Universität für Bodenkultur Wien
Interuniversitäres Department für Agrarbiotechnologie, IFA-Tulln
Konrad-Lorenz-Straße 20 – A-3430 Tulln - AUSTRIA - EUROPE
Tel. +43 2272 66280 402 - Fax +43 2272 66280 403 – officeaz@boku.ac.at
Analytikzentrum
Leiter: Univ.Prof.DI Dr. Rudolf KRSKA

Development and Application of Analytical Methods for the Metabolic Profiling of Volatiles Produced by Grapevine

Dissertation

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Georg Weingart

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kann ich nur zu all den vielen Menschen sagen, die dazu beigetragen haben, dass diese Arbeit angefangen, durchgeführt und auch beendet worden ist.

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Wenn Gott Deine Meinung verändert, dann ist das Glaube.
Wenn Fakten Deine Meinung verändern, dann ist das Wissenschaft.

Tulln, September 2011 Georg Weingart

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Preface

"Science is built up with facts, as a house is with stones. But a collection of facts is no more a science than a heap of stones is a house."

Jules Henri Poincare´ (1854-1912) La Science et l'hypothe´se.
(in Brown et al., 2005)

1 Biological background and aims of this thesis

The major goal of my thesis was the development and application of analytical methods for the detection and identification of grapevine metabolites. The techniques used can be summarised with the keyword “metabolomics”. Since metabolomics covers much more than only analytical techniques but also e.g. the biological experiment or statistical data analysis, I will start in this first chapter with the description of the biological background and the major goals of this thesis. The metabolomics concept is discussed in chapter 5 including a discussion of the techniques used during this thesis and their role in metabolomics.

Biological background

After decades of simplifying and reducing investigations in plants to one or a small number of genes, proteins, metabolites, etc. researchers changed their view to look at the whole genome, transcriptome, proteome, and metabolome. The –omics era begun and whole organisms were investigated. The availability of the –omics techniques together with the knowledge that “the whole is more than the sum of the parts” led to the concept of systems biology.

“But one thing is clear: to understand the whole, one must study the whole.” (Kacser, 1986)

In practice, systems biology frequently means to investigate the biological response of a cell, an organism or a biological system to e.g. genetic or environmental changes. The functions of the biological system can be analysed on various levels, i.e. genes, proteins, metabolites, etc. The newly developed –omics techniques – genomics, transcriptomics, proteomics, and metabolomics – are combined to obtain a comprehensive view into the biochemical and molecular processes. Systems biology demands the combination of all these techniques to an integrative approach (Fukushima et al., 2009).

Metabolomics is defined as the qualitative and quantitative analysis of the whole metabolome of an organism (Fiehn, 2001). This cannot be achieved with a single analytical technique or method at the moment, therefore a combination of different sample preparation and analysis methods is necessary. Metabolomics is not reduced to the technical analysis of biological systems but covers a complex multi-step process.

A typical metabolomics workflow, as it will be presented in this thesis, consists of several steps (Figure 1.1). It begins with a scientific biological question

followed by the setup and realisation of an appropriate experiment. Thereafter, samples have to be taken (e.g. plant or animal tissue, biofluids), the metabolism interrupted (quenching) and the samples prepared for further analysis. Metabolites sometimes have to be extracted from the samples, the samples sometimes need to be purified, concentrated and then analysed, e.g. with a chromatographic system coupled to a detector. The last step is processing of the data and interpretation of the results in relation to the initial biological question.

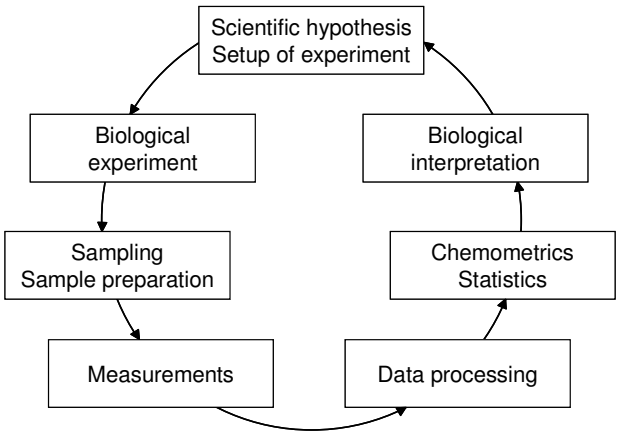

Figure 1.1: Typical metabolomics workflow

The biological background of this the presented research work was the project “Physiological Fingerprinting in Viticulture”.

This project mainly aimed at the development of tools to identify and measure indicators for grapevine vitality. Such tools can help to improve vineyard management and hence wine quality. Former studies used mostly grape samples for quality analysis. Total soluble solids, acidity and colour parameters were measured to determine the grape quality. However, these existing parameters alone are insufficient for assessment of the plants vitality. Therefore, the “Fingerprint-project” aimed at the development and application of techniques for the detection of physiological (water potential, photosynthesis activity) as well as biochemical indicators (sugar and polyphenol content, secondary plant metabolites) for plant vitality. While two other PhD students investigated well-known stress markers from the substance classes of polyphenols and sugars with a targeted analytical approach, my challenge was to find yet unknown stress markers in the metabolome of grapevine.

To this end, techniques from the rather new metabolomics research area were established and used at the Center for Analytical Chemistry, IFA-Tulln.

Aims of this thesis

This thesis describes the development and application of a workflow for metabolite profiling of grapevine metabolites. Individual steps of the presented workflow are discussed in view of the intended and realised applications. Moreover, existing challenges in the field of metabolomics for plant volatiles will also be described.

The following aims were defined at the beginning of this work:

1. **Comprehensive literature survey** to get an overview about the substances detected so far in grapevine plants.
2. **Development of an analytical method** for identification of grapevine leaf metabolites with the focus on volatile metabolites.

   Development of a **GC-MS method** for measurement of **volatiles produced by grapevine leaves.** Optimisation of method parameters and evaluation of the method.
3. **Automation of** metabolite annotation with the aim to minimise the number of false positive annotations.
4. **Integration** of the analytical **method in a workflow** covering all steps from **sampling** to detection of **biomarkers.**
5. **Application** of the developed workflow **to selected biological samples** of **grapevine** with the specific aims to:
   a. **identify volatile metabolites** of Pinot Noir leaves
   b. **test if** leaf samples originating from different **sampling dates can be differentiated**
   c. **identify metabolites**, differentially expressed due to **drought** and **UV-B stress** and might serve as **biomarkers** for this type of stress
   d. **identify root metabolites** the concentrations of which are directly **influenced due to insect attack** (grapevine louse phylloxera)

2 Abstract

This thesis presents the development and applications of a workflow for the investigation of the volatile metabolome of grapevine plants and metabolite profiling of volatile constituents of settled floor dust samples. The rather new scientific area of metabolomics offers a couple of techniques, which are suited for such investigations. The challenge was to choose the appropriate methods and settings for the given task. The workflow consists of the following steps:

Grapevine leaves were harvested in the field and metabolism was quenched by immediate insertion of the leaves into liquid nitrogen. Frozen leave samples were homogenised with a ball mill under cooled conditions, weighed into headspace (HS)- vials, and stored at -80°C until analysis. For the extraction of volatile metabolites, I used headspace solid phase microextraction (HS-SPME) as this technique needs no further sample preparation and additionally offers the possibility of analyte concentration on the fibre. Furthermore, by means of an autosampler the extraction as well as desorption process into a chromatographic system can be fully automated. I made use of the excellent separation efficiency of gas chromatography (GC) in combination with high selectivity and the potential of structure elucidation by mass spectrometry and realised a GC-MS method for the analysis of volatile metabolite. Additionally, retention time indices (RIs) on two GC columns with different polarity (apolar DB-5MS, polar Optima-WAX) were used to increase the quality of metabolite identification. For automated mass spectra deconvolution and comparison of both mass spectra and RI with library entries I applied AMDIS software. Strict criteria were used for metabolite identification. Minimum mass spectral match factor was 90 and maximum relative RI deviation was ±2% from reference value. After its successful development the analytical workflow was applied in various studies.

The investigation of open field samples of Pinot Noir grapevine leaves resulted in the annotation/identification of 63 metabolites. Several standards were available and the identity of about 47 metabolites has been proven. I evaluated the analytical (below 40% RSD for the majority of metabolites) as well as the biological variability (7-119% RSD) with grapevine leaves. The data analysis was done with multivariate statistics (principal component analysis) and showed for leaves from two different sampling dates two clearly separated clusters. This application and the workflow development and method evaluation are described in paper #3, which has been submitted to *Phytochemical Analysis*.

To compare the results with former studies, a literature survey was carried out resulting in the compilation of a database for metabolites detected in grapevine by GC. The database covers now data from 39 research studies using GC. In total 1619 entries referring to *Vitis* metabolites are contained in this database.

A further application was the investigation of the metabolic response of grape vine roots to the grapevine louse phylloxera in cooperation with Nora C. Lawo who kindly provided the root tissue. The applied workflow was nearly identical as described above but smaller sample amounts had to be handled. Univariate statistics showed 14 differentially expressed metabolites between control and phylloxera attacked roots, 32 metabolites were described in grape vine roots for the first time. Evaluation of the differentially expressed metabolites indicated that defence related pathways such as the mevalonate and/or alternative isopentenyl pyrophosphate-, the lipoxygenase- and the phenylpropanoid pathway are affected in root galls as a response to phylloxera attack. The re sults have been published recently in *Plant Physiology and Biochemistry* (paper #1).

Based on this experiment, I participated in writing a book chapter “Study of the volatile metabolome in plant-insect interactions” in “The Handbook of Plant Metabolomics’” which is currently in the process of editing and shall be published soon. The book chapter represents a general description of a typical metabolomics workflow suited for the investigation of plant-insect interactions. Furthermore, it contains a protocol exactly describing how to sample, treat, and analyse plant tissue followed by data processing and statistics to find volatile metabolites with different concentrations in plants attacked by insects compared to non-attacked plants.

Additionally, the developed workflow was the basis of another application, which investigated settled floor dust samples. Together with a PhD colleague, Vinay Vishwanath, we used the workflow for the detection of fungal metabolites in dust samples. Although dust is not a biological system per se, it consists of substantial amounts of organic material, therefore the application of metabolomics techniques was appropriate. The results of this study are presented in paper #2.

To sum up, the presented metabolomics workflow is well-suited for the annotation/identification of volatile metabolites of plants but also other samples. The detected metabolites are able to separate different sample groups if data is processed with multivariate statistics. The method is sensitive and accurate enough to provide significant differences in the metabolite concentrations in differentially treated plants. I tried to use only simple and well established techniques in the workflow to enable a broad range of applications.

The results of this thesis contribute to the rather new field of grapevine metabolomics in the future. It enables the easy investigation of grapevine plant tissue for metabolite identification and biomarker detection. I was able to describe several metabolites for the first time in grapevine leave or root tissue. The compiled grapevine metabolite database serves as valuable basis for future research of grapevine plants.

3 Kurzfassung

Die vorliegende Arbeit stellt die Entwicklung und Anwendung eines Arbeitsablaufes (workflow) für die Untersuchung der flüchtigen Metabolite der Weinrebe und flüchtiger Bestandteile von Staubproben (settled floor dust, SFD) dar. Ein relativ neuer wissenschaftlicher Forschungszweig, die Metabolomik, hält eine Reihe von Techniken bereit, die bestens für diese Aufgabenstellung geeignet sind. Die besondere Herausforderung in dieser Arbeit war das Auswählen der geeigneten Methoden und Techniken sowie die Optimierung der zahlreichen Verfahrenschritte zur Probenahme, Probenaufbereitung, Analyse und Datenauswertung. Der entwickelte Arbeitsablauf besteht aus folgenden Schritten:

Blätter der Weinrebe wurden geerntet und sofort in flüssigen Stickstoff getaucht, um möglichst alle Stoffwechselprozesse zu stoppen (Quenching). Die gefrorenen Blätter wurden in einer Kugelmühle homogenisiert, wobei darauf geachtet wurde, dass alle Gegenstände (Spatel, Mahlbecher, Mahlkugel) immer mit flüssigem Stickstoff gekühlt wurden, um ein Auftauen der Probe zu verhindern. Nach dem Vermahlen wurde das Blattpulver in 20-mL Glasgefäße eingewogen und im Kühlschrank bei -80°C bis zur weiteren Analyse gelagert. Für die Extraktion der flüchtigen Metabolite wurde die Festphasen-Mikroextraktions-Technik (solid phase microextraction, SPME) verwendet. Mit dieser Technik kann die manuelle Probenvorbereitung auf ein Minimum reduziert werden. Bei der SPME erfogt die Anreicherung der Analyte auf einer Faser und es besteht die Möglichkeit, den ganzen Extraktionsprozess zu automatisieren. Für die Trennung und Detektion der Metabolite verwendete ich einen Gaschromatographen (GC) in Verbindung mit einem Massenspektrometer (MS). Die Gaschromatographie bietet eine sehr gute Auftrennung der Metabolite und das MS bietet die Möglichkeit einer Annotierung der detektierten Substanzen aufgrund der erhaltenen Massenspektren. Zur besseren Absicherung der Identifizierung der Metabolite wurden zusätzlich Retentionsindices (RIs) verwendet. Diese wurden auf zwei Säulen unterschiedlicher Polarität (apolare DB-5MS, polare Optima-WAX) ermittelt. Die Dekonvolution der gemessenen Massenspektren, deren Vergleich mit Spektren einer Referenzdatenbank sowie der Vergleich der gemessen RIs mit Literaturwerten erfolgte vollautomatisiert mit dem Programm AMDIS. Für eine sichere Identifizierung (es sollten möglichst wenig falsch positive Resultate erhalten werden), wurden strenge Kriterien angelegt: Der Vergleich zwischen gemessenem und Referenzmassenspektren musste mindestens eine Ähnlichkeit von 90 (minimum match factor) ergeben und der gemessene RI durfte nicht mehr als ±2% vom Referenzwert abweichen, um einen Metaboliten

als annotiert (vorläufig identifiziert) bezeichnen zu dürfen Der Arbeitsablauf ist detailliert in den Publikationen #3 und #4 beschrieben).

Die Untersuchung von Weinblättern (*Vitis vinfera* cv. Pinot Noir 18 Gm) aus einem Versuchsweingarten ergab die Annotierung 63 flüchtiger Metabolite. Die Identität von 47 dieser Metabolite konnte mittels authentischer Standards abgesichert werden. Die Variabilität der analytischen Methode lag für einen Großteil der Metabolite unter 40 % (relative Standardabweichung, RSD), die biologische Variabilität zwischen den Pflanzen reichte von 7 – 119% RSD. Die Daten wurden mit multivariater Statistik (Hauptkomponentenanalyse, HKA) weiter ausgewertet. Ich konnte zeigen, dass die Blätter zweier verschiedener Probenahmetermine in der HKA zwei entsprechende Häufungen bilden. Diese Anwendung und die Entwicklung der Methode sowie des ganzen Arbeitsablaufes wurden zur Publikation bei der Fachzeitschrift *Phytochemical Analysis* eingereicht (Publikation #3).

Zum Vergleich der eignen Ergebnisse mit der Literatur wurde eine intensive Literatursuche durchgeführt. Das Ergebnis ist eine Datenbank mit 1619 Substanzeinträgen, die als Inhaltsstoffe der Weinpflanze mittels GC in insgesamt 39 ausgewerteten Studien gefunden wurden. Die Datenbank ist der Publikation #3 als zusätzliches Material beigefügt.

Die zweite äußerst interessante Anwendung war die Untersuchung von Wurzeln der Weinrebe, die von der Reblaus (Phylloxera) befallen waren. Der analytische Arbeitsablauf war beinahe identisch mit dem oben beschriebenen, lediglich die Probenmenge der Wurzeln war sehr viel kleiner im Vergleich zu den Blättern. Es wurden 38 Metabolite annotiert, wovon 32 zum ersten Mal in Wurzeln der Weinrebe beschriebenen wurden. Mittels univariater Statistik (t-test) wurden 14 Metabolite gefunden, deren Konzentrationen in befallenen Wurzelspitzen signifikant höher oder niedriger waren, als in nicht-befallenen Wurzelspitzen. Diese Substanzen weisen darauf hin, dass die Aktivität einiger Stoffwechselwege (z.B. Mevalonat und/oder alternative Isopentenyl pyrophosphat -, Lipoxygenase -, Phenylpropanoid Stoffwechselweg), die bei der Verteidigung der Pflanze gegen Fraßfeinde eine Rolle spielen, durch den Reblausbefall verändert wurde. Die Ergebnisse wurden in *Plant Physiology and Biochemistry* veröffentlicht (Publikation #1).

Auf Basis dieses Experiments entstand auch das Buchkapitel „Study of the volatile metabolome in plant-insect interactions“, welches in „The Handbook of Plant Metabolomics“ veröffentlicht wird. In dem Buchkapitel ist eine allgemeine Beschreibung eines typischen Metabolomik-Arbeitsablaufes beschrieben. Weiters findet sich eine Schritt-für-Schritt-Anleitung für die Untersuchung von Pflanze-Insekt Interaktionen. Diese reicht vom Aufbau eines biologischen Experimentes über Probenahme und -vorbereitung, Analyse der Metabolite bis hin zur statistischen Datenauswertung (Publikation #4).

Eine weitere eher unkonventionelle Anwendung der in dieser Arbeit etablierten Methoden war die Analyse von Staubproben gemeinsam mit meinem Kollegen Vinay Vishwanath. Obwohl Staub kein Organismus ist, konnte der Metabolomik-Arbeitsablauf angewendet werden, besteht Staub ja zu einem Teil aus organischem Material. Durch die Analyse der Pilzmetabolite in Staub kann auf die An- oder Abwesenheit von Schimmelpizen im betreffenden Gebäude geschlossen werden. Die Ergebnisse der Studie wurden mittlerweile in *Talanta* veröffentlicht (Publikation #2).

Zusammengefasst lässt sich sagen, dass der entwickelte und hier vorgestellte Arbeitsablauf sehr gut geeignet ist, um flüchtige Metabolite von Pflanzen aber auch die Inhaltsstoffe anderer Proben zu annotieren/identifizieren. Mittels multivariater Statistik konnte gezeigt werden, dass die gefundenen Metabolite charakteristisch für verschiedene Probengruppen sind. Die Methode ist sensitiv und genau genug, um signifikante Unterschiede in den Metabolitkonzentrationen verschieden behandelter Pflanzen zu finden. Ich legte Wert darauf, etablierte und robuste Methoden zu verwenden, um eine breite Anwendung des entwickelten Arbeitsablaufes zu ermöglichen.

Die vorgelegte Arbeit hat das Potential, zu einem besseren und tieferen Verständnis des Weinrebenmetaboloms beizutragen. Der präsentierte Arbeitsablauf ermöglicht eine einfache Untersuchung von Weinreben zur Identifizierung und Detektion von Biomarkern. Ich konnte einige Metabolite von Blättern und Wurzeln der Weinrebe zum ersten Mal beschreiben. Die Datenbank flüchtiger Inhaltsstoffe von Weinreben stellt ein weiteres hilfreiches Werkzeug für zukünftige Forschungen im Bereich der Weinrebe dar.

4 List of publications

International peer-reviewed publications

Paper #1 Phylloxera attacked grapevine roots

Lawo* NC, **Weingart* GJF**, Schuhmacher R, Forneck A **2011** The volatile metabolome of grapevine roots: First insights into the metabolic response upon phylloxera attack. *Plant Physiology and Biochemistry* 49:1059-1063.

* shared first authorship

Paper #2 Settled floor dust samples

Vishwanath V, Sulyok M, **Weingart G**, Kluger B, Täubel M, Mayer S, Schuhmacher R, Krska R **2011** Evaluation of settled floor dust for the presence of microbial metabolites and volatile anthropogenic chemicals in indoor environments by LC-MS/MS and GC-MS methods. *Talanta* 85:2027-2038.

International peer-reviewed publications, revised version submitted

Paper #3 Method development, grapevine leaves

Weingart GJF, Kluger B, Forneck A, Krska R, Schuhmacher R **2011** Establishment and application of a metabolomics workflow for identification and profiling of volatiles from leaves of *Vitis vinifera* by HS-SPME-GC-MS. *Phytochemical Analysis.*

Other publications

Paper #4 Plant-insect interaction, book chapter, submitted

Weingart GJF, Lawo NC, Forneck A, Krska R, Schuhmacher R **2012** Study of the volatile metabolome in plant-insect interactions. In: Weckwerth W, Kahl G, editors. The Handbook of Plant Metabolomics. Wiley-Blackwell-VCH.

