSTs

In order to identify the remaining MSTs we used the retention index, as this is a useful measure to map peaks from one chromatographic system to another. Often, adhesives are used as retention index markers. However, they do not ionize under atmospheric pressure chemical ionization conditions. Consequently, we had to switch to fatty acid methyl esters and estimated the retention index according to Pawliszyn. Besides the retention index we consulted the molecular ion for the MST mapping as long as it was available.

6. Elemental Composition

6.1 Retention Index + Molecular Ion

6.2 Molecular ion (FAMES) + Retention Index

6.3 Molecular ion (GC-APCI-QTOF-MS) + Retention Index

7. Conclusions

8. References

The authors thank Aiko Barsch as well as Thomas Arthen-Engeland from Bruker Daltonics for technical support during the setup of the system.

9. Acknowledgement

The authors thank Aiko Barsch as well as Thomas Arthen-Engeland from Bruker Daltonics for technical support during the setup of the system.

10. Acknowledgement

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11. Acknowledgement

The authors thank Aiko Barsch as well as Thomas Arthen-Engeland from Bruker Daltonics for technical support during the setup of the system.
Improved method for characterization of phenolic compounds from rosemary extracts using ESI-Qq-TOF MS

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1 CSIC, Granada, Spain
2 Bruker Daltonics, Bremen, Germany

Introduction
Rosemary (Rosmarinus officinalis L.) is a shrub traditionally used as culinary spice, as well as in folk medicine, being a greatly valued medicinal herb. In fact, the plant exerts a great number of pharmacological activities: hepatoprotective, antibiotic, antitumor, antithrombotic, antidiabetic, antiviral, antiinflammatory, antispasmodic, antiammoniuretic and antioxidant activities. Most of these observed effects are linked to its phenolic content. The characterization of phenolic compounds (Fig. 1) in the literature reveals a high variability in terms of extraction and analysis methods.

Recently there has been a growing interest in the use of natural antioxidants in the food industry as a preservation method. Due to its high antioxidant capacity, rosemary was used as a natural antioxidant because of its value for preservation and its beneficial effects mentioned above.

Methods
Two rosemary extracts obtained using supercritical fluid extraction (SFE, CO2 at 150 bar) and pressurized liquid extraction (PLE, RJO at 205°C) were devided in ethanol at 10 mg/mL. Chromatographic separation was performed on a Kinetex C18 column (2x100 mm, 2.6 µm particles) using as mobile phase A 0.1% aqueous formic acid and as mobile phase B acetonitrile. A 30 min linear gradient (from 0 to 100%) was used. Extracts were analyzed in ESI positive and negative mode using a maXis impact ESI-Qq-TOF mass spectrometer (Bruker Daltonics) in the mass range 50-1500m/z operated at 3Hz, in MS full scan and MS/MS mode. Compounds were characterized by taking into account retention time, accurate mass and isotopic pattern information (retention time, elemental formulae derived from accurate mass and isotopic pattern information (calculated in the DataAnalysis software) as well as both in-house and public databases (SciFinder, ChemSpider, MassBank) and literature.

Results
The work aims for the comprehensive characterization of a wide range of phenolic compounds in rosemary extracts by using ESI-Qq-TOF MS. Table 1 shows the summary of identification results.

Conclusions
The novelty of this work is the development of a faster and more sensitive method for characterization of rosemary phenolic extracts compared to previous studies.

The presented methodology has proven to be a useful tool for the separation of a wide range of phenolic compounds in rosemary extracts. Phenolic diterpenes, flavonoids, phenolic acids, among other classes of phenolic compounds obtained from rosemary extracts were identified and tentatively characterized using ESI-Qq-TOF MS.

