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Novel Analytical Workflow for Comprehensive Non-targeted Phytochemical Metabolic Profiling

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Abstract: The understanding and interpretation of pharmacological properties on a molecular level is of great importance for many different fields of research. Our study provides a novel model work-flow for comprehensive metabolic profiling by structural identification of relevant metabolites not limited to phytochemistry applications. High resolution liquid chromatography mass spectrometry LC-MS/MS data can be directly correlated with pharmacological test results on a molecular level. Thus the understanding and interpretation of pharmacological properties is supported by structural and chemical information.

Keywords: Mass spectrometry · Metabolic profiling · Natural products · Statistical data analysis · Structural characterization

Introduction

Bamboo is an important plant for a large part of the world's population. The uses of bamboo are manifold, ranging from construction materials, energy source, nutriment, textiles and cosmetics, to traditional Chinese medicine applications. Bamboo is a rich natural source of promising phytochemicals including flavonoids and other secondary plant metabolites. These metabolites exhibit a broad range of health-promoting effects including anti-inflammatory, anti-oxidant, anti-viral and anti-aging properties.[1,2] However, there is still a lack of information on the metabolites responsible, present in the many different bamboo species around the world. Thus, there is a great need for detailed information on the metabolite level of different bamboo species influenced by genus, age and geographical origin.

Despite the most modern analytical instrumentation and high quality databases, the step to transform a measured high resolution mass spectrum into an identified chemical compound with known structure is still tedious, cumbersome, time-consuming and often not successful by mass spectrometry data alone.

In this research project, we describe a novel analytical work-flow for non-targeted metabolic profiling. These data were filtered with efficient open source algorithms such as seven golden rules[3] to generate qualified elemental sum formulae. These qualified sum formulae were consolidated by matching MS/MS fragmentation pattern with focused libraries, *e.g.* the Dictionary of Natural Products (DNP) or Metlin.[4] Proof of structural identity was finally achieved by comparison with reference compound data, based on retention time and high resolution MS- and MS/MS data.

Experimental Setup

In order to investigate the metabolome of bamboo, extracts of young and old leaves of *Phyllostachys edulis* were prepared. About 1.5 g of dried leaves were milled with a Retsch MM400 ball mill for 15 min at 30 Hz. 500 mg of milled leaves were extracted with a Dionex ASE 350 accelerated solvent extraction system using methanol/water 50:50 (v:v) at 70 °C in 25 min, applying heat for 5 min. Yellow clear solutions were obtained. Solvents were evaporated to dryness with a Genevac EZ-2 Plus Personal Evaporator at reduced pressure for 4 h at 60 °C. 10 mg of the crude extract were purified on Waters Oasis HLB SPE cartridges (30 mg). Cartridges were conditioned with 3 ml methanol, equilibrated with 3 ml water/methanol 95:5 (v:v) and eluted with 3 ml methanol. Eluates of SPE cleaned extracts were used for injection into LC-MS/MS system. The sample preparation protocol was performed three times independently for young and old *Phyllostachys edulis* leaves respectively. An aliquot of 1 µl of each extract was injected in four replicates on the Agilent 1290 Infinity binary UHPLC system coupled to an Agilent 6540 UHD Accurate Mass Quadrupole Time-of-Flight (Q-TOF) system. Separation was performed on a Zorbax SB-Phenyl column 3.0 mm × 150 mm packed with 1.8 µm particles. A gradient elution with methanol/water each with 0.1% formic acid was performed within 45 min at a flow rate of 0.4 ml/min. Agilent Jet Stream Electrospray in positive mode was used for ionization. Q-TOF spectra were measured from 100 to 1000 m/z with an acquisition rate of 4 Hz. The system was running under MassHunter B.06.