Oral presentations

Weingart GJF, Lawo NC, Schuhmacher R, Forneck A **2010** Metabolomic profiles of phylloxerated gall tissues of root tips, 5th Phylloxera Symposium, September 19th – 23rd, 2010, Vienna, Austria

Weingart GJF, Schoedl K, Krska R, Forneck A, Schuhmacher R **2010** GC-MS based metabolomics of volatile metabolites produced by leaves of *Vitis vinifera.* International Metabolomics Austria, August 29th - September 3rd, 2010, Vienna, Austria

Weingart GJF, Krska R, Forneck A, Schuhmacher R **2010** Identifying grapevine leaf metabolites with GC-MS - just a routine application? MassSpec-Forum, February 16th - 17th, 2010, Vienna, Austria

Weingart GJF, Schumacher R, Krska R, Forneck A **2009** A bottom up metabolic profiling approach for the detection of potential stress markers in Pinot Noir grapevine leaves. 5. ASAC JunganalytikerInnen Forum, September 5th-6th, 2009, Innsbruck, Austria

Weingart GJF, Schuhmacher R, Krska R, Forneck A **2009** Preliminary study for the identification of stress induced volatiles of *Vitis vinifera* leaves – analytical method development and evaluation. 27th Informal Meeting on Mass Spectrometry, May 3rd – 6th, 2009, Retz, Austria

Poster presentations

Weingart GJF, Schoedl K, Krska R, Schuhmacher R, Forneck A **2010** Finding of stress related metabolites in *Vitis vinifera* by GC-MS, 28th Informal Meeting on Mass Spectrometry, May 2nd-6th, 2010, Köszeg, Hungary

Weingart GJF, Schuhmacher R, Krska R, Forneck A **2009** A bottom up metabolic profiling approach for the detection of potential stress markers in Pinot Noir grapevine leaves. Euroanalysis, September 6th – 10th, 2009, Innsbruck, Austria

Weingart GJF, Schuhmacher R, Krska R, Forneck A **2009** A bottom up metabolic profiling approach for the detection of volatile metabolites of in Pinot Noir grapevine leaves. Metabomeeting, July 5th – 8th, 2009, Norwich, Great Britain

Weingart GJF, Schuhmacher R, Krska R, Forneck A **2008** Metabolic profiling of volatile compounds in leaves of *Vitis vinifera* cv. Pinot noir by GC-MS. Advances in Metabolic Profiling, October 16th – 17th, 2008, Lisbon, Spain

Lawo NC, **Weingart G**, Schuhmacher R, Forneck A **2011** First insights into a metabolic fingerprint of grapevine roots upon phylloxera attack. Entomology Congress, March 21st – 24th, 2011 in Berlin, Germany

Schöfbeck D, **Weingart G**, Lemmens M, Krska R, Schuhmacher R **2010** Profiling of Volatiles of Wheat Ears Using Headspace SPME-GC-MS. Metabolomics and More: The Impact of Metabolomics on the Life Sciences, March, 10th – 12th, 2010, München, Germany

Metabolomics

“Progress in science depends on new techniques, new discoveries and new ideas, probably in that order.”

Sydney Brenner (in Robertson, 1980)

5 Introduction

"When a thing was new, people said, 'It is not true'. Later, when the truth became obvious, people said, 'Anyway, it is not important.' And when its importance could not be denied, people said, 'Anyway, it is not new."

William James (1842-1920) (in Goodacre et al., 2004)

This chapter will give a short overview about definitions and history of the rather new and important metabolomics area. A typical metabolomics workflow as it was developed and used in this thesis is presented. Further on, the steps of the workflow and the techniques used in this thesis are presented. The particular challenges in metabolomics applications are discussed.

Definition

The term "metabolome" is derived from ancient Greek" metabolē" which means "change" whereas "-omics" refers to the Greek "oma" which means "indicating process, action" and "-ics" forms nouns referring to fields of knowledge or practice. According to the Metabolomics Society homepage metabolomics is "concerned with the comprehensive characterisation of the small molecule metabolites in biological systems" (http://www.metabolomicssociety.org/metabolomics.html, access 22.8.2011). One of the first definitions by Oliver Fiehn (2001) describes metabolomics as "a comprehensive and quantitative analysis of all metabolites" in a biological system. Today metabolomics covers a whole scientific area with several sub-areas. Table 5.1 gives some general definitions of terms that are frequently associated with metabolomics and related techniques. In the beginning of the metabolomics era, metabolomics was regarded to be an enhancement of genomics and proteomics. Hence, the first definitions (Fiehn, 2002) connect metabolomics closely with investigations of the functions of genes in organisms. Nowadays the definitions are more general (e.g. Krastanov, 2010). Due to the impossibility of analysing all metabolites of an organism simultaneously, different approaches have evolved.

Metabolomics is one of the younger –omics techniques consequently following other high-throughput analytical approaches as genomics, transcriptomics or proteomics. Due to the limited variety of the building blocks of genes or proteins they can be investigated by using just a small number of analytical techniques (Brown et al., 2005). In contrast to genes or proteins, metabolites occur in a wide range of substance classes (ionic, neutral, polar, apolar). Concentrations cover several orders of magnitude within an organism or one single cell. Hence, in metabolomics, we have to deal with an unknown number of metabolites. It is

estimated that there are about 200000 metabolites present in the plant kingdom, the metabolome of a single plant is estimated to consist up to 5000 different metabolites (Bino et al., 2004). At the moment, no analytical technique is capable to face this challenge and cover the whole metabolome of an organism. Therefore, it is necessary to combine different extraction, separation, and detection methods for full comprehensive studies.

Table 5.1: Definitions of important terms in the field of metabolomics (Fiehn, 2001; Harrigan and Goodacre, 2003; Goodacre et al., 2004; Dettmer et al., 2007; Allwood et al., 2008).

Metabolome	All small molecules produced by an organism, tissue, cell.
Metabolomics	An unbiased identification and quantification of all (low molecular weight) metabolites of a biological system, organism, tissue, or cell under well-defined conditions.
Metabonomics	Quantitative analysis of metabolites of an organism changing in response to pathophysiological stimuli (stress, disease, toxic exposure, specific dietary or drugs) or genetic modification.
Metabolic profiling*	Identification and (approximate) quantification of a predefined set of metabolites, e.g. belonging to a substance class (lipids, carbohydrates, isoprenoids, volatiles) or to a particular pathway or all metabolites ascertainable with a specific analytical technique.
Global metabolomic profiling	
Metabolite fingerprinting	Classifying (a large number) of samples whose metabolites alter due to e.g. disease, environmental or genetical perturbations. Aims in the identification of the discriminating metabolite but qualitative and quantitative assignment of metabolites is initially not necessary.
Metabolite footprinting	Analysis of all emitted/secreted metabolites. Offers the advantage of non-invasive measurement.
Targeted metabolite analysis	Identification and precise quantification of a single metabolite or small set of target compounds. Based on existing knowledge or after unbiased metabolomics analysis, offers the advantage of selective extraction separation and detection techniques.

* This approach is described and discussed in this thesis.

Despite the above mentioned analytical challenges in metabolomics, there are several reasons why metabolomics studies have advantage over other –omics studies and are well suited for system-wide characterisation of biological systems (e.g. Fiehn, 2001; Dunn and Ellis, 2005; Goodacre, 2005; Álvarez-Sánchez et al., 2010a):

- Smaller number of metabolites per organism compared to genes and proteins, reduced sample complexity.
- Metabolomics techniques are cheaper, more robust, mature and frequently available by most labs dealing with analytical chemistry. The instruments used are faster and allow high-throughput analysis. Additionally, metabolomics analysis are cheaper than the application of genomics or proteomics techniques.
- A large number of metabolites are exactly the same in different organisms whereas genes and proteins are frequently organism specific. This eases the availability of standard compounds and facilitates the study of different biological systems with the same metabolomics platform.
- A large number of metabolites can be affected by one or only a few genes (Figure 5.1a). Hence, metabolomics studies have great potential to illuminate the function/influence of genes.
- Although the change in concentration of enzymes may be not significant due to a treatment of the organism, the change in metabolite concentrations might well be.

Finally no –omics technique alone can answer biological questions completely. Realisation of full comprehensive approaches needs the integration of several -omics techniques. As a result, of such combined approaches e.g. pathways are today not longer considered to be linear but complex metabolic networks (Figure 5.1b).

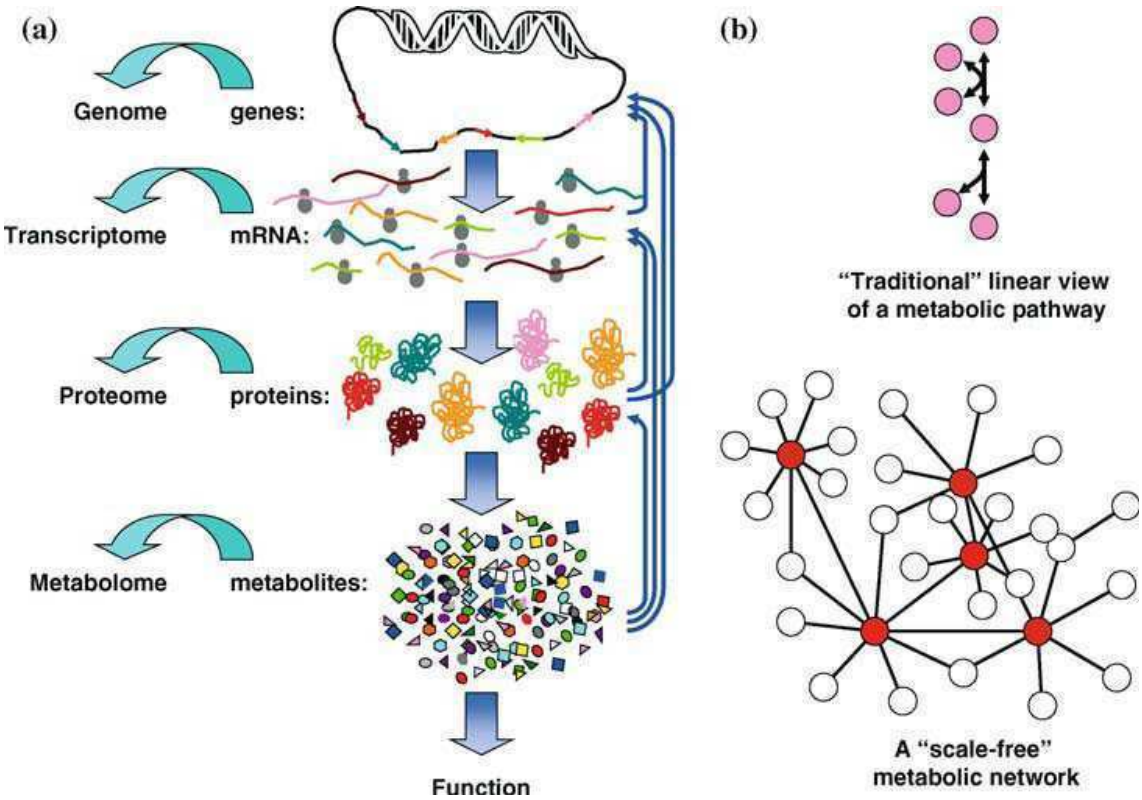

Figure 5.1: *(a) Systems biology needs the investigation of all levels of a biological system. Information from the genome is written to the transcriptome, which codes for the proteome resulting in many chemically different metabolites. All levels feedback and influence each other.*
(b) The traditional linear pathways as models for the processing in organisms are out of date. Modern approaches/models use so-called scale free metabolic networks. It is one of the challenges in metabolomics to find all metabolites participating in a particular pathway (from Goodacre, 2005).

History

The concept of detecting as much metabolites as possible in a sample is rather old (Table 5.2). For example Dalgliesh et al. reported 1966 "A Gas-Liquid-Chromatographic Procedure for Separating a Wide Range of Metabolites occurring in Urine or Tissue Extracts" in which they identified about 100 metabolites simultaneously.

The term "metabolic profiling" appears in scientific literature for the first time in an article title 1975 (Thompson and Markey, 1975) according to SciFinder (access 8.8.2011). A review from the same year about "Clinical Chemistry" mentions several studies about metabolic profiling (Gochman and Young, 1975). Clinical chemistry and medicine were the birth place for all metabolomics related approaches. Oliver et al. (1998) first mentioned the term "metabolome" in literature. At the website of the Scripps Center for Metabolomics and Mass Spectrometry a timeline about important steps towards metabolomics is given (Table 5.2).

Table 5.2: Timeline presenting milestones in the development of today's metabolomics. (modified from http://masspec.scripps.edu/metabo_science/timeline.php, access 3.8.2011).

Contributions	Year
Body fluids used to predict disease Ancient Greeks recognise the value of examining body fluids (at this time called humors) to predict disease.	**300 BC**
Quantitative basis of metabolism Santorio Sanctorius, considered to be the founding father of metabolic studies, publishes his work on 'insensible perspiration' in De Statica medicina, and determined that the sum total of visible excrement (urine, feces, sweat) was less than the amount of substance ingested. This work is considered the first effort to obtain physiological data and provide a quantitative basis to pathophysiology via meticulous study and precise instrumentation.	**1614**
First mass spectrometer J.J. Thomson at the University of Cambridge constructs the first mass spectrometer (then called a parabola spectrograph).	**1905**
GC development by Martin and Synge	**1940's**
NMR introduced Felix Bloch at Stanford University and Edward Purcell at Harvard University simultaneously publish the first NMR spectra in the same issue of Physical Review.	**1946**
Mass spectrometry-based metabolomics 1966 Dalgliesh et al. perform the first GC/MS Metabolomic experiments and report it in Biochemical Journal 101, 792. 1971 Mamer and Horning publish an approach to Human Metabolite Mass Spectrometry Profiling in Clinical Chemistry 17, 802-809	**1966**
NMR based metabolomics Observation of tissue metabolites using 31P nuclear magnetic resonance. Hoult DI, Busby SJ, Gadian DG, Radda GK, Richards RE, Seeley PJ. Nature. 1974 Nov 22;252(5481):285-7	**1974**
First use of the word "metabolome" SG Oliver, MK Winson, DB Kell and F Baganz use the term 'metabolome' for the first time, published in Trends in Biotechnology.	**1998-9**
Introduction of SPME by Arthur and Pawliszyn	**1990**
1st meeting of the Metabolomics Society	**2005**
XCMS bioinformatics platform for untargeted metabolomics A bioinformatic platform XCMS was developed for untargeted mass spectrometry based metabolomics.	**2006**
metaXCMS meta-metabolomics analysis approach A major challenge in interpreting metabolomics data is distinguishing metabolites that are causally associated with the phenotype of interest from those that are unrelated but altered in downstream pathways as an effect.	**2010**

In 1999 Trethewey et al. (1999) reported only two publications in the field of plant metabolomics. In the following years, the application of GC-MS for the profiling of plant metabolites, mainly non-volatiles after derivatisation, increases steadily (Allwood et al., 2008). The investigation of the plant volatile metabolome is still rare. Koek et al. (2011) give an overview of plant

metabolomics studies using GC-MS. The authors report 27 studies, three of them extracted the volatiles from the plant sample headspace whereas two useed SPME, the method which has been used during this work.

Metabolomics workflow

A typical metabolomics experiment consists of not only the analytical part but begins with the biological question (hypothesis), followed by the appropriate experimental setup, sampling, sample preparation, measurement, data handling, interpretation of results and finally answering the initial biological questions (Figure 5.2).

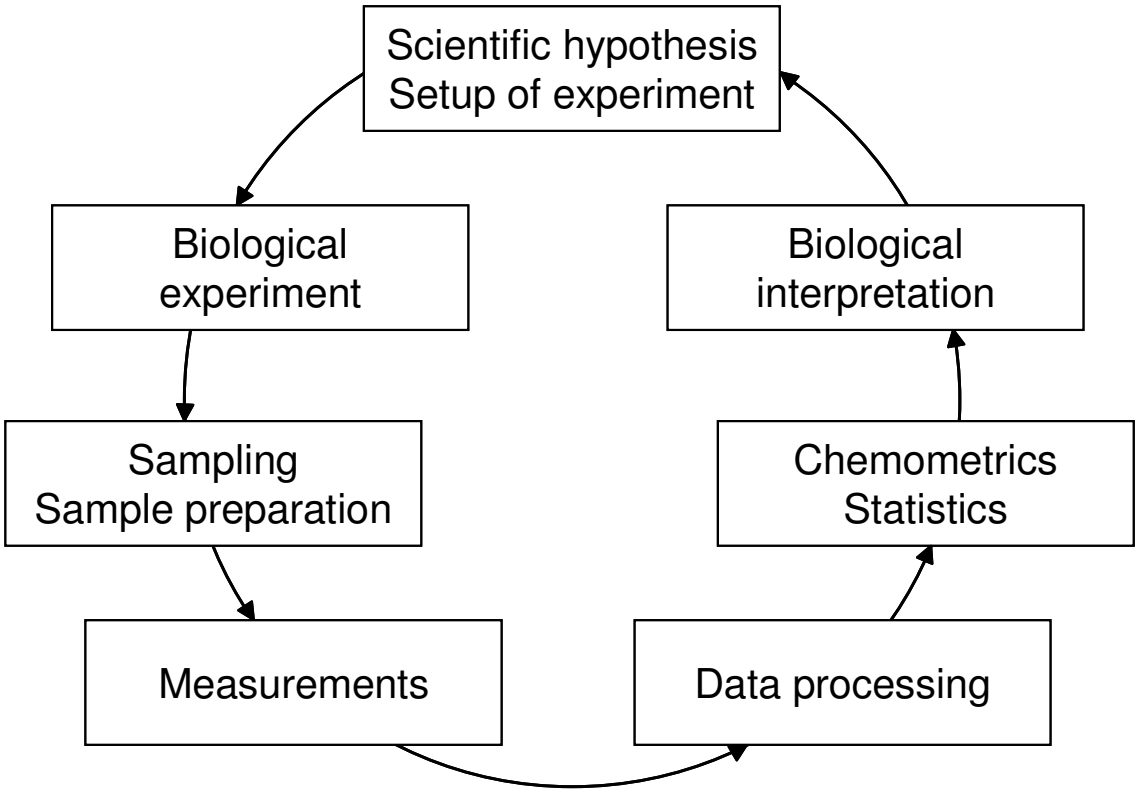

Figure 5.2: Typical metabolomics workflow

Often new questions appear which require new experiments to be answered (hypothetic-deductive reductionistic approach, Figure 5.3). On the other hand, the hypothesis can be generated out of the data (inductive approach to knowledge discovery, holistic approach, Figure 5.3). Since there are several scientific disciplines involved, metabolomics needs a close cooperation of e.g. biologists, analytical chemists, and bioinformaticians.

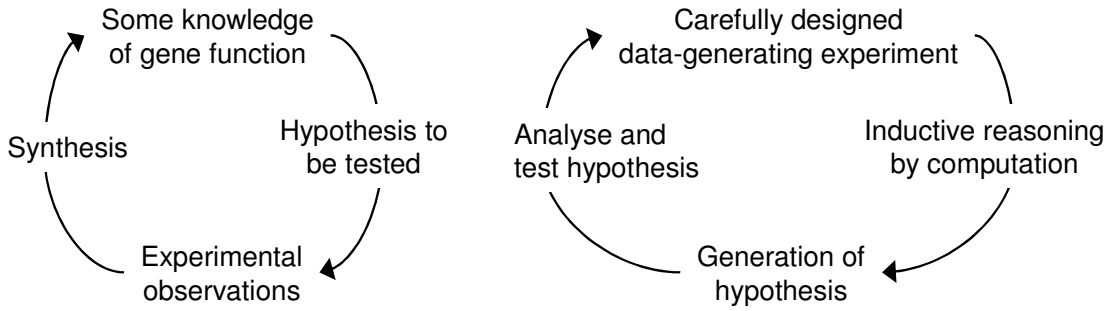

Figure 5.3: How hypothesis and data are connected in science. Left: Existing knowledge leads to a hypothesis, which is tested experimentally. The resulting data either verifies or falsifies the starting hypothesis. Right: Analysis of the data via "active learning" computation algorithms provides ideas for hypothesis which can then be tested like in the left approach (adapted from Goodacre et al., 2004).

Comparative studies

As shown in the definitions section above, different approaches can be chosen depending on the biological question or the hypothesis. The majority of the published metabolomics studies deal with the investigation of gene or protein functions or aim in the detection of biomarkers. Therefore, a comparative approach is suitable. Scientists compare the metabolome of e.g. wild-type and mutant or a stressed group of plants with a unstressed control group. For the detection of the metabolites (biomarkers) which are affected by e.g., genetical mutation, stress or insect attack, two procedures are reported:

- bottom up or targeted: The data handling starts with the identification of the metabolites. Subsequently the concentrations (or if not quantified the peak areas) of these metabolites are compared between the two sample groups with the aim to find significant differences.
- top down or untargeted: the data handling starts with an annotation of all peaks followed by the search for differentially expressed peaks. Only those were further considered e.g. for identification/structure elucidation.

Independent of the chosen approach, the bias in the steps before data analysis shall be as low as possible.

Database

The targeted approach requires a list of substances (positive list) , which shall be searched for in the GC-MS files. This positive list can either be derived from known literature data or from an untargeted approach. Thereby, as many metabolites as possible are identified and these results used as positive list. Best solution will be to combine both procedures, as it was done in this work.