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References:
[1] Bruker Daltonik, Bremen, Germany
P lants accumulate human beneficial metabolites such as amino acids, flavonoids and sulfur-containing metabolites (S-metabolites) with a wide range of biological activities related to antioxidation, platelet aggregation inhibition, and anticancer benefit. Profiling methods of S-metabolites with high accuracy and throughput are being required for efficient phytochemical functional genomics and crop breeding. Here, we describe transformation cyclotron resonance mass spectrometry (FT-ICR-MS) with U-13C-labeled onion bulb. Natural abundance of 32S (31.972072 Da) and its isotope 34S (33.967868 Da) is 95.02 % and 4.21 %, respectively. These facts are obviously reflected on molecular ion peaks of S-metabolites. Thus, the use of such information allows us to efficiently and accurately extract the peaks related to S-metabolites. By using this method, finally, S-alk(en)ylcysteine sulfoxides together with their intermediates were chemically annotated in onion bulb. It means to realize S-omics using LC-FT-MS for screening of biomarkers in any other organisms.

**Peak picking of S-metabolite**

**Sulfur-containing metabolite-targeted analysis using LC-FT-MS in non-labeled and 13C-labeled onion bulb**

**Conclusions**

- FT-MS is a strong tool for extraction and characterization of S-metabolites.
- Onion bulb includes quite rare S-metabolites which have strong anti-inflammatory activity.
- Strategy of biomarker screening will be changed by this method with approaches of natural products chemistry.

**Acknowledgements**

- Strategic International Collaborative Research Program (SICORP) for JP-NZ and JP-US, JST
- Japan Advanced Plant Science Network
Metabolic Characterization of Sod1 null mutant flies using Liquid Chromatography/Mass Spectrometry

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Introduction

We are characterizing the metabolic impact of knocking out (KO) members of the superoxide dismutase (Sod) family in Drosophila melanogaster using the Sod1 KO allele cSODn108. Sod1 is responsible for scavenging the O$_2^-$ ion, a highly reactive chemical that contributes to oxidative stress(1). cSODn108 mutants have a variety of phenotypes including an accumulation of oxidative damage, decreased lifespan, male infertility and an overall enfeebled phenotype(2). Here we describe our initial steps to use a liquid chromatography coupled mass spectrometry (LC-MS) based approach to characterize the broad metabolic impact of this KO.

Methods

Adult Sod1 null (cSODn108) and transgenic rescue control Drosophila melanogaster were homogenized in cold methanol and proteins were removed by centrifugation. Supernatant was brought to approximately 80% water before injection into a microOTOF Q2E mass spectrometer (Bruker Daltonics) with an ESI source coupled to a ULTRA 3000 rapid separation liquid chromatography system (Dionex). The LC was run with diamylammonium acetate at pH 4.95 using a water-methanol multi-step gradient on a Kinetex C18 RP 100x2.1mm, 2.6 μm column. The mass spectrometer was operated in negative ionization mode. Chromatograms were analyzed using SmartFormula feature uses accurate mass measurement, isotopic pattern and, potentially, fragmentation pattern to generate a candidate formula. Fig 3 C reveals the SmartFormula result.

PCA scores plot (Fig 2 B), PCA vs PC2 demonstrates separate grouping between the two fly sample types. As expected, generic differences between the groups result in downstream alteration of the metabolic profile.

Results

• Polar molecules are notoriously difficult to separate with C-18 reversed phase columns and an alternative, hydrophilic interaction chromatography (HiLC), generates less than desirable results. We therefore focused on establishing a C-18 reversed phase column based separation with a basic ion pairing reagent to improve resolution. Fig 1 A and Table 1 show a series of polar standards we are currently able to resolve. Polar basic compounds are still poorly retained.

• LC-MS raw data from fly samples was subject to peak finding using the ProfiKrawler software (PKS) algorithm which can efficiently deconvolute real signals and background noise. PKS features from all samples were aligned to buckets, a combination of time and m/z, in a bucket table as basis for PCA or t-test analysis.

• PCA loadings plot (Fig 2 A) and bucket statistics (Fig 2 D) reveal how the PKS compounds contribute to the variation between groups. Each point in the loadings plot represents a bucket and the bucket statistics display the levels at which each sample expresses the bucket.

• PCA scores plot (Fig 2 B): PC1 vs PC2 demonstrates separate grouping between the two fly sample types. As expected, generic differences between the groups result in downstream alteration of the metabolic profile.

Conclusions

These are preliminary results from LC-MS analysis aimed at characterizing the metabolic impact of Sod-null genotypes and oxidative stress in D. melanogaster. The poster also demonstrates the applicability of LC-MS analysis for D. melanogaster metabolomics.