Results and Discussion

An important criterion for utilization of bamboo as a therapeutic crop is a thorough understanding of the metabolite inventory and its variation with genus and age as well as with geographic origin. First investigations on bamboo leaf extracts focused in a targeted analysis on flavonoid metabolites and revealed different patterns according to genus and species. However, the targeted LC-MS/MS based analysis of the major flavonoid composition of bamboo leaf extracts did not correlate with anti-oxidative and anti-inflammation properties. Therefore, samples of different bamboo leaf extracts of genera *Phyllostachys, Fragesia* and *Sasa* as well as young and old leaves were analyzed by a non-targeted metabolic profiling approach to reveal structural characterization of the significant metabolites. The major hurdle in non-targeted metabolic profiling so far is that measured numerical MS data have to be transformed into a unique chemical compound with a defined structure. Looking at the molecular formula space of common organic molecules with more than 8×10^9 possible elemental formulae for molecular weights below 2000 *u* seems to be more hopeless than searching for a needle in a haystack.[3]

Our novel non-targeted metabolic profiling workflow combines statistical data analysis tools with smart filtering algorithms, database searches in focused libraries, and MS/MS hit confirmation. The outline of this workflow is illustrated in Fig. 1.

In a first step after data acquisition, statistical data analysis

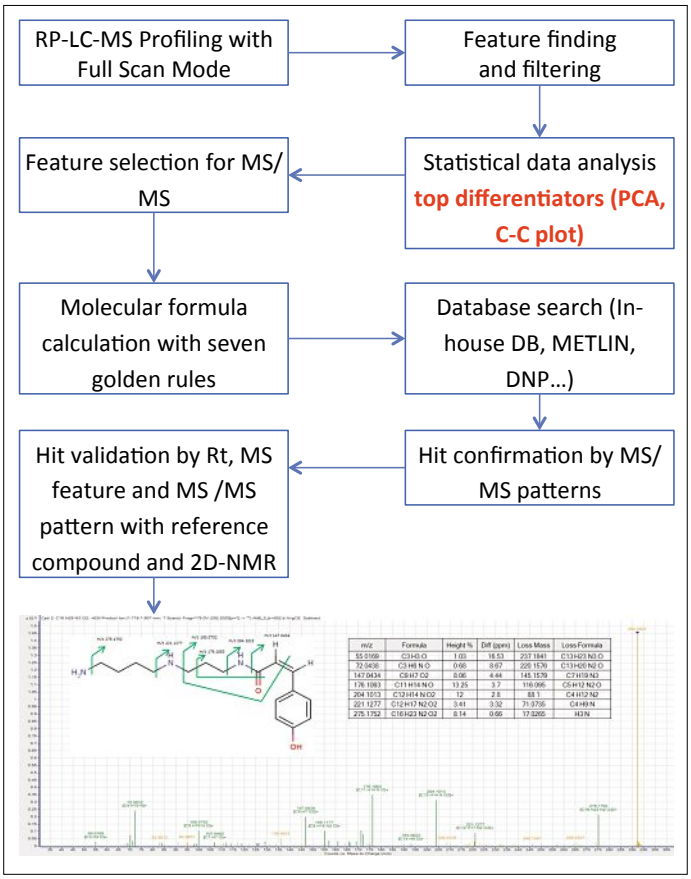

Fig. 1. Novel non-targeted workflow with LC-QTOF.

was performed with mass profiler professional (MPP version 12.5, Agilent) including, amongst others, principal component analysis (PCA). Significant separating features identified from PCA analysis were selected based on high resolution MS. Subsequently, data were extensively filtered by application of seven golden rules.[3] This step guided and directed the assignment of relevant consolidated structural features. These consolidated structural features, characterized by high resolution MS/MS patterns, were selected and searched against focused databases. The integrated database search features of MPP software supported and facilitated the transformation of features into first hits. Main sources for hit confirmation were found in dedicated libraries, *e.g.* Dictionary of Natural Products and METLIN.[4] In the event that no database match was obtained, hits were rejected and the process was repeated with the next hit. Confirmed hits were validated by comparison with measured compound reference data, based on retention time and high resolution MS- and MS/MS patterns.