Before I started with the method development, I wanted to gather an overview about the substance classes I would have to expect in grapevine leaves. Therefore, I started an intense literature research about previous studies of

grapevine leaves and other parts of this plant species. This investigation resulted in the compilation of a database with about 1619 entries of substances found in 39 studies referring to the investigation of grapevine using GC. If available, the CAS number and the systematic name were added as well as part of the plant where the substance was detected and the corresponding reference. The database is available as supplementary material to paper #3.

Additionally, in all grapevine studies the recorded mass spectra were searched against combined Wiley NIST 08 reference MS spectra library. Details about the annotation and identification process can be found in chapter 9.4, paper #1, and #3.

Standardising metabolomics experiments

"The nice thing about standards is that there are so many to choose from." Andrew S. Tannenbaum (in Quackenbush, 2006).

As mentioned above, one and the same metabolite always has the same chemical structure independent of its origin (plant, animal, fungi). Thus, the results of metabolomics experiments can complement each other and therefore it makes sense to standardise metabolomics experiments. In most cases, authors refer to the standardisation of the reporting of the experiments. Additionally, there are suggestions to standardise the experiments or single steps in the metabolomics workflow itself. There are several reasons for standardising or at least document accurately metabolomics experiments in detail including all steps and all factors possibly influencing the results (Fiehn et al., 2006). Standardisation is required to

- make the studies repeatable
- have the possibility of judging metabolomics experiments
- enable the finding of new correlations, pathways and/or networks of metabolites as the results are colleted in databases

One of the first suggestions for "Minimum Information About a METabolomics experiment" (MIAMET) was described by Bino et al., 2004. In the same year Jenkins et al. (2004) presented ArMet (architecture for metabolomics). Several publications describing standards for every step in metabolomics experiments were initialised by the Metabolomics Standards Initiative (MSI) (Fiehn et al., 2006, 2007a,b, 2008; Goodacre et al., 2007; Morrison et al., 2007; Sumner et al., 2007). There exist now suggestions for minimum reporting standards for:

- biological study design, biological context metadata
- chemical analysis
- data acquisition, data exchange

- data processing/analysis and interpretation relative to the biological hypotheses

The knowledge about the metabolites resulting from metabolomics studies ought to be compiled into databases whereby also mass spectra of unidentified substances are stored. The final aim of metabolomics is to reduce “terra incognita” in biological pathways and systems.

LC-MS or GC-MS for metabolite profiling?

Originally the “fingerprint project” aimed in the detection of secondary metabolites in grapevine plants, which can be conducted with different analytical techniques e.g. liquid chromatography (LC) or GC coupled to a MS detector. Both techniques have their own advantages and disadvantages.

LC covers a wider range of metabolites than GC but identification of metabolites is more challenging. LC is frequently coupled to a MS detector allowing MS/MS, which leads to fragmentation spectra characteristic for the fragmented metabolite. Hence, LC provides the *m/z* of the metabolite ion and a spectrum of ion fragments. Nevertheless, the fragmentation pattern depends strongly to the instrument settings and also to the instrument itself. Therefore, the spectra cannot be compared easily and only small spectra library exist.

GC-MS instruments are frequently equipped with a single quadrupole (see 8.2) and provide standardised fragmentation spectra but only in rare cases the metabolites ion *m/z*. However, the standardised substance spectra have been collected for many years in commercial available mass spectra databases. Hence, identification of metabolites is much easier than with LC-MS instruments. Additionally, GC offers a higher reproducibility of the elution behaviour of the metabolites, which enables the usage of retention time indices for further metabolites identification. Since volatiles are a major group of the secondary metabolites, application of GC-MS is a good alternative to LC-MS. In the project, the identification of potential biomarkers for stress was demanded, which GC-MS can provide more easy than LC-MS. All in all GC-MS was the method of choice for this thesis.

Volatile organic compounds

According to Wenke et al. (2010) volatile organic compounds (VOCs) are mostly lipophilic substances with a high vapour pressure (0.01 kPa or higher at 20 °C) and a low molecular mass (≤ 300 Dalton). Therefore, they easily evaporate. Their volatility makes VOCs well suited for the investigation with GC.

The investigation of volatiles is of great interest since VOCs are produced and emitted by plants and hence contribute to a high extent to the communication of plants with their environment. VOCs participate e.g. in plants defence, attraction of pollinators or seed-dispersing animals (Pichersky and Gershenzon, 2002).

Among plant volatiles nearly all substance classes can be found, mainly C_6-compounds, terpenoids, aromatic compounds, alcohols and n-alkanes.

In this thesis, I ended up with a bottom up metabolic profiling approach. Using HS-SPME and GC-MS the metabolites to be detected are limited to volatiles. I took care that this was the only restriction. The workflow was developed and evaluated using open field grapevine leaf samples and later on applied to grapevine roots. The set of metabolites for investigation of two differently treated sample groups was derived from the samples itself by identifying as much metabolites as possible (see paper #1 and #3).

6 Sample handling

Samples are the basis of each analysis. This chapter describes, what plant parts and how they can be sampled, discusses quenching of metabolism, sample storage and sample preparation for further analysis.

6.1 Selection of biological samples, sampling

In plant metabolomics studies, it is important to have well defined plant material. Especially in comparative studies, the external influences shall be under control to avoid effects beside the intended treatment to the plants. This can be largely achieved with plants grown in the greenhouse or an artificial growth chamber (Fukusaki and Kobayashi, 2005). On the other hand, some plants cannot be grown under greenhouse conditions simulating the real world. For example trees are simply to big and growing too slow. In general, perennial or indeciduos plants might show different behaviour depending on their age but in greenhouse studies mostly young plants, growing for some weeks or months are used. Although the environmental influences cannot be controlled in field studies, they can be at least monitored and will reflect the natural metabolome more realistically.

While the most published literature deals with improvement of metabolite detection, identification and data handling, only a few papers deal with the starting point of every analytical process, the selection of the appropriate sample for the given task (e.g. Álvarez-Sánchez et al., 2010 a). In target analyses the sampling and in particular the sample treatment are very important steps, which are frequently optimised and evaluated intensively. The sampling has to provide a representative part of the whole sample. Also in metabolomics the aim is to sample a representative part of the organism. This is easy for small organisms like e.g. *Arabidopsis thaliana*, where the whole plant can be sampled but difficult for larger plants as for example grapevine or even trees. Strategies for sample pooling and homogenisation are necessary. Unfortunately, it is not possible to verify whether a sampling step is appropriate due to missing reference methods yet.

Samples can be obtained by invasive or non-invasive methods. While DNA, RNA or primary metabolites are not accessible without damaging plant tissue, volatiles can be sampled without any damage. Non-invasive methods allow frequent sampling of one and the same plant but require a more complex equipment than invasive sampling techniques. A plant provides several parts, which can be sampled: roots, stems, leaves, stalks, sap, tubers, flowers, fruits, and the derived products as oils, juices, etc. (Álvarez-Sánchez et al., 2010a).

Depending on the biological question, different plant parts can be sampled. Sampling xylem or phloem sap e.g. can provide knowledge about molecules serving as signalling substances. Leaves for example are one of the favourite parts for investigation of the volatile plant metabolome as the leaves can easily be harvested and are available over the most time of the year. Roots are more difficult to sample as they are surrounded by soil, which has to be removed before analysis. Therefore, the plant has to be taken out of the soil, which can cause stress to the plant and hence change the metabolome. Or the soil has to be replaced with a soil-less culture system (Fukusaki et al., 2003).

The biological experiment shall provide an adequate number of samples. Plants grown in the greenhouse may be less influenced by environmental factors than plants in open field experiments. The latter might show a higher biological variability, which complicates the detection of differentially expressed metabolites (biomarkers). Taking more samples and/or pooling samples can help to overcome these problems. In addition, the size of the plant and the number of samples harvested has to be taken into account. Every invasive sampling can lead to a change of the metabolome. Therefore, in the ideal case, plants, which have been sampled once, shall be excluded from the further experiment. This demands a high number of plants for the whole experiment. For bigger plants or field experiments or if it can be guaranteed that the control and the treated group experience the same sampling treatment it might be acceptable to take several samples from the same plant at different time points.

During this thesis, only detached plant material was analysed. The grapevine plants in the fingerprint project used for method development (paper #3) were grown in the field. Hence, we judged the environmental influences higher than the influence of the sampling of four leaves per plant and sampled the same plants a second time a month later.

In the phylloxera experiment (paper #1), we assumed that the small number of roots which were cut did not have such a large influence to the metabolome as the feeding insect. In addition, roots were also cut from plants of a control group (identical sampling procedure) so that eventually appearing changes due to the sampling process are expected to be the same in both groups.

The dust samples (paper #2) were non-living material, therefore this particular aspect of sampling had not to be considered in this case.

6.2 Quenching

It is necessary to “freeze” (quench) the actual status of a sample immediately upon sampling. The quenching process has to fulfil certain needs (Fiehn, 2002; Dettmer et al., 2007; Álvarez-Sánchez et al., 2010b):

- The quenching has to be very fast, before the metabolome can change. Turnover rates of some primary metabolites e.g. have been reported to be in the range of 1mM/s. D’Auria et al. (2007) reported a maximum of emitted green leaf volatiles (GLV) 30-45 seconds after plant tissue wounding. For terpenoids, it has been described to last several hours until a change can be observed (Arimura et al., 2009).
- During sampling, the damage of cells must be minimised as this may lead to undesirable reactions between metabolites, which are normally separated in different cell organelles.
- The quenching technique itself must not lead to any change in terms of chemical properties.
- After quenching, the sample shall be available for further sample treatments.

The following quenching techniques are reported in literature, all are based on the fast change of sample conditions (Álvarez-Sánchez et al., 2010b):

- Change of pH to high (alkali) or low (acidic) values
- Change of temperature: e.g. with cold methanol, lq. nitrogen, hot ethanol

The quenching with cold methanol is very common when the non-volatile metabolites are investigated whereas the volatile metabolome of plants is quenched mostly with liquid nitrogen. It is a simple and fast method, feasible for lab and field samples.

In the presented publications (paper #1, #3, #4) dealing with grapevine tissue the quenching was done with liquid nitrogen. The dust samples (paper #2) were not quenched due to any risk of changes of the metabolites.

6.3 Sample storage

After successful quenching, the samples shall be analysed as fast as possible. Often this is not possible, hence they have to be stored for a certain time period. Especially in comparative studies or time course studies it makes sense to measure all samples within a short time interval to minimise imprecision due to varying instrument performance. The rule is in general the colder the better but freezing does not stop but just slow the chemical processes in the plant tissue.

Repeated freezing and thawing cycles have to be avoided (Al-Jowder et al., 1997). Inappropriate storage can lead to conversion and degradation of metabolites (Peng and Jay-Allemand, 1991; Teahan et al., 2006). Because liquid nitrogen is well suited for quenching it is as well suited for storage. Due to high costs of liquid nitrogen storage, most laboratories store the plant samples at -80°C.

Freeze-drying is often reported to be well suited for stabilising plant samples for long-term storage. It reduces the water content and therefore enzymes can no longer be active. For the investigation of the volatile metabolome it has turned out that freeze-drying leads to the loss of volatile metabolites (Aprea et al., 2011). Therefore, no samples were freeze-dried within the presented work. The grapevine leaves and roots were stored at -80°C whereas the dust samples were stored at -20°C until analysis.

6.4 Sample preparation

The goal of sample preparation in target analysis, e.g. by extraction and subsequent purification, is to separate matrix compounds from the analyte(s) of interest and to increase their concentration to facilitate the measurement. The sample preparation in metabolomics studies is supposed to lead to no or as little changes in the metabolome as possible. The following sample preparations prior to analysis are reported (e.g. Kim and Verpoorte, 2010; Álvarez-Sánchez et al., 2010 b):

- Freeze-drying
- Homogenisation
- Liqud extraction
- Extraction from headspace
- Derivatisation

Either a single method or any combination of the listed options can be applied. The extraction step has to fulfil the following requirements (e.g. Álvarez-Sánchez et al., 2010 b):

- Extraction of all metabolites in the same relation, as they exist in the sample. This is not possible at the moment with the available techniques.
- Preparation of the sample for subsequent analytical analysis.
- Eventually it might be necessary to concentrate the metabolites in order to enable the detection of traces.

- If only part of the metabolome is under investigation, it might be useful to remove disturbing substances without changing the metabolites of interest.

Prior to HS extraction, homogenisation of the plant tissue is recommended due to two reasons. First, if an aliquot of the sampled plant tissue is used for analysis a representative and defined amount has to be transferred to HS vials. Second, to increase extraction efficiency which can be achieved by larger sample surface (Villas-Boas et al., 2007, p 51). The breaking of the cell walls carries the risk of undesirable reactions (Álvarez-Sánchez et al., 2010b). I suppose, adding antioxidants or inert gas to the sample in the HS-vial might reduce the risk of oxidation products.

Homogenisation can be done e.g. with mortar and pestle, ball mill, vibration mill, UltraTurrax, ultrasonic probe or thermomixer (Álvarez-Sánchez et al., 2010b). Since thawing has to be avoided all steps have to be carried out under cooling e.g. with liquid nitrogen. For this purpose, ball mills are available which allow the milling container to be cooled during the milling process.

In the presented grapevine studies (paper #1, #3, #4), a ball mill (Retsch MM 301) was applied for leaf and root sample homogenisation. The used equipment did not offer the possibility of cooling the milling containers during the milling process. Therefore, I cooled the stainless steel beakers in liquid nitrogen before and after milling. I achieved always a fine powder and no thawing of the samples was observed. Immediately after the homogenisation process, the samples were weighed into 20-mL HS-vials and stored at -80 °C till analysis.

No further sample preparation was applied to the dust samples (paper #2). Samples were weighed into 20-mL HS-vials prior to analysis.

7 Solid phase microextraction SPME

SPME is a technique for the simultaneous extraction and enrichment of metabolites (Arthur and Pawliszyn, 1990). It is well established in routine analytical chemistry nowadays, applied in food chemistry, drug analysis, forensic, wastewater, and environmental chemistry. SPME can be applied for either screening, qualitative or even quantitative challenges. It is a rather cheap technique (about 100 EURO per PDMS fibre) and the fibre can be used up to several hundred times in HS mode. No solvents are necessary and the HS vials can be reused. For high-throughput analysis, which is neccessary in metabolomics, an autosampler with a needle heater and a vial heating station is available.

Different coatings of the fibre are available the selection of which has to be adjusted to the properties of the metabolites of interest (Table 7.3). Larger molecules can be sampled with a fibre with a thin coating whereas small metabolites need a thicker fibre coating otherwise they will desorb to fast.

Table 7.3: Different fibre coatings, sorbtion mechanism and polarity (Pawliszyn, 2009, p 91).

Coating	Thickness [µm]	Mechanism	Polarity
Polydimethyl-siloxane (PDMS)	7, 30, 100	Absorbtion	Apolar
Polyacrylate (PA)	85	Absorbtion	Polar
Polyethylene glycol (PEG)	60	Absorbtion	Polar
Carbopack Z/PDMS	15	Adsorbtion	Bipolar
PDMS/Divinylbenzene (DVB)	65	Adsorbtion	Bipolar
Carboxen (CAR)/PDMS	85	Adsorbtion	Bipolar
DVB/CAR/PDMS	55/30	Adsorbtion	Bipolar

The coating is fixed on a fused silica core and in case of PDMS can be considered as a thin liquid film like the coating in a GC column. Depending on the coating material, the metabolites are ad- or absorbed (Table 7.3). In case of mixed coating materials, both processes can occur. In SPME, a multiphase equilibrium occurs: between sample and gas phase and between gas-phase and fibre. The initial concentration of a metabolite in a sample C_0 is the sum of the equilibrium concentrations C^{∞} of this metabolite in the sample, the headspace and the fibre coating after reaching the equilibrium state (Equation 7.1). Also the volumes of the three phases play a role. The concentration of the analyte in the fibre is related to the concentration of the analyte in the sample. It depends on the distribution constant (diffusion of the metabolites between the phases) which is temperature dependent (Equation 7.2). With this knowledge, the SPME parameters time and temperature for equilibration and extraction and

fibre coating have to be optimised for best extraction results. The multiphase equilibrium can be expressed with the following equation (simplified, Pawliszyn, 2009, p 25):

$$C_0 V_S = C_f^{\infty} V_f + C_{HS}^{\infty} V_{HS} + C_S^{\infty} V_S \qquad (7.1)$$

defining $K_{fHS} = \frac{C_f^{\infty}}{C_{HS}^{\infty}}$ and $n = C_f^{\infty} V_f$ leads to

$$n = \frac{K_{fHS} K_{HSS} V_f C_0 V_S}{K_{fHS} K_{HSS} V_f + K_{HSS} V_{HS} + V_S} \qquad (7.2)$$

C_0	Initial concentration of metabolite in sample
C^{∞}	Equilibrium concentration
C_f	Equilibrium concentration of metabolite in fibre coating
C_{HS}	Equilibrium concentration of metabolite in headspace
C_s	Equilibrium concentration of metabolite in sample
V_s	Volume of sample
V_f	Volume of fibre coating
V_{HS}	Volume of headspace
K_{fHS}	Distribution constant between fibre coating and headspace
K_{HSS}	Distribution constant between headspace and sample
n	Mass of analyte in fibre coating

For most reproducible SPME results, metabolites shall be extracted after equilibrium between sample and headspace has been reached. The extraction itself shall last until equilibrium between the metabolites in/on the fibre and the HS has been reached (Figure 7.4). The time interval necessary to reach this equilibrium is substance dependent and can range from a few minutes to hours. Typically, for the whole SPME procedure (equilibration plus extraction time) a time range from a few minutes up to 1 hour is reported in literature.

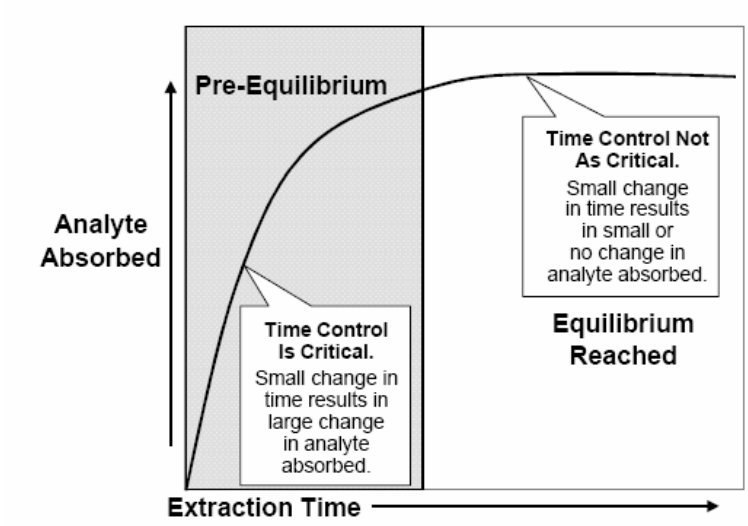

Figure 7.4: Saturation curve, the amount of analyte on the fibre reaches a maximum after a certain time period (Supelco Bulletin 929).

Two problems have to be solved regarding the equilibrium: first, some substances reach the equilibrium after hours, which will lead to unacceptable long analysis time. Second, different substances show different time intervals until the equilibrium is reached.

The first problem can be solved by heating the sample. The metabolites will evaporate faster and the equilibrium is reached faster. Regarding the second aspect, it is not necessary to wait until all substances have reached an equilibrium state. If one can reproduce the time and temperature for each extraction precisely, repeatability of the extraction yield will also be acceptable. This can be achieved by usage of an autosampler.

The theory above is valid in case of a single analyte in the sample with no matrix (at least no volatile matrix). In case of a complex matrix and many metabolites as in plant tissue samples, the matrix generally influences the equilibrium process heavily.

This leads us directly to one of the major disadvantages using SPME in metabolomics. It is nearly impossible to quantify absolutely and precisely a large number of metabolites with different polarities or volatility simultaneously with SPME. Additionally, in comparative studies differently treated plants are sampled which might have to some extent different metabolomes. Therefore also the matrix, which influences the metabolites' equilibrium between the phases is different. Complex biological samples such as e.g. plant leaves contain volatile metabolites of varying boiling points and polarity. Hence, the simultaneous analysis can always be regarded as a compromise. The chosen SPME conditions cannot be ideal for all metabolites simultaneously.

In my experience with SPME the most important SPME parameters to be optimised are the temperature for equilibration and extraction as well as the extraction time. These parameters were optimised with grapevine leave samples. I aimed to optimise the before-mentioned parameters in a way to achieve a high number of peaks (metabolites) and at the same time a high abundance of the peaks (paper #3). In this work, for the extraction of grapevine metabolites, a DVB/CAR/PDMS fibre was used because it covers the largest range of metabolites. During method development for grapevine leave samples the SPME parameters equilibration time, extraction time, equilibration temperature, and extraction temperature were optimised. The optimised conditions were: equilibration time: 30 minutes, extraction time: 60 minutes, equilibration and extraction temperature: 90°C (paper #3).