References


For more information please see our research publications.

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Caenorhabditis elegans is a widely used model organism, that was introduced in the 1960s by Sydney Brenner. Its sequenced genome, the short reproduction cycle and transparency makes it an ideal model organism.

It is routinely used for studying host-pathogen interactions, neurobiology, physiology, ecology and many more. Despite its popularity, surprisingly few metabolomics studies, mostly using NMR have been carried out using C. elegans [1]. Here we present the setup of a MS based metabolomic platform for C. elegans studies.

Invertebrate Metabolomics: *Drosophila melanogaster*

Non-targeted metabolomics using UPLC-MS:

**Sample preparation:**
Metabolite extraction from worm pellets (~1000 worms) with 50% MeOH at -80°C

**Non-targeted metabolomics using UPLC-MS:**
RP method for non- and mid-polar metabolites:
- Acquity BEH C8, 1.7 µm particles, 150 x 2.1 mm
- A: 10% MeOH + 0.1% formic acid
- B: 100% MeOH + 0.1% formic acid

**HILIC method for polar metabolites:**
- Acquity BEH Amide, 1.7 µm particles, 150 x 2.1 mm
- A: 95% ACN + 10 mM ammonium acetate + 0.1% formic acid
- B: 50% ACN + 10 mM ammonium acetate + 0.1% formic acid

Calibration of individual UPLC-MS runs through injection of standard mix via 6-port valve at the beginning of each run and lock mass calibration

Data analysis / Bioinformatics:
- Internal spectra recalibration, mass list export and automated metabolite annotation using MassTRIX server [2,3]
- Unsupervised methods (PCA and HCA) and supervised methods (PLS) for pattern recognition and identification of masses contributing to groups separation

**Multivariate statistic / Data analysis**
- Comparison and Combination of ICR-FT-MS and UPLC-MS results
- Collection and identification of unknown peaks using NanoMate based fraction collection, exact mass from ICR-FT-MS, MS/MS experiments and NMR annotation using MassTRIX server

**Data pre-processing II:**
- Raw data import into MZmine 2.1 [4], chromatogram deconvolution, peak picking, identification of isotopes and adducts, alignment and database searching for metabolite annotation
- All steps can be automated by the MZmine batch function

Further development:
- Development of metabolite extraction method using 96-well plate format and "fast" UPLC-MS for high throughput screening and deep phenotyping
- Identification of novel compounds using a LC-SPE-NMR-MS system with a 800 MHz NMR (Bruker BioMag Prodigy)
- Mass difference networking [5]
- Mass spectrometry based metabolomic platform allows the measurement of both, polar and non-polar, metabolites in one platform. The developed methods are currently applied to different projects using C. elegans as model organism in the fields of host-pathogen interactions, nutritional studies, probiotic research and the analysis of knock-out mutants.

Ongoing developments in sample preparation and chromatographic methods, in combination with the implemented automated data pre-processing will allow us to use this platform for high throughput metabolite analysis of bigger sample amounts and studies.

**Litterature:**
[3] Voulhoux Romé, 3

**UPLC-MS:**
- Waters Acquity UPLC
- Bruker maXis UHR-ToF-MS
- Resolution > 50,000
- error < 2 ppm
- Advion NanoMate for Chip-ESI and fraction collection

**ICR-FT-MS:**
- Bruker solarX with 12 T magnet
- Resolution > 300,000
- error < 100 ppb
- Gilson 223 sample changer, 6-port valve and Agilent 1100 quad pump for automated sample infusion

**Data pre-processing I:**
- Automated internal recalibration using standard mix and lock mass and export to net-CDF format using Bruker Data Analysis 4.0 and VB scripting

**Further development:**
- Development of metabolite extraction method using 96-well plate format and “fast” UPLC-MS for high throughput screening and deep phenotyping
- Identification of novel compounds using a LC-SPE-NMR-MS system with a 800 MHz NMR (Bruker BioMag Prodigy)
- Mass difference networking [5]

**Conclusion:**
Our presented MS based metabolomic platform allows the measurement of both, polar and non-polar, metabolites in one platform. The developed methods are currently applied to different projects using C. elegans as model organism in the fields of host-pathogen interactions, nutritional studies, probiotic research and the analysis of knock-out mutants.