The investigated bamboo species were differentiated by principle component analysis of high resolution MS data. In addition, not only for different bamboo genera but also for *Phyllostachys edulis* young and old leaves, significant separating features were identified. By application of the non-targeted metabolic profiling workflow, several compounds of different classes including nucleosides, coumaroyl derivatives, fatty acids or aminoalkyltriols were characterized (Fig. 2 and 3). Flavonoids not included in the targeted analysis were also found in significantly different concentration levels in *Phyllostachys edulis* young and old leaves. Thus, our workflow has been proven to be very effective and leads to a valid structural identification of the most important metabolites within a short time. The combination of high resolution MS/MS experiments with high mass accuracy (≤2 ppm), as well as Product Ion Scan data was key to implementing the analytical workflow.

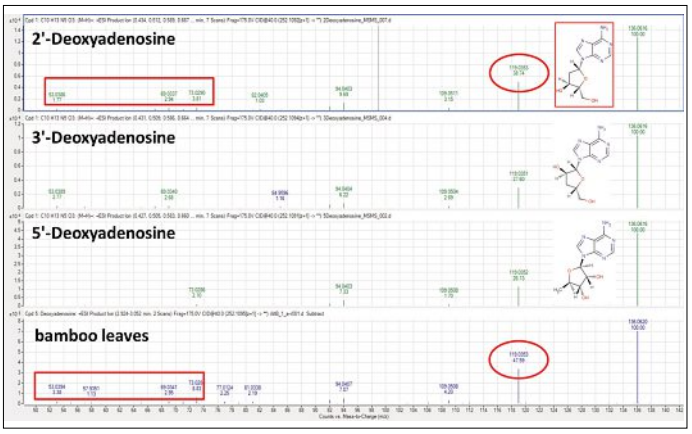

Fig. 2. 2'-Deoxyadenosine as an example of identification of a significant metabolite differentiating young and old *Phyllostachys edulis* leaves.

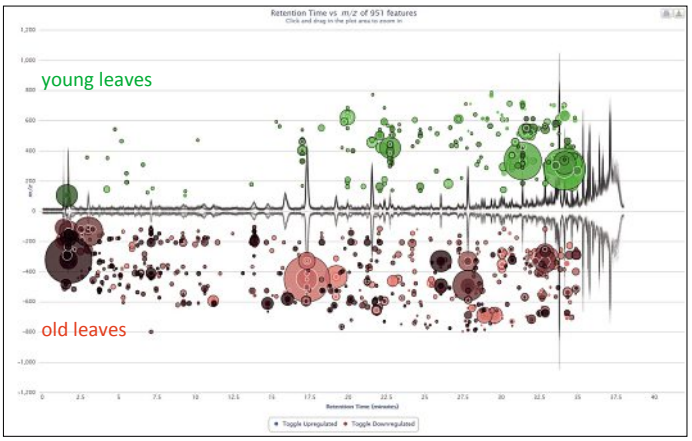

Fig. 3. LC-MS chromatogram of significant features in young and old *Phyllostachys edulis* leaves with the XCMS online tool.[5]

Conclusions

Non-targeted metabolic profiling in combination with integrated software-directed feature selection was extremely helpful for rapid structural assessment of differentiating features between young and old leaves of *Phyllostachys edulis*. The implementation of this integrated but focused workflow significantly accelerated the process from statistical feature identification and molecular feature identification (sum formulae) to structural characterization of relevant metabolites. Pharmacological studies with the same bamboo extracts exhibited positive effects on the anti-inflammatory and wound-healing assays for young leaf extracts of *Phyllostachys edulis*. The novel non-targeted metabolite profiling workflow structure supported identification of the significant metabolites and enabled understanding and interpretation of pharmacological properties on a molecular level. Our study provides a novel model workflow for a comprehensive phytochemical assessment combining high resolution LC-MS/MS data with pharmacological testing.

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