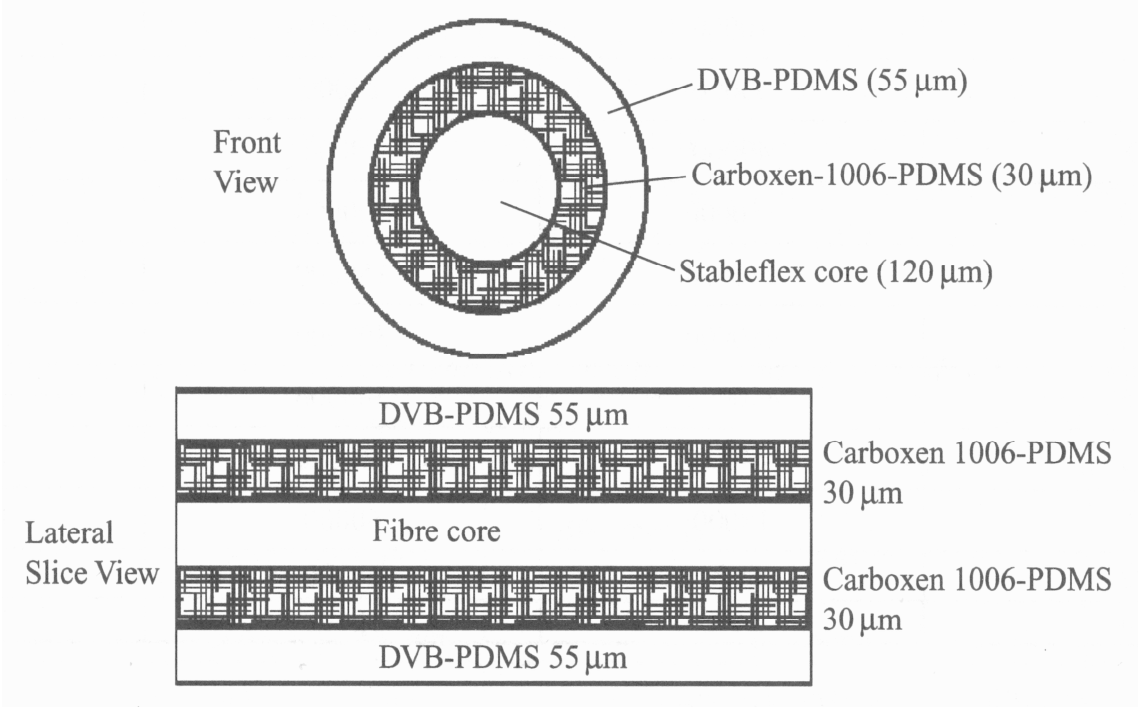

Figure 7.5: Assembly of a multicoating fibre (Pawliszyn, 2009, p 101).

In case of dust samples, different fibre types were tested and the extraction conditions optimised. It turned out that the CAR/PDMS fibre extracted most metabolites from the dust samples. Optimum extraction time was 30 minutes at 90 °C and no equilibration of the samples was necessary.

8 Separation and detection of metabolites

One of the major goals of this thesis was to develop a method providing potential markers indicating stress in grapevine plants. Therefore, it was necessary to identify these potential markers. The ideal detector would be able to provide the metabolites unambiguous identity. A separation system in front of the detector will provide two benefits. First, concentration of the single metabolites resulting in higher detector signals. Second, separation of the metabolites will lead to consecutive arrival at the detector. Since this will avoid detecting substance mixtures, the identification of metabolites is more reliable. Unfortunately, no such ideal system is available at the moment. As I was interested in the volatile metabolome of grapevine leaves and wanted to identify potential stress markers, GC-MS was chosen for separation and detection, a technique, which fulfils most of the above-mentioned demands.

8.1 Gas chromatography

Gas chromatography (GC) is a widespread and well-established technique for separation of low boiling, low mass compounds even in complex mixtures. A gas chromatograph (GC) consists of a sample inlet, an oven compartment holding the chromatographic column, and a detector (Figure 8.6 and Figure 8.6).

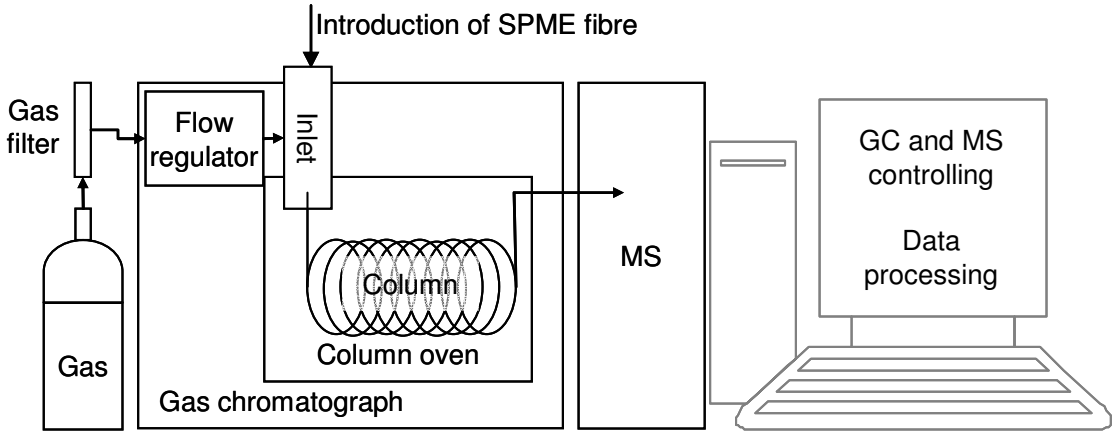

Figure 8.6: Scheme of a GC-MS instrument

For successful GC separation, analytes have to evaporate at the temperature applied in the GC inlet without being destroyed. The sample is evaporated in the hot inlet (200 - 400°C) and further on transported with the mobile phase to the chromatographic column where the metabolites are separated. An inert gas (nitrogen, hydrogen or helium) is used as mobile phase. The column is a thin

(0.1 – 0.5 mm inner diameter) 10 – 100 meter long fused silica capillary which is coated at the outer side with polyimide to make the column flexible. The stationary phase is located on the inner side of the capillary as a liquid film with a thickness of typically 0.1 – 5 µm (Figure 8.7). Stationary phases are available in different polarities to address different separation challenges. The column is placed in an oven, which can be heated either to a constant temperature (isothermal) or in a gradient mode with high precision. Compared to isothermal mode, the usage of the oven by applying a temperature gradient to the column has several advantages: it fastens the separation and therefore the whole analysis, it leads to narrower peak shapes, which is combined with a better separation and it enables the detection of substances with different boiling points within one run.

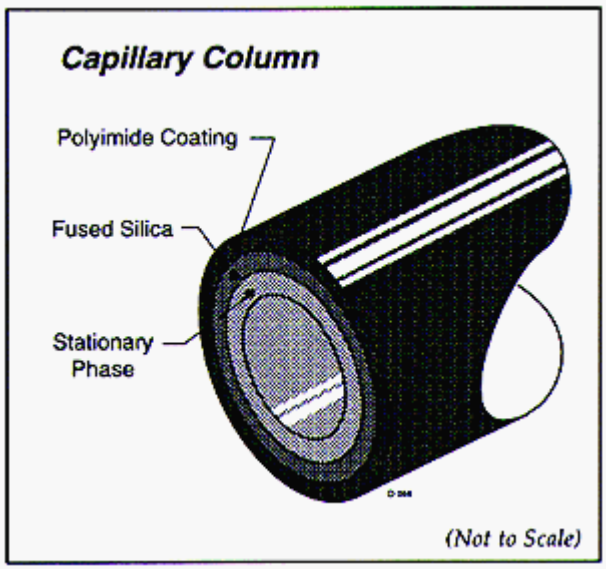

Figure 8.7: Assembling of a GC column (from www.chem.agilent.com/cag/cabu/capgccols.htm, access 28.8.2011)

Although GC shows a very good chromatographic separation efficiency, which means a high number of theoretical plates and high peak capacity, it is not possible to achieve a complete separation of all analytes in metabolomics. Typical peak capacity in GC is 150 – 250 peaks in a 5 – 60 minutes run (Medina et al., 2001). In a typical chromatogram of grapevine leaves, more than 350 peaks can be detected (Figure 8.8). Thus, overlapping peaks originating from coeluting substances are unavoidable.

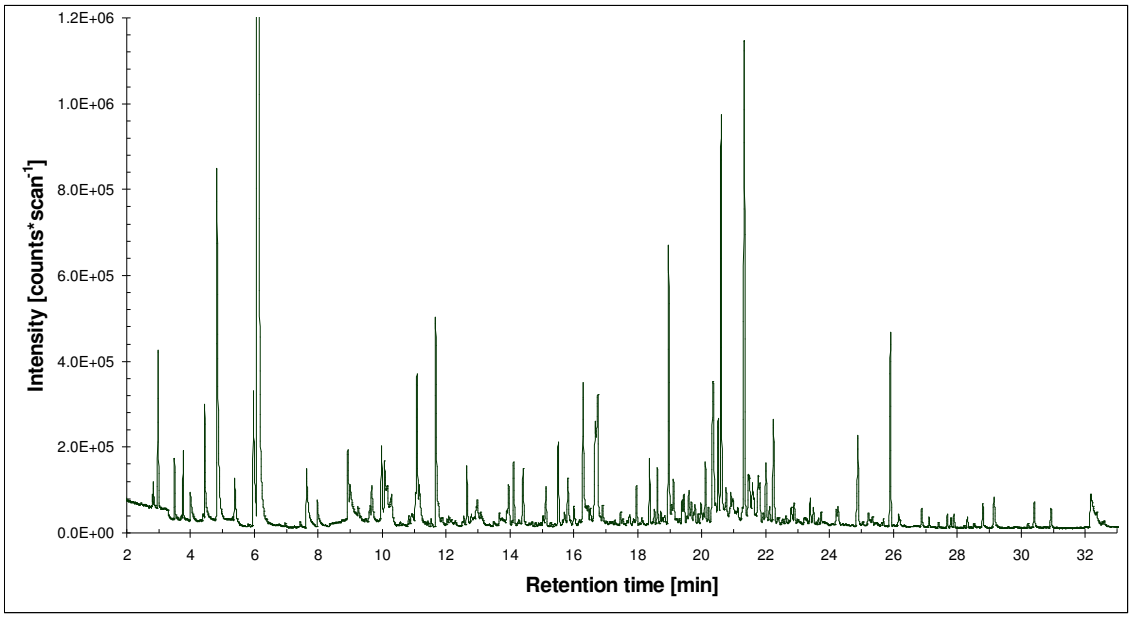

Figure 8.8: A typical GC-MS chromatogram of a leaf sample. Manual inspection shows more than 100 peaks.

Another challenge in metabolomics is to prevent or at least minimise the risk of producing artefacts. It is very important to test which substances origin from the GC inlet, column or SPME fibre. The bleeding of substances from these devices cannot be completely avoided. They contain for example a high number of silicium and can therefore easily be excluded from the results list.

Furthermore, artefacts might be produced from sample constituents during injection into the GC instrument. This can either be tested with authentic standards or by injection of aliquots of the same sample under varying inlet temperatures.

For the experiments carried out in the presented work, an Agilent 6890 instrument was used for GC (Figure 8.9). The injector was set to 250°C which is sufficient for desorption of the majority of the metabolites from the SPME fibre. A flat temperature ramp of 5°C/min was applied with a gas flow of 1 mL/min helium for separation of sample metabolites. This kind of settings is typically described in literature for the separation of a large number of analytes (e.g. Fiehn et al., 2000; Broeckling et al., 2005; Batovska et al., 2008). Metabolites were measured on two columns with different polarity: an apolar DB-5MS and a polar Optima-WAX (chapter 12).

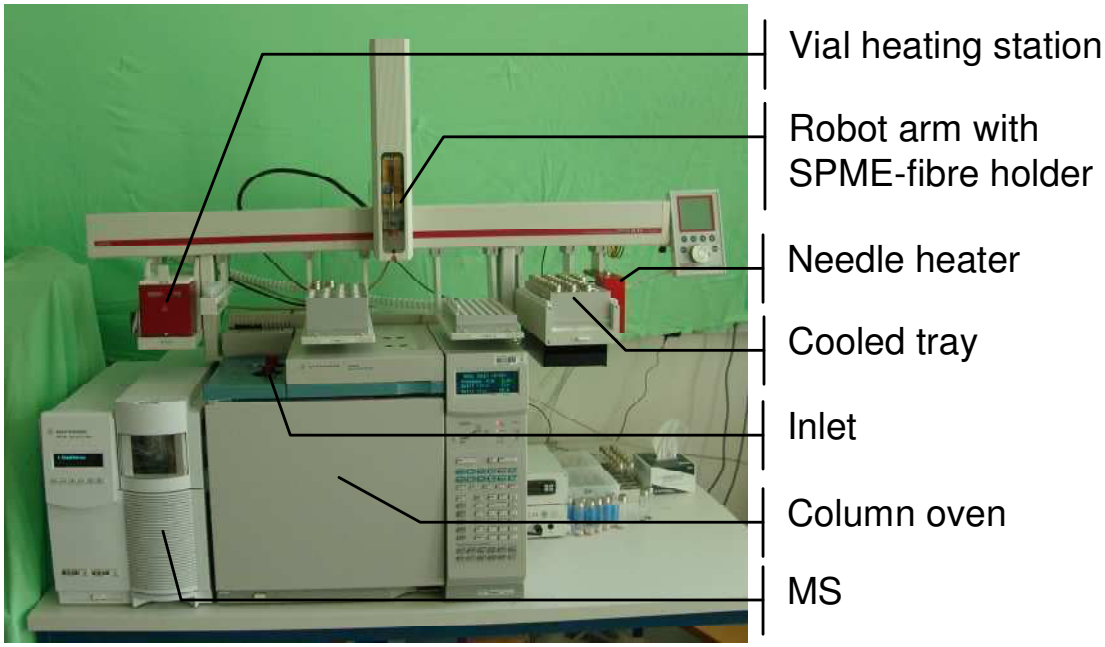

Figure 8.9: GC-MS with autosampler for fully automated SPME. The photo was taken at the Center for Analytical Chemistry. With the shown setup all analysis presented in this work have been done.

8.2 Mass Spectrometry

Mass spectrometry (MS) is a technique to determine the mass-to-charge-ratio (*m/z*) of a substance. Although is not a real spectroscopic technique it is frequently referred to as “spectrometry “or “spectroscopy”. The reason is the similar appearance of the resulting mass spectra compared to real spectroscopic spectra. An MS detector is coupled to GC frequently (GC-MS). MS detectors are very sensitive, show low substance specificity, and allow even the detection of substances in case of coelution. Additionally, MS provides the ability of substance identification by comparison of experimental MS spectra with reference spectra, hence there is not necessarily a need for authentic standards. After separation of the substances in the GC part, they are ionised, and their *m/z* is determined.

A typical mass spectrometer used in GC-MS consists of an ion source, a mass analyser, an ion detector, vacuum pumps and a computer (Figure 8.10). Several mass analysers are mainly used in combination with a GC instrument: ion trap (IT), time-of-flight (TOF), and quadrupole (single quad or triple quad QqQ). All experiments in this thesis have been carried out with a single quadrupole instrument, therefore only this type of mass analyser will be explained in detail.

The ion source, the mass analyser and the detector are under extremely low pressure (about $6*10^{-9}$ bar) to remove the mobile phase and avoid collisions of analyte molecules. Since the mass analyser can only deal with charged

molecules, an ion source is needed in front. Two types of ion sources are common in GC-MS: chemical ionisation (CI) which is a soft ionisation technique providing mainly intact charged molecules (parent ions) with nearly no fragmentation. Or electron ionisation (EI) which was applied in the instrument used for the thesis' experiments.

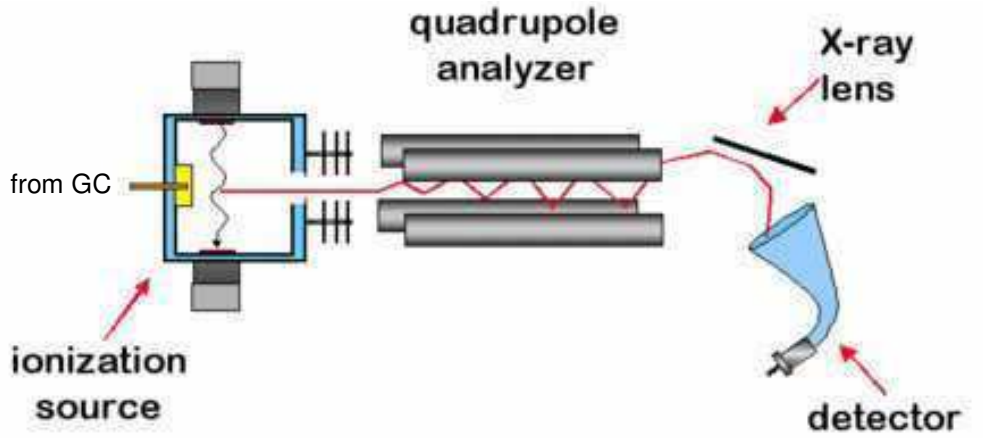

Figure 8.10: Scheme of an MS instrument (www.ull.chemistry.uakron.edu)

Electron ionisation

In the ion source the analytes are bombarded with electrons and subsequently the analyte molecule looses an electron resulting in a positive charged parent ion ($[M]^+$, Figure 8.11).

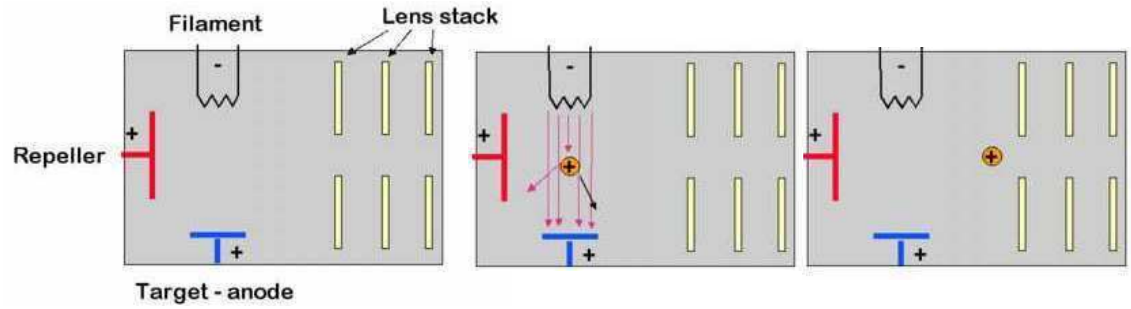

Figure 8.11: Electron ionisation (EI). An analyte molecule passes the electron beam, looses one electron, is therefore positively charged and repelled to the mass filter (quadrupole). Lenses serve for focussing the ions (www.ull.chemistry.uakron.edu).

Due to the high ionisation energy (70 eV) most parent ions carry an excess of internal energy and consequently charged and uncharged fragments are generated. In a mass spectrum the *m/z* of the intact molecule ion and the corresponding fragments (x-axis) as well as the intensity (or abundance, y-axis) are displayed (Figure 8.12). The fragment pattern is characteristic for each substance and provides information about the structure of the analyte. One

disadvantage of the fragmentation in EI is the loss in sensitivity since the initial intensity of a molecule is distributed over all fragments.