Ongoing developments in sample preparation and chromatographic methods, in combination with the implemented automated data pre-processing will allow us to use this platform for high throughput metabolite analysis of bigger sample amounts and studies.

**Litterature:**
Introduction

Non-targeted GC-EI-MS profiling is a core metabolomics technique for studying central metabolism in a wide range of organisms. However, many detected metabolites cannot be identified and unknown identification is mostly impossible due to lack of molecular ion information. By contrast, GC-APCI-TOF-MS readily yields pseudomolecular ions, facilitating generation of molecular formulae and thereby partial or complete compound identification. As a test case, we studied primary metabolism in the nematode worm Caenorhabditis elegans, a popular model organism for aging processes and mitochondrial dysfunction. Profiling with GC-EI-MS revealed biochemical differences between control and mitochondrially deficient animals, but many metabolites including important ones for phenotype differentiation could not be identified. We therefore compared GC-APCI-TOF-MS to GC-EI-MS profiles of C. elegans with respect to unknown identification and also phenotype differentiation.

Methods

For metabolite profiling, worms were homogenized in cold methanol/methanoic acid (m.wt 96% and 4%) and extracted metabolites were derivatized with methansulfonyl chloride and MSTFA (fig. 1). For derivatization, worms were mixed with derivatization reagent and incubated for 1 h at 80°C. Subsequently, worms were suspended in 80% methanol and 20% water by bead beating and extracted metabolites were obtained from the reaction mixture by GC-APCI-TOF-MS instruments (Fig. 4), respectively. Identical molecular formula generation based on accurate mass preserves the precursor ion information and enables confirmation was possible for 57 out of 60 test metabolites. By comparing peak elution order, EI-MS peaks without database match were used for molecular formula generation and structure proposal.

Results

Initial profiling with GC-EI-MS yielded 212 reconstructed mass spectra, of which 134 could be assigned structures at a match factor of ≥ 800. To evaluate GC-APCI-TOF-MS as an identification platform, we tested if EI matched metabolites could also be confirmed via their GC-APCI- TOF-MS mass traces, of which 80% could be assigned structures at a match factor of ≥ 800. Fig. 3 shows discrimination of biochemical phenotypes by GC-EI-MS and GC-APCI-TOF-MS in C. elegans. Differentiation could not be identified. We therefore compared GC-APCI-TOF-MS to GC-EI-MS profiles of C. elegans with respect to unknown identification and also phenotype differentiation.

Conclusions

• GC-EI-MS and GC-APCI-TOF-MS were successfully applied as complementary platforms for metabolic profiling and identification in C. elegans.
• The potential of GC-APCI-TOF-MS for the identification of unknown metabolites has been demonstrated with an unidentified compound from EI-MS measurements.
• Further investigations with GC-APCI acquiring high resolution Q-TOF MS/MS data will extend the capabilities for structural elucidation.

GC-APCI-TOF-MS

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The Fastest Way to a Molecular Formula: How fast can it be? TLC-UHPLC-ESI-QTOF MS Coupling for Molecular Formula Determination of New Natural Products

Introduction

The coupling of (U)HPLC with high resolution QTOF mass spectrometers allows fast determination of sum formulae for both known and unknown compounds in a Natural Product Screening. This approach is often applied to crude extracts or enriched fractions. The combination of UHPLC systems with fast scanning QTOF mass spectrometers has the potential to perform the analysis in a few minutes. Here, we investigated if the analysis time could be further reduced by coupling TLC-MS with direct sum formula determination from TLC spots by high resolution MS analysis.

Methods

Mass Spectrometer: Bruker Daltonik maSIA 4G and maSIA IMPACT
HPLC: Dionex RSLC
Column: Waters, BEHC18, 1.7 µm, 21.5 x 0.2 mm
A: H2O, B: ACN, both 0.1% HCOOH
TLC-MS: CAMAG TLC-MS Interface Plates: Merck, TLC Silica 60 F254 Software: SmartFormula3D; Compound Crawler and Fragmentation Explorer, Bruker Daltonik.

Results

Several Natural Products were analyzed by a combination of (U)HPLC-MS and TLC-MS: Reserpine, curcumin, emetine, bafilomycin, several iromycines, collinolacton, collinoketon, hexacyclinic acid, arylomycin, PKS products of LLp-series and three Streptomyces crude extracts.

- TLC-MS analysis only 30 sec
- Semi-automation with TLC-MS
- Mass accuracy of TLC-MS as good as with (U)HPLC-MS: ~ 1 ppm
- Sum Formula determination as reliable as with (U)HPLC-MS
- TLC-MS analysis requires high sample amounts (5-50 µg on plate)
- Only few compounds found by TLC-MS could be further reduced by coupling TLC-MS with direct sum formula determination from TLC spots by high resolution MS analysis.
- Only main compound ionizes, difficulties in detection of side products
- TLC-MS analysis well suited for pure compounds and samples of low complexity.
- Only few compounds found by TLC-MS of crude extract, but many by (U)HPLC-MS (separation, less ion suppression)
- Software Fragmentation Explorer helpful for MS/MS spectrum interpretation
- Structure elucidation of compounds with similar sum formula as iromycin. Are they all derivatives of iromycin?

Outlook

- Structure elucidation of additional arylymycines found by HR-MS/MS.
- Addition of a short column between TLC and MS for salt removal.

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Automated Tissue State Assignment for High Resolution MALDI-FTMS Imaging Data

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**Introduction**

Multivariate feature extraction methods such as principal component analysis (PCA) and segmentation methods are routinely used in MALDI-TOF imaging to provide a concise overview of the main structure in MALDI imaging datasets.

High mass resolution imaging datasets generated from MALDI-FTMS imaging typically are much more complex than MALDI-TOF data, containing thousands of mass signals. Although FTMS imaging data should, therefore, benefit from concise representations, not much work has been done in this field so far.

Here we investigated the use of PCA and hierarchical clustering for FTMS imaging data.

**Methods**

The MALDI imaging dataset of a whole body rat section (19275 pixels, 500 µm spatial resolution) was acquired on a Solarix 9.4T mass spectrometer (Bruker). The sample was sectioned at 20 µm thickness and mounted on a polished steel target. Matrix (CHCA, 40 mg/mL in 50% methanol) was applied using the HTX Sprayer (HTX).

The peak lists (mass range 400 Da to 900 Da) of the individual spectra were binned into one bucket table (bin width = 0.02 Da). In all calculations Pareto scaling on the peak intensities was used. Data were normalized to RMS intensity. All calculations were done in R (www.r-project.org). The results of the PCA and hierarchical clustering were saved in the ClinProTools format and imported into flexImaging 3.0 (Bruker) for visualization.

To assess the parameters for the PCA and the clustering, calculations were performed on a subset of data (each 64 spectra from selected organs). Figure 2 shows the score plots for three parameter settings in the PCA. It is seen that PCA on the raw data leads to scattered and overlapping clusters (Fig. 2a). After normalization, the clusters are better separated, but still a certain amount of noise (Fig. 2b). Spatial smoothing (all peak intensities are replaced with the average of a 3x3 pixel window) leads to well separated clusters (Fig. 2c).

This effect is also visible in the whole dataset. The scores of the first PC align well with the anatomy of the sample (Fig. 3a) as is expected when the main variance in the data comes from real features in the sample. However, a slight granularity (salt-and-pepper-noise) can be observed. This noise leads to the fact that the clusters of spectra are not perfectly separated. The clustering shows main anatomical features (such as liver, brain, cecum) but with a lot of granularity that prevents a perfect segmentation (Fig. 3b).

Spatial smoothing can effectively remove the noise. The PC scores show a much smoother distribution after spatial smoothing of the dataset (Fig. 3c).

Hierarchical clustering of the dataset after normalization and smoothing leads to a very detailed and good segmentation, ... and in general one cluster per organ or region of an organ (such as medulla and cortex of the kidney) is observed.

The calculation of a PCA on the whole rat dataset with almost 20k pixels typically took around 30 minutes. The calculation of the clustering around 3 hours on a well-equipped state-of-the-art workstation. Since these calculations can run unattended, these timeframes are compatible with a routine workflow.