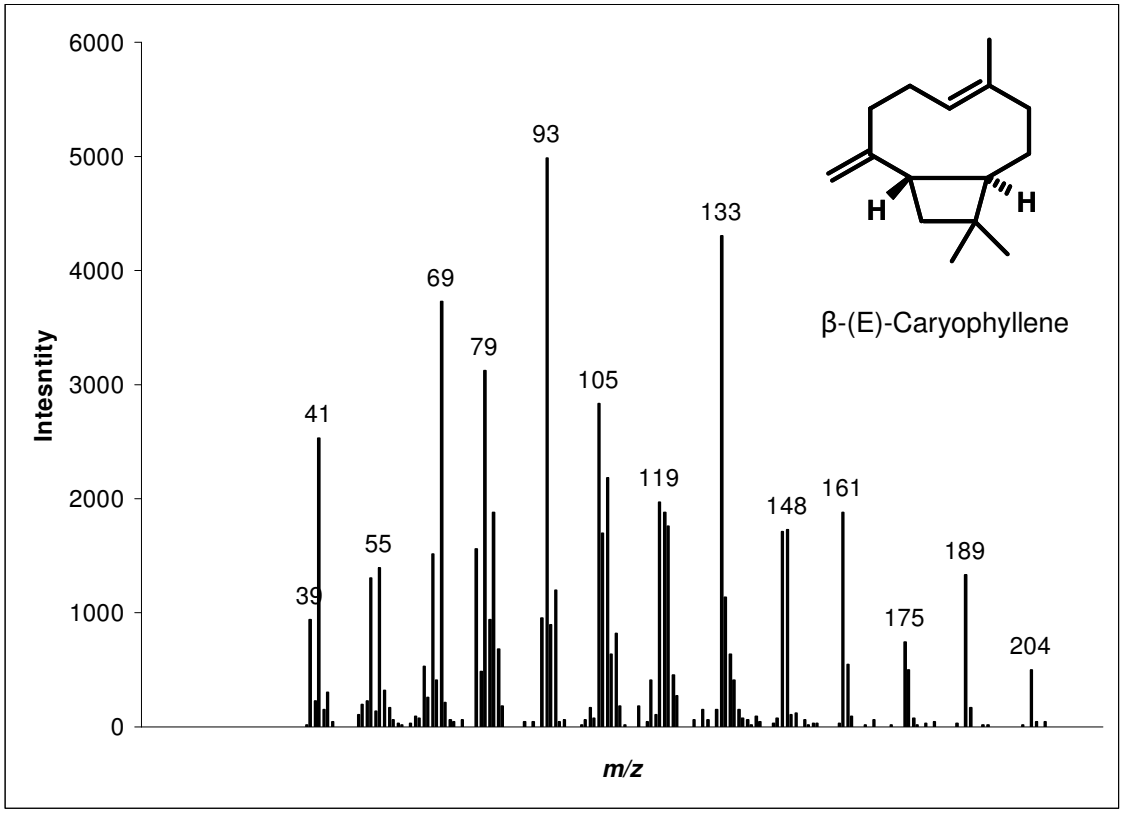

Figure 8.12: Complex mass spectrum of beta-caryophyllene recorded after extraction and separation of a phylloxera- infested grapevine root tip. m/z 204 represents the parent ion.

8.2.1 Quadrupole mass analyser

A quadrupole consists of four parallel rods (Figure 8.14). The opposite rods are under the same (one pair positive, one negative) direct current (Figure 8.13). This is overlaid by a radio frequency. Entering a high frequency field, the ions are stimulated to follow oscillating trajectories. The electromagnetic field in the quadrupole stabilises only the trajectory of a certain *m/z* ratio – only ions with this *m/z* can pass the quadrupole and reach the detector. All other ions with other *m/z* ratios leave the quadrupole on the side or collide with the rods and therefore do not reach the detector (Figure 8.14).

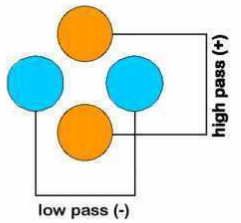

Figure 8.13: "High-pass" filters ions with too low m/z-ratios, "low pass" filters ions with too high m/z-ratios (www.vias.org, accessed 28.8.2011).

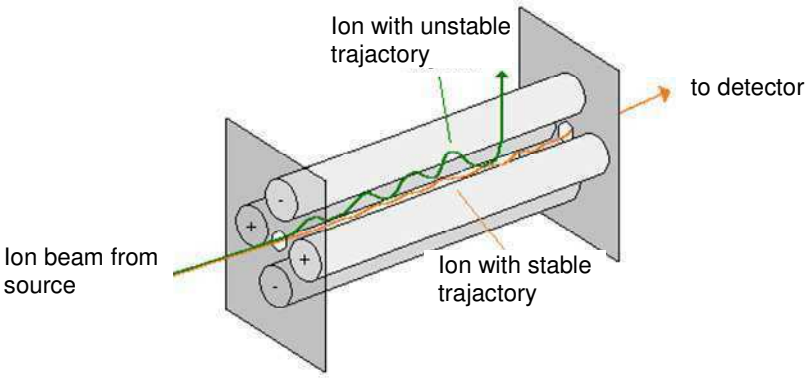

Figure 8.14: Only ions with a predefined m/z-ratio can pass the quadrupole (www.vias.org, accessed 28.8.2011).

An Agilent 5975B MSD mass analyser was used for the presented studies in full scan mode. The recorded mass range was 35 – 500 *m/z*. Three scans per second were measured. The MS was calibrated weekly with PFTBA. For further settings see chapter 12.

The detector counting the ions passing the mass filter was an electron multiplier (Figure 8.10).

9 Annotation and identification of metabolites

Comparative metabolomics studies frequently aim at the detection of metabolites whose concentration is different in samples that were treated differently. Further on the results shall be interpreted in relation to the starting hypothesis (biological question). Therefore, it is necessary to assign the metabolites to their biological role or pathways. This can only be done correctly if these metabolites are unambiguously identified. To detect significant differences between the sample groups it is necessary to at least annotate the metabolites, i.e. to give them either a preliminary name or a code. It is also extremely important to compare relative concentrations of always the same substance between the samples otherwise false results will be obtained which might lead to wrong interpretations.

I want to point out here that the use of data processing software can only provide annotated metabolites. To term a metabolite identified the validation of the annotation with authentic standards is required (Fiehn, 2008). GC-MS offers two features, which ease the annotation/identification enormously:

- mass spectrum
- retention index

These two analytical characteristics are briefly described in the following chapters. The descriptions always refer to AMDIS software, which was used for raw data processing throughout this thesis. Other software packages providing similar features are e.g. MetaboliteDetector (Hiller et al., 2009) or Tagfinder (Luedemann et al., 2008).

9.1 Mass spectrum

Since the ionisation energy in GC-MS is standardised (70 eV), for a certain substance mass spectra with nearly identical fragment patterns are obtained independent of the instrumentation. These fragmentation patterns are collected in so called mass spectra (MS) libraries, e.g. Wiley (McLafferty, 2008), NIST (NIST/EPA/NIH, http://www.nist.gov/srd/nist1a.cfm, access 23.8.2011) or the Eight Peak Index of Mass Spectra (Royal Society of Chemistry, 1991). By means of appropriate software the automated comparison of the measured spectra with the library entries and hence, the annotation of the metabolites is possible. Additionally, self-made MS libraries can be compiled. For the automated comparison two approaches are reported in literature: the first simply treats the mass spectra as row vector, consisting of intensity values for each m/z of the scan range. The second more complex approach tries to reproduce

the physical properties underlying each spectrum (Hertz et al., 1971; PBM probability based matching: McLafferty, 1976). For the former, three possibilities have been reported to measure the similarity of the spectra: the Euclidean distance, the absolute value distance and the dot-product between measured and reference spectrum (Stein and Scott, 1994). I want to briefly describe the principle of the algorithm for the automated comparison of measured and reference spectra, which is applied by AMDIS software used for spectra comparison during this work (Stein, 1999).

The algorithm aims at the comparison of the similarity of the measured spectrum (m) and the reference spectrum (r). If m is generated from a single pure compound, all peaks in m and r have to match for a good match factor (pure spectrum match factor, weighted match factor). If there are signals in m which are not present in r and e.g. originate from other substances or from background ions, the algorithm has to ignore these signals in m (impure spectrum match factor, reverse match factor). AMDIS combines both approaches in a 70:30 ratio (net match factor = 70*weighted+30*reverse). The function used by AMDIS for the comparison is the normalised dot-product of the spectra chosen for comparison (Equation 9.3, modified from Stein, 1999).

$$100\frac{\left(\sum w\left(\frac{m}{z}\right)\left(A_m A_r\right)^{1/2}\right)^2}{\sum\left(\frac{m}{z}\right)A_m \sum\left(\frac{m}{z}\right)A_r} \qquad (9.3)$$

w	Weighting factor penalising uncertain peaks
A_m	Vector with abundances in measured spectrum
A_r	Vector with abundances in reference spectrum

The whole algorithm includes several corrections and a weighting term (e.g. for spectra with few major peaks). Further details can be found in Stein (1999).

The mass spectrum also contains information about the structure of the corresponding metabolite. If a metabolite cannot be found in a MS library, the information about the structure can help to narrow down the possible number of candidates. If no library entry matches a recorded mass spectrum, it can be added to a self-made library. Afterwards, the other samples are searched for matching mass spectra. Hence, unidentified compounds can be taken into account for comparison between differently treated samples. If it turns out, that an unknown compound has the potential to be a biomarker the structure might be elucidated with the help of the recorded mass spectrum.

It is worth to mention, that a successful and correct identification of a metabolite requires mainly two things: a “clean” measured mass spectrum and most

important an entry in the reference database of this metabolite. Therefore, large and comprehensive spectra libraries are necessary. The new 2011 version of the NIST mass spectra library contains spectra of 212961 substances. As this number includes also inorganic substances and the number of plant metabolites is estimated to about 200000 (Bino et al., 2004) it is obvious that not all metabolites can be annotated at the moment by their mass spectra. Nevertheless, the libraries can help to find at least similarities of unknown metabolites to referenced substances and therefore enable partly structure elucidation.

Metabolites with similar structure can provide nearly identical mass spectra. Therefore, a metabolite identification based only on the mass spectrum is not always reliable (Wagner et al., 2003). To avoid confusion, the retention index (RI, see below) can be used as an additional and complementary parameter to improve the quality of the identification.

9.2 Retention index

The concept of retention index was developed by Kováts in 1958 for isothermal GC measurements. It was then adapted for temperature ramps by Van den Dool and Kratz (1963) by introducing the linear temperature programmed retention time index (LTPRI, Equation 9.4). Figure 9.15 shows the principle. A series of alkane standards (or other homologues, e.g. fatty acid methyl esters) is either added to every sample or at least included once in a measurement sequence. Every metabolite is bracketed by two alkanes, which allow the calculation of the corresponding RI. In principle, the RI is the ratio of the retention time interval between the metabolite and the earlier eluting alkane to the retention time interval between the two bracketing alkanes.

$$RI = 100\left(n + \frac{t_{Ri} - t_{Rn}}{t_{R(n+1)} - t_{Rn}}\right) \qquad (9.4)$$

t_{Ri}	Retention time of metabolite
t_{Rn}	Retention time of earlier eluting alkane
$t_{R(n+1)}$	Retention time of later eluting alkane
n	Number of C-atoms of earlier eluting alkane

Although the retention index is characteristic for a substance, it is not sufficient for an unambiguous identification when used as a single criterion. In some cases, not even the combination of MS data with RI provides a reliable identification. Therefore, the RI shall be determined on two GC columns with different polarities (Royal Society of Chemistry, Analytical Methods Committee, 1981). The use of a second stationary phase is useful, since substances, which

coelute on one column and therefore have the same or very close RI can be expected to be clearly separated (in the most cases) on a column with different polarity.

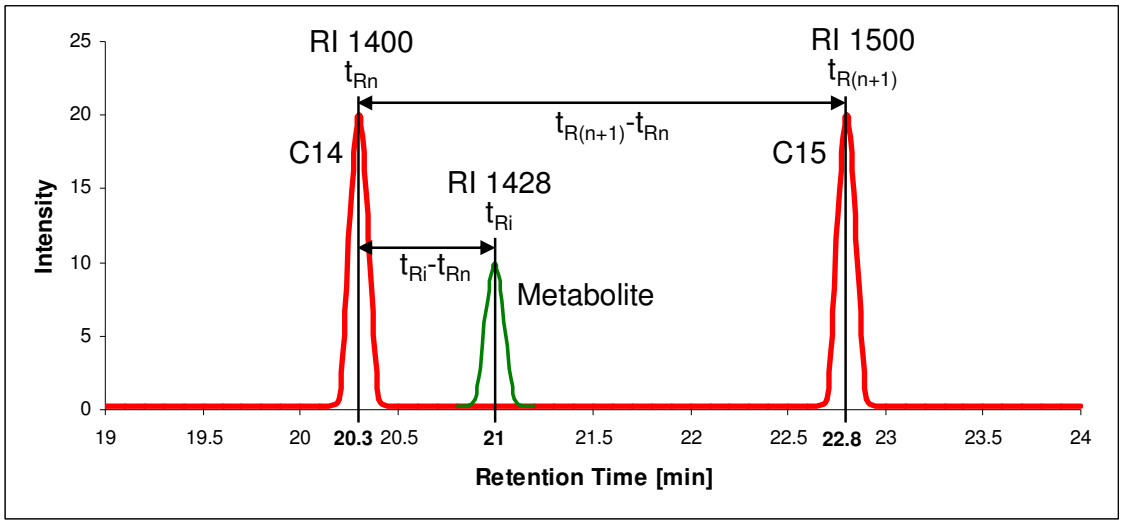

Figure 9.15: Calculation of LTPRI. C_{14} tetradecane, C_{15} pentadecane.

The LTPRI was used throughout this thesis. Alkane standards were included in every measurement sequence and ranged from C_7 – C_{30}. The RI was automatically calculated by AMDIS software (paper #4). Details about the applied metabolite annotation/identification workflow are discussed below (9.4).

9.3 Deconvolution

In case of (partly) coeluting substance peaks the recorded mass spectrum shows ions of two or more substances. Hence, spectrum comparison with library entries will result in no, false or low quality matches. Biller and Biemann (1974) presented the first solution for this problem. Their algorithm for deconvolution of mass spectra chose only *m/z*-intensity pairs for comparison with library entries, which show maximum abundance in the selected or the adjacent scan (maximising peaks concept). The concept of maximised peaks works fine for overlapping substances, which do not have common ions in their mass spectra. Problems occur when there are some ions in the same scan originating from both (or more) overlapping substances. Dromey et al. (1976) reported an algorithm based on the comparison of the peak shapes of all ions. They assumed, that the chromatographic peaks of individual *m/z* traces (extracted ion chromatogram EIC) originating from the same substance have the same peak shape (model peak concept). The more recent and sophisticated algorithms from AMDIS (Stein, 1999) or MetaboliteDetector (Hiller et al., 2009) are based on this idea.

During this thesis, I applied the AMDIS software to the measured mass spectra. AMDIS stands for Automated Mass spectral Deconvolution and Identification System and was developed at the National Institute for Standards and Technology NIST (Stein, 1999). This software is freely available (www.amdis.net) and provides not only the possibility of deconvolution of recorded mass spectra but also their automated comparison with MS library entries. The following section describes the principles of the deconvolution algorithm implemented in the AMDIS software. The deconvolution process consists of three main steps:

- noise analysis
- component perception
- extraction of “purified” spectra

which are briefly described below following the publications of Stein (1999) and Mallard (2001).

9.3.1 Noise analysis

Signals produced by ion-counting detectors show a certain amount of fluctuation. In average, this fluctuation is proportional to the square root of the signal abundance (Peterson and Hayes, 1978). In AMDIS this average is expressed as median of random deviation (Equation 9.5). The proportionality factor (noise-factor N_f) can be obtained from regions with constant signal abundance to be found in every GC-MS chromatogram.

$$N_f = \frac{\text{median of random deviation}}{\sqrt{\text{signal}}} \tag{9.5}$$

For these regions, a local abundance mean is calculated for every EIC and the TIC. Then the algorithm tries to find these regions in the EIC (and TIC) where one-half of the signal abundances are below the signal-level corresponding to the mean abundance and the other one-half shows abundance above the mean. This is termed “crossing” as figuratively the signal “crosses” the mean abundance frequently in such regions (Figure 9.16). Subsequently, the median deviation of all signals from the calculated mean abundance within this region is calculated. Dividing this median value by the square root of the mean abundance of the region gives the noise factor of this region. The noise factor for the whole GC-MS run is the median of noise factors of all regions.

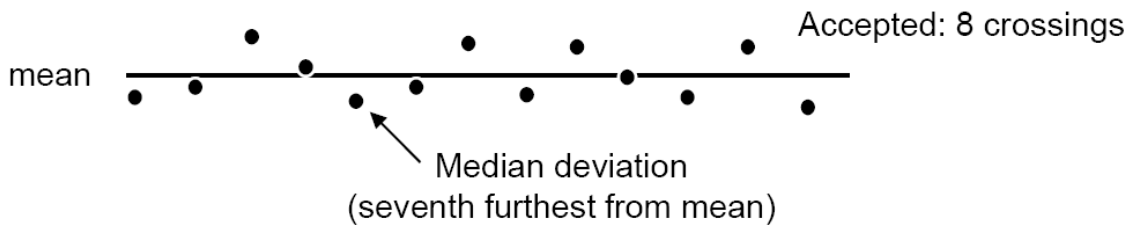

Figure 9.16: Illustration of how to find segments in a chromatogram, which can serve for noise-factor calculation. The AMDIS algorithm uses regions with a width of 13 scans which have to be crossed at least seven times by the signal (from Stein, 1999).

The "median of random deviation" from equation (9.5) then equals one noise unit and corresponds to the detector-derived scan-to-scan variation in the signal. Analysis of noise is a crucial part of every algorithm dealing with the detection of peaks. On basis of the noise level it is decided which maxima in an EIC chromatogram (or TIC) were caused by a substance and which are just by random. In AMDIS e.g. a peak is assigned when it shows minimum abundance of 5 noise units.

9.3.2 Component perception

This is a very complex but important step. Errors in this step can lead to false results in metabolite identification. Briefly, a component is perceived when the detector signals for a lot of m/z values with adequate abundance show maxima within a small retention time window. The component perception consists of:

1. **Determination of peaks** in the EIC chromatogram
2. **De-skewing** of mass spectra, **retention time correction** (i.e. calculation of true EIC peak maximum)
3. **Determination of sharpness values** for detected EIC peaks
4. **Binning** of sharpness values, final determination of components
5. Determination of **model peak shape**

All steps are carried out for each EIC and the TIC.

Ad 1 Determination of peaks in the EIC chromatogram The recognition of a peak is based on the detection of local maxima in the EIC chromatogram and further the peak height, which has to be at least 5 noise units. This value can be changed in the AMDIS deconvolution settings under "Sensitivity" (Figure 9.17). Local (tentative) maxima are found in AMDIS by searching for ion chromatogram points which are at least 5 noise unites above the adjacent scans. Subsequently, the peak width is determined i.e. how many scans on both sides of the local maximum belong to the peak. The maximum number of scans to be assigned to a peak can be defined in the AMDIS settings under "Component width" (Figure 9.17). The value should correspond with the actual

peak width of the GC-MS data. The default value is 12 scans. This equals a peak width of 8 seconds at a scan rate of 3 scans per second, which was the setting of the MS instrument used during this work. Peak widths in the recorded chromatograms ranged from 6 – 20 seconds, therefore I kept the default value. Changes in “Shape requirements” will affect both the tolerated distance to the next maximum and the required peak height.

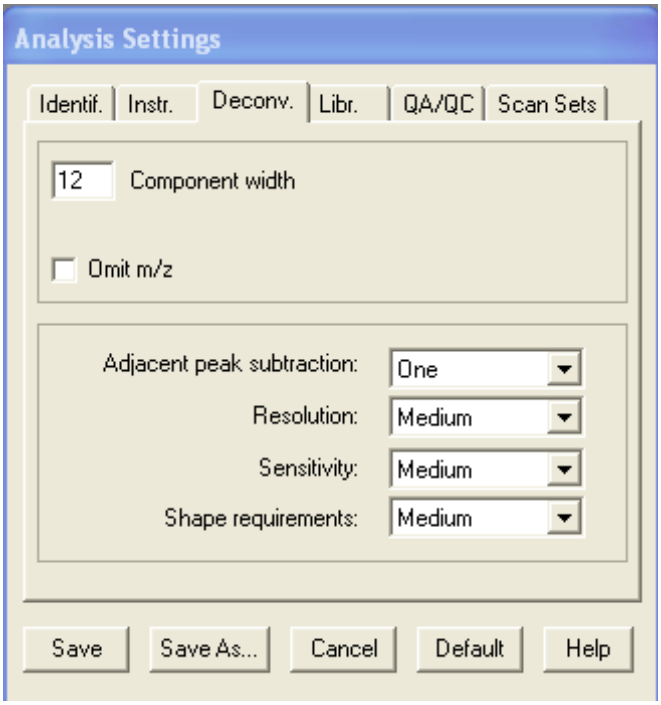

Figure 9.17: Screenshot of deconvolution settings in AMDIS (Analyze, Settings, Deconvolution).

Ad 2 De-skewing of mass spectra, retention time correction Skewing is a phenomenon, appearing in instruments, which scan the *m/z* one at a time as it is the case in a quadrupole, ion trap or magnetic sector field instrument. Since chromatographic peaks in GC are narrow, it occurs that during a single scan (over a pre-defined *m/z* scan range) the concentration of an eluting substance increases (at the beginning of a chromatographic peak) or decreases (after the signal has reached the maximum value). If the concentration of the substance increases during a scan, low *m/z* values will be discriminated (if the scan direction is from low to high *m/z*). This results in a false ratio of the ion abundances in the recorded spectrum. In addition, the local maxima of the EICs originating from a single metabolite can appear in different scans. The retention time which corresponds to a EIC peak maximum) is corrected based on a three-point-quadratic-interpolation of the EIC signal values obtained for the maximum and the two adjacent scans (Figure 9.18).