**Conclusions**

- Hierarchical clustering and PCA of FTMS imaging data
- Automated assignment of tissue states
- Spatial smoothing and segmentation
- Detailed annotation of anatomical features

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References – Further Reading

Original papers

Plant Metabolomics

Development and validation of a liquid chromatography-electrospray ionization-time-of-flight mass spectrometry method for induced changes in Nicotiana attenuata leaves during simulated herbivory.

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Fragmentation Pathways of Acylated Flavonoid Diglucuronides from Leaves of Medicago truncatula

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Differential metabolic response of narrow leafed lupine (Lupinus angustifolius) leaves to infection with Coletotrichum lupini

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Microbial Siran Pronation Using Metabolomics Tools for the Discovery of Natural Products

Metabolic fingerprinting as an indicator of biodiversity: towards understanding interspecific relationships among Homoscleromorphs chondroids

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GC-APCI based Metabolomics

Gas chromatography/atmospheric pressure chemical ionization-time of flight mass spectrometry: analytical validation and applicability to metabolic profiling.

Evaluation of GC-APCI/MS and GC-FID As a Complementary Platform.

Performance Evaluation of Gas Chromatography Atmospheric Pressure Chemical Ionization Time-of-Flight Mass Spectrometry for Metabolic Fingerprinting and Profiling

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Combined Reversed Phase HPLC, Mass Spectrometry, and NMR Spectroscopy for a Fast Separation and Efficient Identification of Phospholipidocholines.

Time-Dependent Oxidation during Nano-Assisted Laser Desorption Ionization Mass Spectrometry: A Useful Tool for Structure Determination or a Source of Possible Confusion?

Metabolomics

Unraveling different chemical fingerprints between a champagne wine and its aerosols.

Expressing forest origins in the chemical composition of cooperage oak woods and corresponding wines by using FTICR-MS.

Exploratory analysis of human urine by LC-ESI-TOF MS after high intake of olive oil: understanding the metabolism of polyphenols.

Automated identification of phenolics in plant-derived foods by using library search approach.

Scope and limitations of principal component analysis of high resolution LC-TOF-MS data: the analysis of the chlorogenic acid fraction in green coffee beans as a case study

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Ultra high performance liquid chromatography-time of flight mass spectrometry for analysis of avocado fruit metabolites: Method evaluation and applicability to the analysis of ripening degrees

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CE-MS for metabolic profiling of volume-limited urine samples: application to accelerated aging TTD mice.
Cross-platform analysis of longitudinal data in metabolomics
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Explorative Analysis of Urine by Capillary Electrophoresis-Mass Spectrometry in Chronic Patients with Complex Regional Pain Syndrome
Metabolic profiling of human urine by CE-MS using a positively charged capillary coating and comparison with UPLC-MS
Metabolite profiling of blood plasma of patients with prostate cancer
Potential Effect of Diaper and Cotton Ball Contamination on NMR- and LC/MS-Based Metabonomics Studies of Urine from Newborn Babies
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Explorative Analysis of Urine by Capillary Electrophoresis-Mass Spectrometry in Chronic Patients with Complex Regional Pain Syndrome
Accurate molecular formula determination and identification of molecules with > 1100 m/z with UHR-TOF
ET-22
Novel approaches for small molecule identification in metabolomics research
ET-29
Screening for novel natural products from myxobacteria using LC-MS and LC-NMR
ET-30
Metabolic profiling of Arabidopsis thaliana secondary metabolites using a maxis impact
TN-23
Certainty in small molecule identification by applying SmartFormula3D on a UHR-TOF mass spectrometer
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Using gas chromatography – tandem mass spectrometry to improve the reliability and accuracy of organic acid analyses in urine
Braker Daltonics – Application Notes
ET-19
Metabolic profiling of tea extracts by high resolution LC in combination with maxis UHR-TOF MS analysis
ET-21
Challenges in metabolomics addressed by targeted and untargeted UHR-Q-TOF analysis
ET-22
Metabolic profiling of a Corynebacterium glutamicum ΔprpD2 by GC-APCI high-resolution Q-TOF analysis
ET-23