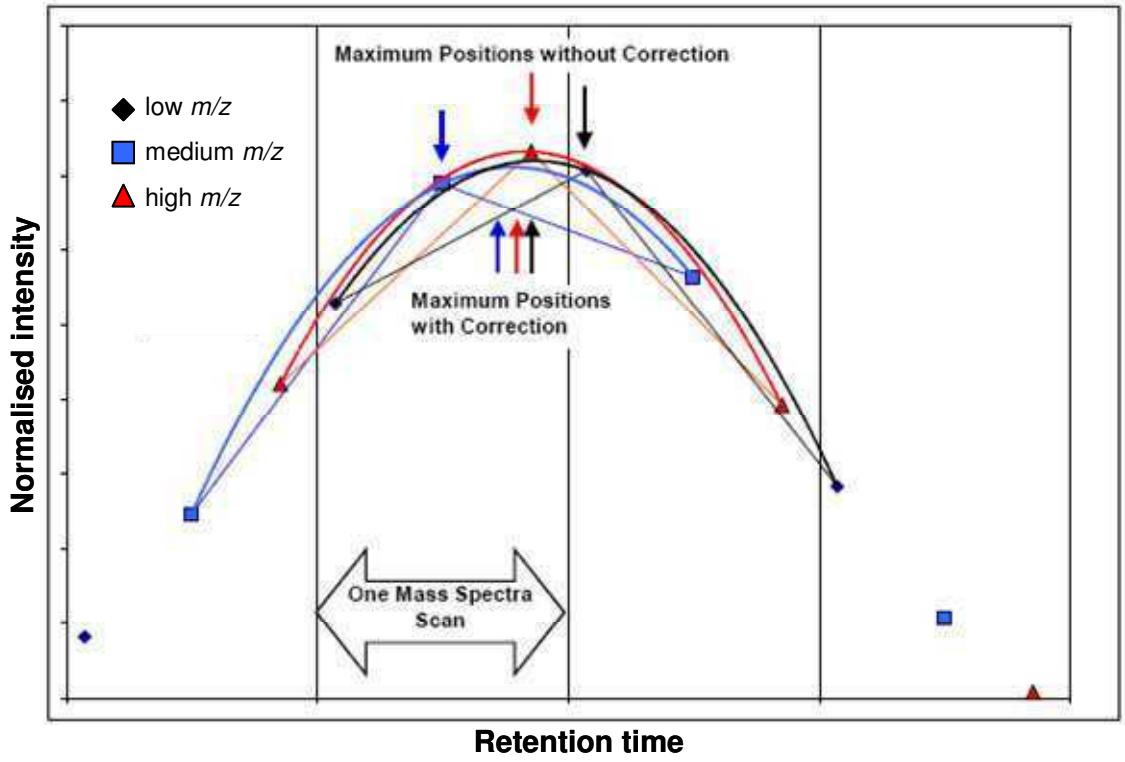

Figure 9.18: Time correction for detected local maxima of the EIC chromatogram. Three ions with low, medium and high m/z are shown. In a quadrupole instrument, these ions do not reach the detector at the same time point. A parabola is fitted into the maximum and the two adjacent scans. This leads to a time corrected peak maximum for every local m/z maximum (adapted from Mallard, 2001).

Ad 3 Determination of sharpness values EIC peaks originating from the same substance are assumed to have similar chromatographic peak shape. This can be described in a simplified way with the peak sharpness value, which is defined as:

$$\frac{A_{\max} - A_n}{nN_f\sqrt{A_{\max}}} \tag{9.6}$$

A_{max} Abundance of maximum
A_n Abundance of n^{th} scan beside maximum
n Number of scan counted from scan representing maximum
N_f Noise factor

Ad 4 Binning, final determination of components On basis of the corrected retention times (step 2) and the sharpness values of the EIC peaks, components are detected. This process is also called binning and is in principle the search for small retention time intervals (AMDIS: one-tenth of the duration of a scan) where many peaks with high sharpness values c. Only at such retention time points a component is perceived. A detailed explanation of this process is beyond the scope of this thesis. I want to mention one detail because the AMDIS user can manipulate this particular setting. According to Figure 9.17, the

setting for “Resolution” can be changed. Resolution refers to the minimum distance (in time) between two perceived components. Setting of resolution to “low” allows more components to be determined within a certain retention time window than “high”.

Ad 5 Determination of model peak shape Generally, a model peak is defined to rise and fall fastest. In AMDIS, the model peak shape is based on all EICs corresponding to a component and which have sharpness values within 75 % of the maximum sharpness of this component.

9.3.3 Extraction of “purified” spectra

So far, the algorithm provides the ions (*m/z*-values) which belong to a certain component but no information about the ion abundance. To compare the purified mass spectrum with library entries additionally the abundance of each ion has to be assigned. On basis of a least-squares method, which fits each EIC peak of a component to the model peak shape and the so detected maximum abundance, the abundance of every individual *m/z* value is calculated. The result, a purified mass spectrum is also termed component in AMDIS software and is indicated with a blue triangle in the graphical output of the deconvolution process.

9.4 Final identification criteria

According to the pre-work of a colleague at our institute, I applied the following criteria for metabolite annotation/identification (Stoppacher et al., 2010) throughout all experiments during this thesis. For a positive identification, a minimum (mass spectral) match factor of 90 and a maximum RI deviation of ±2% from the reference value were tolerated. Additionally, these criteria had to be fulfilled on two GC-columns of different polarities (apolar DB-5MS, polar Optima-WAX) to specify a substance as annotated. Moreover, the identity of several metabolites has been proven with authentic standards if available.

After the annotation/identification of metabolites all samples of an experiment were analysed in batch job mode with AMDIS software. Therefore a library containing the before annotated/identified substances mass spectra and RI was compiled. Different libraries for grapevine leaves, roots and the dust sample analysis were created.

To include low abundant metabolites or peaks with poor deconvolution I lowered the minimum match factor to 60 but increased the RI deviation to max. 5 RI values. These settings were applied only to the grapevine samples (paper #1, #3, #4).

10 Data analysis, statistics, chemometrics

"Errors using inadequate data are much less than those using no data at all" Charles Babbage (1792-1871) (in Brown et al., 2005)

In this thesis, the term data analysis is used to describe the statistical treatment of the AMDIS processed data. Chemometrics is an umbrella term including development and application of formal methods (statistics, informatics) with the aim to extract a maximum of relevant information from the data derived by chemical analysis (Varmuza and Filzmoser, 2009, p 1).

In metabolomics studies, large amounts of data are produced. A manual data analysis will be too time consuming and is often not possible (e.g. PCA). Therefore, several data analysis software tools have been developed, which are especially suited for metabolomics studies using GC-MS, e.g. Spectconnect (Styczynski et al., 2007), MetaboliteDectector (Hiller et al., 2009), Tagfinder (Luedemann et al., 2008) or XCMS (Smith et al., 2006). Of course, any software providing statistical tools can be used for further treatment of data, e.g. R (www.r-project.org/, access 26.8.2011), Matlab (www.mathworks.de/products/matlab/, access 26.8.2011) or Unscrambler (www.camo.com/rt/Products/Unscrambler/unscrambler.html, access 26.8.2011).

The biological question (hypothesis) in a metabolomics experiment frequently refers to the difference in the metabolome of a control (untreated) and a treated sample group. This can be answered by searching for patterns in the data, which can be found with multivariate statistical methods as e.g. principal component analysis (PCA), multivariate calibration or multivariate classification. Unsupervised methods use only the dependent variables (e.g. concentrations of metabolites) for calculating the model. These methods show the whole variation of the variables used to describe the respective samples independent from the origin of the variations. Supervised methods try to describe the part of the variance which originates from the applied treatment of the biological samples.

PCA is an unsupervised method commonly used in metabolomics (Fukusaki and Kobayashi, 2005; Hall, 2006) to simplify/enable the illustration of the n-dimensional data by reduction to two or three dimensions. PCA is a linear data transformation in n-dimensional data space, which aims to choose a new coordinate system for the dataset. The new axes are termed principal components (PC). The first PC represents the direction of maximum variance in the data. The second PC is orthogonal to the first PC and again represents the maximum variance in this direction of the data. Typically, the result of a PCA is presented with two graphs. One, the loadings plot, shows the samples (objects).

If the variance in the data results from differences between groups of differentially treated biological samples this leads to clusters in the plot corresponding to the different sample groups. The other graph, the scores plot, shows the contribution of each metabolite (variable) to the variance in the data (for an example see paper #3). However, no information about significance of the metabolites' contribution to the variance in the data is given.

Another unsupervised method for classification is hierarchical cluster analysis (HCA) which is well suited for discriminatory analysis (Fukusaki and Kobayashi, 2005). It is based on the similarity or distance between sample pairs. Typical for HCA are the dendrogram (tree) plots. No information about the contribution of the metabolites to the result is obtained.

As an example for multivariate calibration methods, I want to mention partial least-squares regression (PLS), a supervised method. PLS like PCA uses a linear transformation of a given set of variables to a new set of variables, which are linear combinations of the original variables. In contrast to PCA, PLS is a supervised method thus allowing correlation of the dependent y-variables (e.g. concentrations of metabolites) to the x-variables (different treatment of sample groups) whereas PCA only describes the variation in the x-variables. PLS can also be used to prove whether the determined set of metabolites is selective for the different states of the samples (Hall, 2006).

Although rarely used in metabolomics studies also univariate statistical methods considering only one variable (e.g. concentration of a metabolite) can be used for data analysis. E.g. to compare the means of relative metabolite concentrations between two sample groups. T-test and Mann-Whitney-U-test are suited for this purpose. Both methods provide a measure whether a substances' relative concentration is significantly different between two sample groups.

In general, for metabolomics studies robust statistical methods are necessary. Robust methods do not require normally distributed data, such methods are unsusceptible to outliers and can be applied to small datasets.

The statistical treatment of the data produced during this thesis was done with R software using t-test, PCA-, and HCA-function.

T-test was applied in the phylloxera experiment to determine which of the detected metabolites shows significantly different concentrations between the two sample groups (paper #1). 14 out of 38 metabolites showed significant differences.

PCA was used in this thesis for differentiating between grapevine leaves originating from two sampling dates (paper #3).

HCA and PCA were applied to the dust samples (paper #2).

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Results

Data does not equal information; information does not equal knowledge; and, most importantly of all, knowledge does not equal wisdom. We have oceans of data, rivers of information, small puddles of knowledge, and the odd drop of wisdom."

Henry Nix 1990 (in Goodacre et al., 2004)

I have not succeeded in answering all questions. The answers I have found only serve to raise a whole set of new questions. In some ways I feel I am confused as ever. But I do believe I am confused on a higher level and about more important things.

(adapted from Kelley, 1951, p 2)

12 Final analytical workflow

Here, a graphical overview (Figure 12.1) is presented together with some additional remarks on practical aspects to be considered during application of the workflow. All steps refer to grapevine samples. Additional detailed descriptions of the experimental procedure and the workflow are given in paper #3, #4.

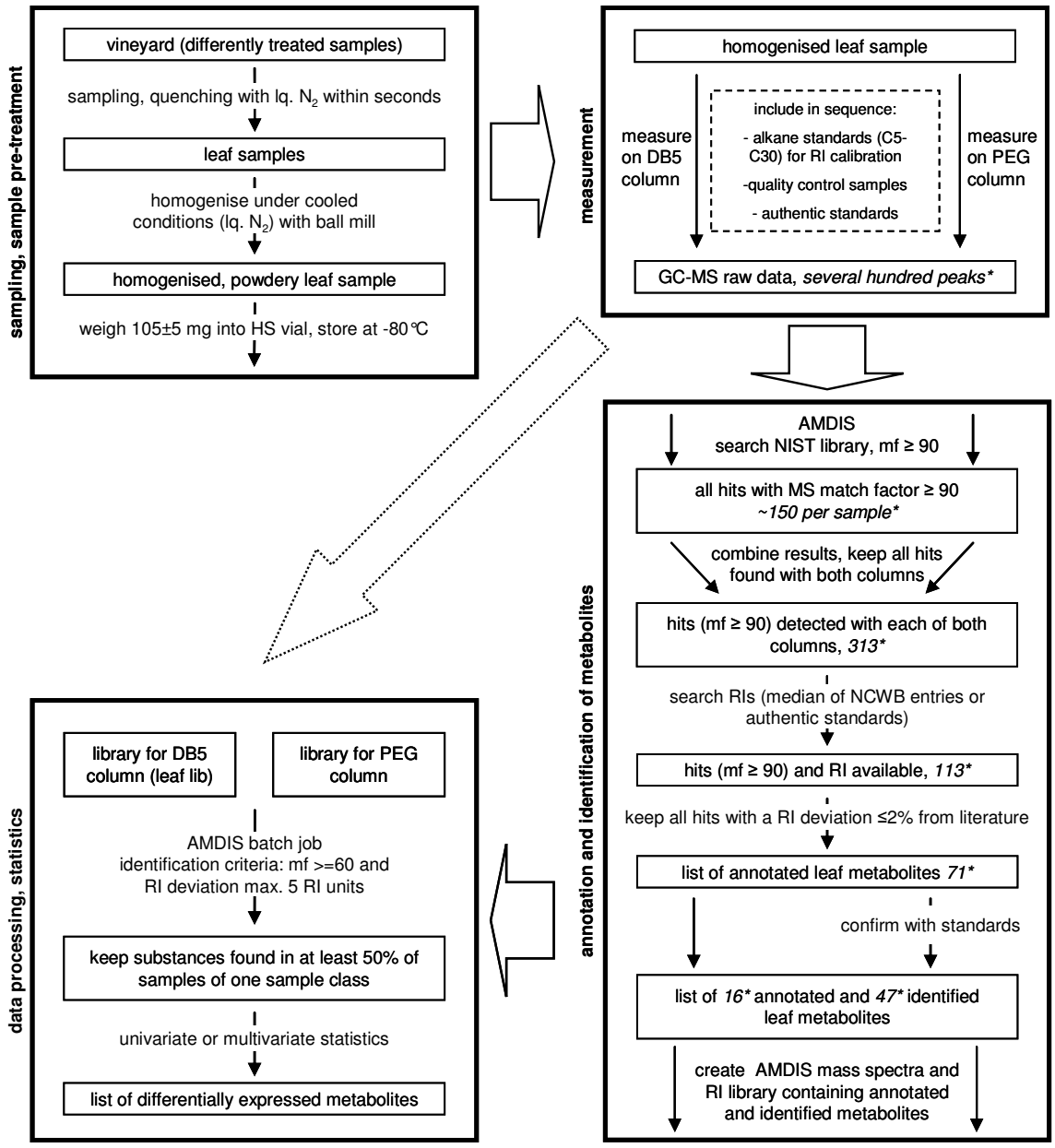

*Figure 12.1: Workflow for investigation of plant tissue samples. * refers to numbers of metabolites detected in the study presented in paper #3.*

Sampling

Leaves were sampled between 10 – 12 o'clock in the morning. They were cut with the stem and inserted in aluminium envelopes or 50 mL plastic tubes with tightly closing screw cap. The envelopes or the tubes are then cooled in liquid nitrogen (lq. N_2) immediately. Sampling and subsequent insertion to lq. N_2 as fast as possible will avoid or minimise changes in the metabolome caused by the sampling itself.

Homogenisation

Homogenisation was conducted with a ball mill (Retsch MM301, Haan, Germany). I used 10-mL stainless steel beakers with 12-mm stainless steel balls. The beakers and balls were pre-cooled in lq. N_2 before filled with leaf tissue.

Leaves in envelopes: Envelopes were removed from lq. N_2, opened, the stem removed and the leaf pieces transferred via a funnel into the pre-cooled milling beakers.

Leaves in plastic tubes: tubes were placed in lq. N_2, the stem removed, leaves crushed with a pre-cooled glass rod and subsequently transferred via a funnel into the pre-cooled milling beakers.

A ball was added to every beaker, the beakers sealed with a screw-lid and cooled in lq. N_2 before the milling process.

Samples were milled for 3 minutes at 30 Hz. After the milling, the beakers were cooled again in lq. N_2.

Weighing

20-mL HS-vials were pre-washed with methanol/water (50/50 v/v) twice. Vials, caps (magnetic with hole) and septa (1.3 mm silicone/PTFE) were baked out at 120°C for at least 60 minutes.

Before weighing, HS-vials were cooled by insertion to an ice/water bath. 105±5 mg of leave leaf powder were weighed into the HS-vials and the exact weight was noted. The HS-vials were tightly closed with screw caps containing septa and stored at -80°C until analysis.

SPME-GC-MS

Materials

- Agilent 6890N GC with 5975B MSD (Agilent, Waldbronn, Germany)
- MPS2XL (Gerstel, Mühlheim a.d. Ruhr, Germany) autosampler with cooled tray, HS-vial heating station, needle heater, SPME holder
- Apolar column DB-5MS, 95% dimethyl-, 5% diphenyl-polysiloxane 30 m length, 0.25 mm inner diameter, 0,25 µm film thickness (Agilent)

- Polar column Optima-WAX, polyethylene glycol, 30 m length, 0.25 mm inner diameter, 0,25 µm film thickness (Machery-Nagel, Düren, Germany)
- Alkanes for RI calibration covering the range of C_7 – C_{30}: C_5-C_{10}: in-house mix, C_8-C_{20} (40 mg each in hexane, Fluka, Sigma-Aldrich, Vienna, Austria), C_{21}-C_{40} (40 mg each in toluene, Fluka)
- Quality control samples: pooled leaf samples
- SPME-liner 0.75 mm inner diameter
- SPME-fibre 2 cm 50/30 µm Carboxen/ Divinylbenzene/ Polydimethyl-siloxane (CAR/DVB/PDMS, Supelco, from Gerstel) for autosampler, conditioned to suppliers recommendation
- Mobile phase GC: He 5.0

SPME-method

- Equilibration time: 30 min
- Extraction time: 60 min
- Equilibration and extraction temperature: 90 °C
- Needle heater: 270 °C, N_2 flushed, 10 min bake out prior to extraction
- Desorption: 2 min at 250 °C in GC-inlet, splitless mode

GC-Method

- Gas-flow: 1 mL/min He 5.0
- Inlet: 250 °C, splitless for 2 min (during desorption of VOCs from fibre)
- Oven programme: starting temperature: 35 °C hold 2 min
- Temperature ramp: 5 °C/min to 260 °C, hold 5 min
- Transfer line: 270 °C

MS-settings

- Electron ionisation mode (EI), ionisation energy: 70 eV
- Source temperature: 230 °C
- Quadrupole temperature: 150 °C
- Scan range: 35 – 500 *m/z*
- Scan speed: 3 scans/s
- Weekly tuning with PFTBA

Data processing

AMDIS, version 2.65

Substance identification: default settings; Minimum match factor: 90; Type of analysis: Use Retention Index Data; RI window: 1 + 2 x 0.01 RI; Match factor penalties: Level: Infinite; Maximum penalty: 100; No RI in library: 100

Comparative study, Batch job mode: default settings, except: Minimum match factor: 60; Type of analysis: Use Retention Index Data; RI window: 5 + 0 x 0.01 RI; Match factor penalties: Level: Infinite; Maximum penalty: 100; No RI in library: 100; Use the positive list resulting from the substance identification step as target library.

Data analysis

For all data analysis steps R-functions and -scripts have been used. In the following, the R-functions used to perform t-test and PCA will be illustrated. I used R version 2.13.0 available from www.r-project.org (R Development Core Team, 2010).

T-test This script enables the application of a t-test to a matrix with variables (metabolite concentrations) in columns. The matrix was of the following structure (8 rows with control samples, 8 rows with treated samples):

sample info	variabel x	variable y	variable z
control	x1	y1	z1
⇣	⇣	⇣	⇣
treated	x9	y9	z9
⇣	⇣	⇣	⇣

```
> x <- read.table(fileName, header=TRUE, sep="\t")
> sampletype <- x[,1]                    # sample type in 1. column
> y <- x[,-1]                            # delete 1. column
> SubstanceNames <- scan("fileName", what=character(0), sep="\t", nline=1)
> z <- as.matrix(y)                      # create a matrix
> s <- as.vector(SubstanceNames)
> ls <- length(s)
> s <- s[2:ls]
#### loop for t-test calculation of all columns ####
> results2 <- matrix(ncol=4)             # creates a matrix with 4 columns
> count=0
> for(i in 1:length(z[1,])){
>       count=count+1
>       print(count)
>       print(s[i])
>       names <- as.vector(s[i])
>       vec1 <- as.vector(z[1:8,i])
>       vec2 <- as.vector(z[9:16,i])     # both vectors must have same length!
>       cat("vec1: ", vec1, "\n")        #cat (concatenate) writes result in R-console
>       cat("vec2: ", vec2, "\n")
>       if((length(na.omit(vec1)))>1  &&  (length(na.omit(vec2)))>1){
>             print(t.test(vec1, vec2, alternative=c("two.sided"), var.equal=FALSE, conf.level=0.95))
                                                                  # t-test result on screen
>             tres<-t.test(vec1, vec2, alternative=c("two.sided"), var.equal=FALSE, conf.level=0.95)
                                                                  # saves t-test results as "tres"
>             means <- as.vector(tres$estimate)
>             means <- c(means[1], means[2])
>             results <- c(names, tres$p.value, means)     # "$" reads saved values from function
# c...combine
>       } else {
>             results <- c(names, "p-value", "mean control", "mean treated")
>       }
>       results2 <- rbind(results2, results)
>}
```

PCA This script allows calculation of PCA and plotting of loadings and scores plot. The matrix is of the following form (for better readability of the loadings plot, the substance names were replaced by numbers):

sample info	**1**	**2**	**3**
date1	x1	y1	z1
↓	↓	↓	↓
date2	x32	y32	z32
↓	↓	↓	↓

```
##### loading data #####
> require(tcltk)
> fileName <- tclvalue(tkgetOpenFile())
> X <- read.table(fileName, header=TRUE, sep="\t")
> Y <- as.matrix(X)
> Date <- X[,1]                    # read out sample info
> X2 <- Y[,-1]                     # delete sample info, delete 1. column
> Substances <- scan("fileName", what=character(0), sep="\t", nline=1)
> SubstanceNumbers <- as.vector(Substances)
#### autoscale, mean-center ####
> X3scale <- scale(X2)
###### PCA #####
> PCA_X <- svd(X3scale)
> scores <- PCA_X$u %*% diag(PCA_X$d)
> loadings <- PCA_X$v
#### variance #####
> vars <- PCA_X$d^2/(nrow(X2)-1)
> totalvar <- sum(vars)
> relvars <- vars/totalvar
> variances <- 100*round(relvars, digits=3)
> variances[1:5]
##### scores-plot #### plot as tiff ####
#### choose file-name ####
> require(tcltk)
> filesavename <- tclvalue(tkgetSaveFile())

> tiff(filesavename, width=10, height=10, units="cm", pointsize=8, res=1000)
>         plot(scores[,1:2], type="n", xlab=paste("PC 1 (", variances[1], "%)", sep=""),
+                 ylab=paste("PC 2 (", variances[2], "%)", sep=""))
>         abline(h=0, v=0, col="gray")
>         points(scores[,1:2], pch=Date)
>         legend("topleft", inset=0.05, legend=c("June", "July"), pch=c(1,2))
> dev.off()
##### loadings-plot ####
#### choose file-name ####
require(tcltk)
filesavename2 <- tclvalue(tkgetSaveFile())
> tiff(filesavename2, width=10, height=10, units="cm", pointsize=8, res=1000)
>         plot(loadings[,1], loadings[,2], type="n", xlab=paste("PC 1 (", variances[1], "%)", sep="")
+                 ylab=paste("PC 2 (", variances[2], "%)", sep=""))
>         abline(h=0, v=0, col="gray")
>         text(loadings[,1:2], labels=SubstanceNumbers, cex=0.7)
>dev.off()
```

13 Original works

Paper #1 Phylloxera attacked grapevine roots
Lawo* NC, **Weingart* GJF**, Schuhmacher R, Forneck A **2011** The volatile metabolome of grapevine roots: First insights into the metabolic response upon phylloxera attack. *Plant Physiology and Biochemistry* 49:1059-1063.
* shared first authorship

Paper #2 Settled floor dust samples
Vishwanath V, Sulyok M, **Weingart G**, Kluger B, Täubel M, Mayer S, Schuhmacher R, Krska R **2011** Evaluation of settled floor dust for the presence of microbial metabolites and volatile anthropogenic chemicals in indoor environments by LC-MS/MS and GC-MS methods. *Talanta* 85:2027-2038.

Paper #3 Method development, grapevine leaves
Weingart GJF, Kluger B, Forneck A, Krska R, Schuhmacher R **2011** Establishment and application of a metabolomics workflow for identification and profiling of volatiles from leaves of *Vitis vinifera* by HS-SPME-GC-MS. *Phytochemical Analysis*

Paper #4 Plant-insect interaction, book chapter, submitted
Weingart GJF, Lawo NC, Forneck A, Krska R, Schuhmacher R **2012** Study of the volatile metabolome in plant-insect interactions. In: Weckwerth W, Kahl G, editors. The Handbook of Plant Metabolomics. Wiley-Blackwell-VCH.

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Research article

The volatile metabolome of grapevine roots: First insights into the metabolic response upon phylloxera attack

Nora C. Lawo [a,*,1], Georg J.F. Weingart [b,1], Rainer Schuhmacher [b], Astrid Forneck [a]

[a] Division of Viticulture and Pomology, Department of Crop Sciences, University of Natural Resources and Life Sciences, Konrad-Lorenz Str. 24, 3430 Tulln, Austria
[b] Center for Analytical Chemistry, Department IFA-Tulln, University of Natural Resources and Life Sciences, Konrad-Lorenz Str. 20, 3430 Tulln, Austria

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ABSTRACT

Many plant species respond to herbivore attack by an increased formation of volatile organic compounds. In this preliminary study we analysed the volatile metabolome of grapevine roots [Teleki 5C (*Vitis berlandieri* Planch. × *Vitis riparia* Michx.)] with the aim to gain insight into the interaction between phylloxera (*Daktulosphaira vitifoliae* Fitch; Hemiptera: Phylloxeridae) and grapevine roots. In the first part of the study, headspace solid phase microextraction (HS-SPME) coupled to gas chromatography – mass spectrometry (GC–MS) was used to detect and identify volatile metabolites in uninfested and phylloxera-infested root tips of the grapevine rootstock Teleki 5C. Based on the comparison of deconvoluted mass spectra with spectra databases as well as experimentally derived retention indices with literature values, 38 metabolites were identified, which belong to the major classes of plant volatiles including C6-compounds, terpenes (including modified terpenes), aromatic compounds, alcohols and *n*-alkanes. Based on these identified metabolites, changes in root volatiles were investigated and resulted in metabolite profiles caused by phylloxera infestation. Our preliminary data indicate that defence related pathways such as the mevalonate and/or alternative isopentenyl pyrophosphate-, the lipoxygenase- (LOX) as well as the phenylpropanoid pathway are affected in root galls as a response to phylloxera attack.

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1. Introduction

Grape phylloxera, *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae), is one of the most devastating grapevine pests worldwide, causing organoid galls (nodosities) on the root tips. After its introduction to Europe in the second part of the 19th century phylloxera caused major economic losses to the wine industry [1]. The spreading of this pest could be prevented by grafting susceptible European grape varieties onto tolerant rootstocks. However, in the last decades the appearance of more aggressive phylloxera biotypes has been reported (e.g., [2,3]). Thus, understanding the interaction between phylloxera and the grapevine root in more detail would be of utmost interest.

The number of studies, which investigated the physiological and molecular response of grapevine to phylloxera root-infestation are sparse and mainly focus on primary metabolites on detached root tissue. So far, increased concentrations of mono- and disaccharides [4,5], starch [4–6] and amino acids [6] and amides [7] have been found to be present in nodosities compared to uninfested root tips. Additionally, recent studies evaluating the metabolic response of grapevine leaves to a phylloxera root infestation, reported a reduction in the ratio of linoleic acid to linolenic acid [8] as well as the chlorophyll content but an increase in xanthophyll-cycle related pigments [9].

Volatile compounds constitute another important class of metabolites known to be involved in the response of many plant species to various types of abiotic (e.g., [10,11]) and biotic stress (e.g., [12,13]), and it is further well known that plant herbivory is associated with an increased formation of volatile metabolites in leaves [14] or roots [15].

Surprisingly, no reports on the involvement of volatile metabolites in the interaction between phylloxera and grapevine have been published so far. While several publications described the detection and identification of volatiles in leaves (e.g., [16,17]) and berries (e.g., [18,19]) of grapevine plants, to the best of our knowledge, there is only a single report on volatile metabolites produced by grapevine roots. Du et al. [20] investigated the volatile metabolites produced by one phylloxera resistant [5BB (*Vitis berlandieri* × *Vitis riparia*)] and one susceptible [Kyoho (*Vitis*

* Corresponding author. Tel.: +43 1 47654 3439; fax: +43 1 47654 3359.
E-mail address: nora.lawo@gmx.de (N.C. Lawo).
[1] Both authors contributed equally to this work.

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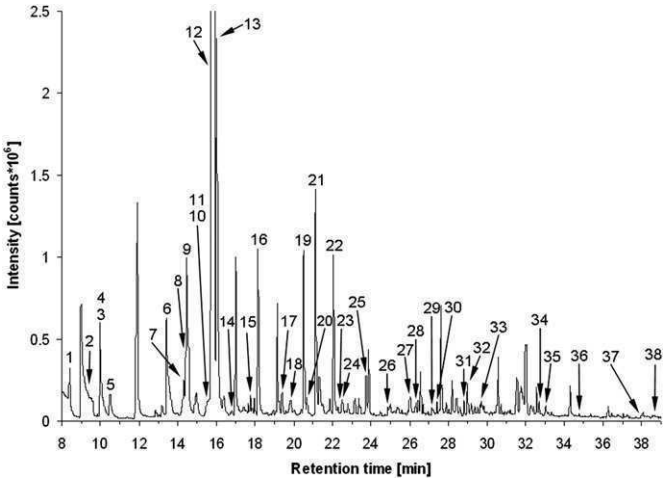

Fig. 1. Typical GC–MS chromatogram obtained after HS-SPME extraction of phylloxera-infested root sample (nodosities). Numbers correspond to substances in Table 1.

vinifera × *Vitis labrusca*)] cultivar and tried to correlate the nymphal preference for the susceptible cultivar Kyoho with the volatile metabolites.

In the present study, the volatile metabolome of grapevine roots of the cultivar Teleki 5C (*V. berlandieri* × *V. riparia*) was investigated by GC–MS. This is the first report on a differential comparison of volatile metabolites of uninfested and phylloxera-infested root tips of grapevine plants.

2. Results and discussion

2.1. Identification of root metabolites

A typical GC–MS total ion current chromatogram obtained after analysis of a phylloxera-infested root tip sample from cultivar Teleki 5C is shown in Fig. 1. This cultivar was chosen due to its phylloxera tolerance [21,22] and its widely use as a model rootstock cultivar in different experiments (e.g., [23,24]).

Manual inspection of the chromatograms resulted in assignment of at least 100 different substances. The automated deconvolution of

Table 1
Volatile substances identified in grapevine root tissue [phylloxera-infested (nodosities) and uninfested]. Numbering of first column corresponds to elution order on DB-5 column, see Fig. 1.

Number in Fig. 1	CAS-Number[a]	Identified substance (trivial name in parentheses)	LTPRI				Formerly described in *Vitis* sp.[e]			
			DB-5MS		Optima-WAX					
			Sample	Reference value	Sample	Reference value	roots	leaves	flowers	berries
1	66-25-1	Hexanal[c]	805	805	1091	1087	x	x	x	x
2	98-01-1	Furan-2-carbaldehyde[c]	842	837	1482	1479				x
3	6728-26-3	(E)-Hex-2-enal[c] (leaf aldehyde)	858	858	1232	1229		x		x
4	928-95-0	(E)-Hex-2-en-1-ol[c]	858	862	1424	1422		x		x
5	111-27-3	Hexan-1-ol[c]	874	873	1370	1370		x		x
6	100-51-7	Benzaldehyde[c]	967	967	1548	1546		x		x
7	123-35-3	beta-Myrcene[c]	994	993	1152	1151				x
8	3777-69-3	2-Pentylfuran[c]	995	994	1227	1223				
9	124-13-0	Octanal[c]	1007	1005	1296	1294				
10	104-76-7	2-Ethylhexan-1-ol[c]	1032	1031	1505	1504				x
11	5981-54-8	Limonene[c]	1034	1032	1192	1191	x		x	x
12	100-51-6	Phenylmethanol[c]	1042	1042	1908	1905		x	x	x
13	122-78-1	2-Phenylacetaldehyde	1048	1047[d]	1668	1648[d]		x		
14	111-87-5	Octan-1-ol[c]	1073	1073	1576	1575			x	x
15	124-19-6	Nonanal[c]	1105	1106	1404	1403	x	x	x	x
16	60-12-8	2-Phenylethanol[c]	1119	1120	1946	1944		x	x	x
17	18 829-56-6	(E)-Non-2-enal	1162	1161[d]	1556	1536[d]		x	x	x
18	124-07-2	Octanoic Acid[c]	1178	1177	2124	2115				
19	119-36-8	Methyl 2-hydroxybenzoate[c] (Methyl salycilate)	1201	1202	1806	1805		x	x	x
20	112-31-2	Decanal[c]	1207	1208	1511	1510	x	x		
21	67-47-0	5-(Hydroxymethyl)furan-2-carbaldehyde[c]	1236	1238	2550	2551		x		
22	106-24-1	(2E)-3,7-dimethylocta-2.6-dien-1-ol[c] (Geraniol)	1257	1258	1870	1869		x	x	x
23	112-05-0	Nonanoic acid[c]	1273	1274	2233	2234		x		
24	141-27-5	(2E)-3,7-dimethylocta-2.6-dienal[c] (Geranial)	1274	1275	1759	1758				x
25	7786-61-0	4-Ethenyl-2-methoxyphenol[c]	1322	1322	2232	2221				x
26	97-53-0	2-Methoxy-4-prop-2-enylphenol[c] (Eugenol)	1364	1365	2202	2195				x
28	87-44-5	beta-Caryophyllene[b,c]	1432	1433	1617	1619	x	x	x	x
29	3796-70-1	(5E)-6,10-Dimethylundeca-5.9-dien-2-one[c] (Geranyl acetone)	1456	1456	1877	1876			x	x
30	6753-98-6	Humulene (alpha-Caryophyllene)	1466	1453[d]	1691	1654[d]			x	
31	39 029-41-9	gamma-Cadinene	1526	1513[d]	1780	1759[d]			x	
32	483-76-1	delta-Cadinene	1533	1524[d]	1773	1747[d]				x
33	143-07-7	Dodecanoic acid[c]	1568	1565	2557	2564		x		
34	629-78-7	Heptadecane[c]	1696	1700	1699	1700	x	x	x	x
35	2765-11-9	Pentadecanal	1716	1714[d]	2050	2041[d]				
36	593-45-3	Octadecane[c]	1797	1800	1800	1800		x		
37	57-10-3	Hexadecanoic acid[c]	1962	1962	2986	2986		x	x	
38	112-95-8	Eicosane[c]	1999	2000	2000	2000		x	x	

[a] Chemical Abstracts Service, SciFinder Scholar 2007.
[b] Only detected in infested samples.
[c] Confirmed with standard.
[d] LTPRI corresponds to the median from NIST Chemistry Webbook.
[e] Substances described in former studies are marked with "x", references see text (2.1).

mass spectra [25] and comparison with MS databases together with evaluation of linear temperature programmed retention index (LTPRI) values [26] on two types of stationary phases led to the positive identification of 38 metabolites (Table 1). Thirty two of these metabolites were additionally confirmed with authentic standards. It shall be noted here that in case of terpenes special caution has to be taken. These substances comprise a very large and diverse class of natural compounds with closely related chemical structures, many of which show similar mass spectra and retention indices. Hence, there is a strong need for the use of authentic standards to avoid false positive identifications.

The identified volatiles comprise numerous chemical substance classes. More precisely, ten aldehydes, nine terpenes five C6-compounds, five alcohols, four acids, three alkanes, one ether and one ester were detected in the investigated samples (Table 1). The chromatograms also contained peaks which have been assigned to dibutyl phthalate and 2,4-di-tert-butylphenol (data not shown). Since these compounds are frequently used as plasticizers and stabilizers of synthetic polymers, they have not been further considered in this study as they represent most likely artefacts.

The majority of the identified substances have already been found with GC–MS in other parts of *Vitis* plants such as leaves (e.g., [12,16,17,27–29]), berries (e.g., [16–18,30–32]) or flowers (e.g., [16,33]). These findings have been summarized in Table 1. To the best of our knowledge, 32 of the metabolites are described for *Vitis* root tissue for the first time, whereas four of those, namely pentylfuran, octanal, octanoic acid and pentadecanal have not been described for *Vitis* spp. at all.

In a recent study Du et al. [20] investigated root volatiles in phylloxera resistant (5BB) and susceptible (Kyoho) rootstock cultivars and detected in total 79 substances. For the resistant 5BB they reported 56 volatile metabolites, whereas 47 volatiles were assigned in total to the cultivar Kyoho. Partly different volatiles were found for the cultivars 5BB and Kyoho with fatty acid methyl esters being the dominating volatile substance classes for both cultivars. In our study, no fatty acid methyl esters were detected. Unfortunately, Du et al. [20] did not describe their extraction method in detail, therefore a direct comparison with our findings is not feasible. Nevertheless, we also found hexanal, limonene, nonanal, decanal, beta-caryophyllene and heptadecane.

2.2. Comparison of volatile profiles obtained for uninfested root tips and nodosities

As presented in Fig. 2 significant differences ($p < 0.05$) in peak areas were found for 14 metabolites. Remarkably, all but one substance (dodecanoic acid) occurred at elevated levels in nodosity samples, whereas beta-caryophyllene was exclusively found in mature nodosity samples. Interestingly, relative standard deviations of peak areas were significantly lower in uninfested root samples compared to mature nodosities (Fig. 2). Furthermore, we detected significant differences for concentration levels of geraniol, eugenol, vanillin and delta-cadinene in roots infested by one 2nd nymphal stage phylloxera (young nodosities) compared to uninfested root tips and elevated levels of phenylmethanol, methyl salicylate, 4-ethenyl-2-methoxyphenol as well as eugenol in mature nodosities compared to young ones (data not shown). Those findings indicate that the metabolic response appears to be highly dynamic and requires closer investigation in the future.

Considering relevant metabolic routes acting on the formation of volatile organic compounds after herbivore attack, we found that at least three different pathways might be affected in phylloxera-infested root tips. The detected and/or elevated terpenoids (beta-caryophyllene, geraniol, beta-myrcene) allow the assumption that the mevalonate (MEV) and/or alternative isopentenyl pyrophosphate

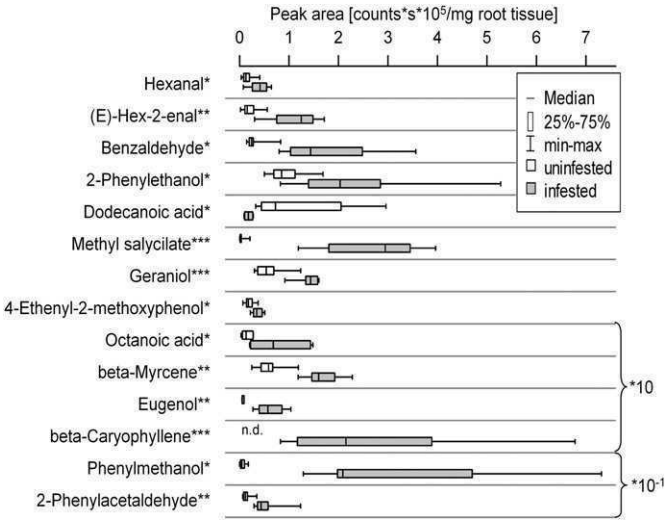

Fig. 2. Overview of differentially expressed volatiles detected in uninfested root tips and mature nodosities (infested by one adult phylloxera producing maximal 5 eggs). Intensities of some metabolite peaks were multiplied by a factor of 10 or 10^{-1} respectively (see graph). Asterisks indicate significant differences between the two sample types: $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$; n.d.: not detected.

(alt. IPP) pathway are modified as a consequence of phylloxera damage. Beta-caryophyllene for example, is well known to be associated with the response of various plants to herbivore root attack and has been reported to attract natural enemies [15]. Geraniol [34] and beta-myrcene [35] are also known to be produced by the green parts of plants after hemiptera attack such as aphids or stink bugs. Further, it can be suggested that the phenylpropanoid pathway is triggered due to a phylloxera infestation. The aromatic compounds, phenylacetaldehyde, methyl salicylate and eugenol, which have been found at elevated levels in phylloxera-infested samples compared to uninfested root tips, have also been described to be produced by plants after leave herbivory [36] or pathogen attack [37]. Mallinger and colleges, for example further suggest that methyl salicylate attracts natural enemies of soybean aphids [38]. Moreover, elevated levels of the C6-compounds (E)-hex-2-enal and hexanal were observed in mature nodosities compared to uninfested root tips which indicates that the lipoxygenase (LOX) pathway might be initiated after phylloxera infestation. C6-compounds result from the oxidative cleavage of linoleic- and linolenic acid. Both substances are well known to be released after herbivore attack [39] as well as C6-compounds from *V. vinifera* [40].

3. Conclusion

Applying strict identification criteria we identified 38 volatile metabolites in grapevine root samples, including C6-compounds, terpenes, aromatic compounds, aldehydes, alcohols and *n*-alkanes. Comparing in a second step mature nodosities with uninfested root tips we identifying twelve substances at significantly increased concentration levels in infested root samples, while dodecanoic acid was found at decreased levels and beta-caryophyllene exclusively in mature nodosity samples. Our preliminary data indicate that several defence related metabolic pathways, namely the MEV and/or alt. IPP, the phenylpropanoid as well as the LOX pathway might be affected as a consequence of phylloxera attack. However, more detailed studies are required to gain deeper insight into the metabolic processes induced upon phylloxera attack e.g., on nodosities still attached to the plant evaluating which volatiles might be involved in plant–plant signalling as well as direct or

indirect defence responses against phylloxera. Furthermore, comparing the metabolic response of rootstocks with different levels of susceptibility to phylloxera and *V. vinifera* will provide a better understanding of the molecular mechanisms mediating resistance against root-feeding phylloxera.

4. Material and methods

4.1. Insect and plant material

Leaf-galling *D. vitifoliae* Fitch (Hemiptera: Phylloxeridae) were collected in Grosshoeflein, Austria in 2007 and maintained since then as a single founder lineage in the greenhouse on the grapevine rootstock Teleki 5C (*V. berlandieri* Planch. × *V. riparia* Michx.).

Samples from different vegetatively propagated cuttings of this rootstock clone were collected during several independent runs during June–October 2009 in the greenhouse. Further details on the experimental setup are given in Lawo et al. [41]. We sampled uninfested root tips and mature nodosities, which were infested by one adult phylloxera producing maximal five eggs. In case of nodosities, the phylloxera was removed and both, uninfested root tips and mature nodosities were immediately cooled with liquid nitrogen and stored at −80 °C until further sample preparation and analysis.

4.2. Sample preparation and HS-SPME–GC–MS analysis

Cooled root tissues were homogenized via a pre-cooled pestle and 25–50 mg of the homogenized sample were weighted into a 20 ml screw cap headspace (HS) vial, sealed with 1.3 mm silicone/PTFE septa (Supelco, distributed by Gerstel, Mühlheim a.d. Ruhr, Germany). Subsequently, samples were incubated for 30 min and extracted for 60 min at 90 °C by headspace solid phase microextraction (HS-SPME) (fibre coating: DVB/CAR/PDMS 50/30 μm, 2 cm stableflex fibre, Gerstel). Thereafter, samples were analyzed by GC–MS according to Stoppacher et al. [42], with the following modifications: apolar column: DB-5MS (Agilent, Waldbronn, Germany); polar column: Optima-WAX (Machery-Nagel, Germany), dimensions for both columns: length 30 m, inner diameter 0.25 mm, film thickness 0.25 μm, oven program: 35 °C (hold 2 min), 5 °C min^{-1} to 260 °C (hold 5 min), no solvent delay, *m/z* scan range: 35–500 amu.

4.3. Analysis of standards and determination of retention indices

Standard substances were purchased from Sigma–Aldrich (Vienna, Austria) (summarised by brand, minimum purity in parentheses): SAFC: (2E)-3,7-dimethylocta-2,6-dien-1-ol (97%), (5E)-6,10-dimethylundeca-5,9-dien-2-one (97%), 4-ethenyl-2-methoxyphenol (98%), beta-caryophyllene (80%), 2-pentylfuran (97%), hexadecanoic acid (98.9%), octanal (92%), Aldrich: (E)-hex-2-en-1-ol (96%), (E)-hex-2-enal (98%), 5-(hydroxymethyl)furan-2-carbaldehyde (99%), dodecanoic acid (98%), furan-2-carbaldehyde (98%), (−)-limonene (96%), nonanal (95%), Sigma–Aldrich: 4-hydroxy-3-methoxybenzaldehyde (99%), benzaldehyde (99%), hexan-1-ol (98%), phenylmethanol (99%), Sigma: decanal (98%), nonanoic acid (97%), Riedel de Haën: octanoic acid (99%), Supelco: octan-1-ol (99.9%), Fluka: 3,7-dimethylocta-2.6-dienal (cis + trans, 95%), 2-ethylhexan-1-ol (99.5%), 2-methoxy-4-prop-2-enylphenol (99.8%), 2-phenylethanol (99%), beta-myrcene (95%), hexanal (97%), methyl 2-hydroxybenzoate (99.5%), alkane standards C8–C20 (40 mg L^{-1} each in hexane), C21–C40 (40 mg L^{-1} each in toluene). Additionally, a C5–C10 alkane standard was mixed from the pure substances (pentane 99% Sigma–Aldrich, hexane Supra-Solv Merck, heptane 99.5% J.T. Baker, octane 99% Sigma Aldrich, nonane 99% Sigma Aldrich, decan p.a. Promochem) in a ratio resulting in narrow peak shapes.

From the original standards (liquids and solids) stock solutions with a concentration of 100 mg L^{-1} in acetonitrile (HPLC gradient grade, VWR, Vienna, Austria) were prepared and stored at 4 °C. Standards and dilutions were always handled with gastight Hamilton syringes.

For determination of linear temperature programmed retention indices (LTPRI, [26]) the standards were combined in a mixture resulting in a concentration of 25 μL L^{-1} in MilliQ-water (in-house device, Millipore, Molsheim, France). Twenty μL of the mix were transferred in a 20 mL HS vial and measured with the same SPME–GC–MS method as the root samples. The alkane standards were measured with the following SPME-methods to achieve narrow peak shapes: C5–C10: 1 μL in 20 mL HS vial, sampling out of tray (10 °C) for 0.01 min, C8–C20: 10 μL in 20 mL HS vial, extraction for 10 min at 90 °C, C21–C40: 30 min equilibration and 60 min extraction both at 120 °C.

4.4. Data evaluation

4.4.1. Detection and identification of metabolites

For identification of metabolites, two pooled samples (one uninfested and one infested root sample) were used. Peak detection, spectra deconvolution, comparison of MS spectra against Wiley/NIST 08 spectra library [43] and LTPRI calculation were carried out with the AMDIS software (version 2.65, www.amdis.net, [25]) with default settings for deconvolution. Putative metabolites found on both columns with a match factor ≥90 and a LTPRI deviation of ≤15 (if determined from a standard) or ±2% (if derived from literature) were put to the results list according to Stoppacher et al. [42]. Median values of those LTPRIs listed in the NIST Chemistry Webbook [44] which corresponded to the same column diameter and film thickness as well as a comparable stationary phase material were used as reference. Substances known to originate from the fibre coating or the stationary phase of the GC-column (e.g., silicium containing substances) were removed from the results list.

4.4.2. Differential comparison of uninfested root tips and nodosities

Metabolites identified according to the criteria listed in 4.4.1, were considered for further comparison of volatile profiles associated with uninfested root tips ($N = 8$) and nodosities ($N = 7$). For this purpose, a sub library containing mass spectra of all identified substances was created. The well defined uninfested and nodosity samples were measured on the DB-5MS column and data were evaluated again with AMDIS with the following settings: minimum match factor = 60, RI-window = 5, match factor penalties: level infinite, maximum penalty and “no RI in library”: 100.

The data were grouped according to sample type. For those metabolites detected in ≥6 out of 7 (nodosities) or in ≥6 out of 8 (uninfested) samples the arithmetic means of each sample type were compared by an unpaired two-sided Welch’s *t*-test with a confidence level of 0.95 (R statistic software version 2.9.2).

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Evaluation of settled floor dust for the presence of microbial metabolites and volatile anthropogenic chemicals in indoor environments by LC–MS/MS and GC–MS methods

Vinay Vishwanath[a], Michael Sulyok[a,*], Georg Weingart[a], Bernhard Kluger[a], Martin Täubel[b], Stefan Mayer[c], Rainer Schuhmacher[a], Rudolf Krska[a]

[a] Center for Analytical Chemistry (CAC), Department IFA-Tulln, Konrad Lorenz Strasse 20, A-3430 Tulln, Austria
[b] Department of Environmental Health, National Institute for Health and Welfare; P.O. Box 95, 70701 Kuopio, Finland
[c] Institution for Statutory Accident Insurance and Prevention in the Trade and Goods Distribution, Mannheim, Germany

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ABSTRACT

This study reports on detection of a large number of biological and anthropogenic pollutants using LC–MS/MS and GC–MS technologies in settled floor dust (SFD). The latter technique was applied to obtain a general picture on the presence of microbial as well as non-microbial volatile organic compounds, whereas the targeted LC–MS/MS analysis focused on identification of species specific secondary metabolites. In the absence of moisture monitoring data the relevance of finding of stachybotrylactam and other metabolites of tertiary colonizers are confined only to accidental direct exposure to SFD. To the best of our knowledge 30 of the 71 identified volatile organic compounds (VOCs) are newly reported in SFD matrix. Coordinated application of "AMDIS and Spectconnect" was found beneficial for the evaluation and identification of prime volatile pollutants in complex environmental samples. Principal component analysis (PCA) of peak areas of 18 microbial volatile organic compounds (MVOCs) resulted in identification of nonanal as potential MVOC marker. Two more volatiles toluene and 1-tetradecanol though had discriminative influence, are not regarded as MVOC markers, considering their probable alternate origin from paints and cosmetics, respectively.

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1. Introduction

The advantages of various methods and matrices for the purpose of indoor evaluation are well documented in earlier reports [1–9]. The suitability of settled floor dust (SFD) for the above mentioned purpose can be best explained on the basis of mode of its formation. SFD is typically formed by the deposition of indoor aerosols, outdoor particles (due to ventilation) and also particles brought by foot trafficking. Moreover criticality and relevance of chosen SFD matrix for the mass spectrometric evaluation are also justified by the reports of Rosas et al. [1] for the detection of antibiotic multi resistance *E. coli* serotypes and reports of SFD surface adsorption of polycyclic aromatic hydrocarbons and other volatile and semi-volatile organic compounds [10,11]. Since the process of degradation indoors is typically slow, settled floor dust is also considered as a well preserved fossil evidence for indoor evaluation [12].

The new findings since reports of sick building syndrome (SBS), indoor pollutants are classified either as biogenic or anthropogenic pollutants. Biogenic pollutants include aerosols of viruses, bacteria, fungal spores and mycelial fragments containing toxic metabolites (e.g.: *Stachybotrys* containing Stachybotrylactam and Satra toxins), pollen, animal dander, dust mite residues and other particles of biological origin [13]. Anthropogenic pollutants are hazardous chemicals which arise as a consequence of their wide spread use in day to day consumer products (phthalate in PVC products). A variety of adverse health effects following human exposure to bioaerosols have been well documented [14]. Some of them are allergy, hypersensitivity, respiratory and toxicological problems, and infectious diseases [15]. Microbial role in indoor pollution and health implications are well known. For instance, fungi as well as bacteria (antigens, structural components, bioactives compounds as endotoxins) in humidifiers have been implicated in "humidifier fever", a disease with both toxic and allergic manifestations [16]. Bacteria in indoor air of houses or offices have also been reported to be associated with extrinsic allergic alveolitis among occupants [17].

Under non obvious moisture complications types and levels of fungal spores in the indoor samples are generally lower to those

* Corresponding author. Tel.: +43 2272 66280 409; fax: +43 2272 66280 403.
E-mail address: michael.sulyok@boku.ac.at (M. Sulyok).

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detected in the outdoor samples. Fungal flora of damp buildings comprises species addition to outdoor fungi that can utilize the nutrients present in building materials and adaptable to particular level of water activity. Based on water activity molds grown on building materials can be divided into primary, secondary and tertiary colonizers. Many of the toxigenic species *Stachybotrys*, *Chaetomium*, *Memnoniella*, *Aspergillus* and *Trichoderma* belong to the class of tertiary colonizers. Indoor isolates of *A. versicolor* from building materials are reported to produce sterigmatocystin, a class 2B carcinogen and biosynthetic precursor of aflatoxin B_1. Prevalence of airborne spores of *Stachybotrys chartarum* in houses with water incursion [18] and satratoxin G–albumin adducts (in vivo) upon human and animal exposures to *S. chartarum* [19] are some evidences for probable health threats due to mycotoxins in water damaged indoor environments. Animal exposure studies of low molecular weight compounds from fungi from the built environment have shown implications on health, such as inflammatory processes [20]. Microbial volatile organic compounds (MVOCs) are an addition to the list of biogenic indoor pollutants and have drawn attention as potential contributors for adverse health effects observed in residents of moisture damaged buildings [21]. In vitro studies of histamine release by bronchoalveolar cells exposed to MVOCs of *Trichoderma viride* [22], is a good example of the experimental evidences of MVOCs connections to clinical aspects.

Studies on house dust have shown for the presence of banned chemicals, and chemicals with endocrine disrupting, carcinogenic, neurotoxic potentials at levels that are considered to be of concern for human health [23,24]. Traditionally, consumption of food has been considered a primary route of exposure to contaminants mentioned above. However, it is becoming clear that exposure through ingestion and/or inhalation of indoor dust may be comparable to corresponding food consumption especially for younger children [24]. Independent studies monitoring for occupational exposure to volatile organic compounds (VOCs) toluene, o-xylene and n-butyl acetate and correlation of these substances to clinical symptoms upon long-term exposure such as deficits in concentration and memory, and an increase in the reaction time are proofs for ill effects of some of the VOCs in adults too [25,26].

State-of-the-art technology GC–MS may be useful for detecting hidden mold [27] and proved to be useful to differentiate between fungal strains [28]. Extremely low MVOC concentrations and the existence of many disturbing concomitants indoor complicate the analysis of microbial VOC in moldy houses leading to false positives [29]. Secondary metabolites being inconsistent in distribution throughout the fungal kingdom are unique as markers for speciation and chemotaxonomy purposes [30]. This was also reported true for fungal species found indoors [31,32]. Hence application of complementary technologies GC–MS and LC–MS/MS for evaluation of complex indoor matrix as SFD can be highly advantageous for comprehensive indoor evaluation and to monitor, e.g., ongoing remediation processes.

Enumeration studies based on microbial viability [1,2], detection of microbial volatiles [3–6] and non volatiles [7–9] in indoor matrices including settled dust are known. To our knowledge this is the first report of comparison of dust matrix from indoors used for different purposes using microbial volatiles and secondary metabolites. Dust samples from vastly differing indoor environments such as waste management and recycling units (WMU), houses with and without any water damage and mold infestation are screened for microbial effectors and subsidiary chemicals. Additionally, we wanted to test feasibility of source recognition/apportionment based on (M)VOC pattern among various indoor dust samples using principal component analysis (PCA) and hierarchical cluster analysis (HCA). Some of the methodological challenges in GC–MS such as sample volume, extraction time, and temperature for the optimal extraction of volatile substances in dust matrix are addressed. Appertain to data evaluation we demonstrated usability of AMDIS deconvoluted chromatograms in combination with Spectconnect [33] for additional verification of sampling and method performance. Practical relevance of the study can be best explained in relation to safety of toddlers in homes with crawling and hand to mouth behavior and workers of WMUs. The extraction temperature condition (max 90 °C) of our GC–MS method is similar considering the working temperature of common home appliances (e.g. surface of a electric bulb (110–160 °C)). This aforementioned fact makes volatile pattern generated under our experimental conditions realistic and comparable to real world scenario. This is the first comparative study of individual SFD samples derived from relatively differing indoor environments in their purposes using both LC–MS/MS and GC–MS methods.

2. Materials and methods

2.1. Study sites and sampling

2.1.1. Waste management facilities

Settled floor dust samples were collected using vacuum cleaner from different waste management units (WMU) in Germany dealing with municipal waste or paper recycling. Samples MWD 1–7 (Municipal waste dust, Group A) were collected from waste handling facilities treating municipal waste with "biological and mechanical" or "biological" methods. Samples PWD 1–8 (Paper waste dust, Group B) were from enclosures used for paper recycling activities such as sorting, storage, or mechanical pressing (Table 1).

2.2. Residential indoors

Settled floor dust samples were collected from houses inhabited by small group of people, generally less than 5. Samples AHD 1–5 (Affected house dust, Group D) were vacuum cleaner dust bag dust samples derived from single family houses located in Eastern and southern Finland. These houses had severe moisture damage/dampness problems that were confirmed by trained engineers upon building inspection. Residents of these buildings typically complained about building related symptoms. Samples CHD 1–2 (Control house dust, Group C) were samples, respectively, from United States of America (SRM 2583) and India (CHD-2). SRM 2583 is a certified reference material (CRM) for 5 elements viz., Arsenic, Cadmium, Chromium, Lead and Mercury. CHD-2 is a self collected house dust where no clinical symptoms of ill health or visible mold growth were observed or reported.

3. Methods for analysis of non volatile and volatile substances

Volatile organic compounds (microbial and anthropogenic) were evaluated using GC–MS technology. Non volatile microbial organic compounds were monitored using LC–MS/MS technology.

3.1. GC–MS screening for volatile and microbial volatile organic compounds

3.1.1. Analytical reagents and supplies

The alkane mixture C_5–C_{10} was mixed in-house. C_8–C_{20} and C_{21}–C_{40} straight chain alkanes of 40 mg L^{-1} concentration in hexane and toluene, respectively, were purchased from Fluka (Buchs, Switzerland). All pure GC–MS standards (substances in Table 2 and Supplementary Table 1 highlighted with "*") used in this study were purchased from Sigma–Aldrich, Vienna, Austria. To avoid artifact originating from GC-column bleeding, SPME fiber coating or from laboratory air, head space vials were left open for 24 h in laboratory

Table 1
Sample description. MW: municipal waste management units, PW: Paper waste recycling units, TA: treatment area, ST: storage, NA: not applicable.

Sample	Nature of sample	Sampling site	Mode of waste treatment at the sampling site	Ventilation of sampling site	Processing load/turn over of waste handling units (kilotons/year)
MWD-1	MW	TA	Biological and Mechanical	Mechanical	62
MWD-2	MW	TA	Biological and Mechanical	Mechanical	62
MWD-3	MW	TA	Biological and Mechanical	Mechanical	62
MWD-4	MW	TA	Biological and Mechanical	Mechanical	62
MWD-5	MW	TA	Biological and Mechanical	NA	62
MWD-6	MW	TA	Mechanical	Natural	400
MWD-7	MW	TA	Mechanical	Natural	200
PWD-1	PW	ST	Paper-Storage	Natural	7.3
PWD-2	PW	TA	Mechanical	Natural	100
PWD-3	PW	TA	Sorting-Mechanical & Manual	Natural	40
PWD-4	PW	TA	Sorting-Mechanical	Natural	20
PWD-5	PW	TA	Sorting-Mechanical	Natural	60
PWD-6	PW	TA	Sorting & Pressing-Mechanical	Natural	10
PWD-7	PW	TA	Sorting & Pressing-Mechanical	Natural	10
PWD-8	PW	TA	Sorting & Pressing-Mechanical	Natural	10

and subsequently analysed. Substances identified this way were excluded during final compilation of data.

3.1.2. GC–MS

Automated sample extraction, chromatographic separation and MS detection was done with an Agilent 6890 GC (Waldbronn, Germany) instrument, coupled to a 5975B MSD detector for recording the mass spectra. The following pair of GC–MS columns were used one at a time during the entire study:

(A) HP-5MS 30 m × 0.25 mm, 0.25 μm f.th. (Agilent, Waldbronn, Germany),
(B) Optima® Wax 30 m × 0.25 mm, 0.25 μm f.th. (Agilent, Waldbronn, Germany).

For both columns Helium (5.0, Messer, Austria, Gumpoldskirchen) was used as carrier gas at a constant flow rate of 1 mL/min. Oven program: HP-5MS: (apolar) 35 °C (hold 2 min), 5 °C/min to 230 °C, 40 °C/min to 260 °C (hold 5 min). Optima® Wax: 35 °C (hold 2 min), 5 °C/min to 250 °C (hold 10 min). The inlet was equipped with a headspace inlet glass liner, 1.5 mm i.d. (Supelco, Bellefonte, USA) and set to 250 °C in splitless mode during desorption (2 min) of analytes from the fiber. The transfer line to MS was at 270 °C. MSD parameters: electron impact ionisation (EI) at 70 eV, source temperature 230 °C, quadrupole temperature 150 °C, full scan mode, mass range 35–500 amu.

3.1.3. HS-SPME

Sampling was done by headspace volatiles extraction procedure fully automated by an auto sampler (MPS 2 XL, Gerstel, Mülheim a.d. Ruhr, Germany).

Fiber selection and extraction optimization were done on a 30 m HP-5MS column using real world settled dust ($n = 5$) and SRM 2583 (certified indoor reference dust) with an empirical sample amount of 0.05 g which was found to be well suited upon validation in due course of the study. The following SPME-fibers with different polarities were tested: polydimethylsiloxane (PDMS), 100 μm; polydimethylsiloxane/divinylbenzene (PDMS/DVB), 65 μm; carboxene/polydimethylsiloxane (CAR/PDMS), 85 μm; polyacrylate (PA), 85 μm and divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS), 50/30 μm (Supelco, Bellefonte, USA). Selection of fiber was on the basis of number of conserved component upon extraction of representative sample of each group using above mentioned 5 fibers. All fibers tested and used were preconditioned according to the manufacturer's guidelines. Dust samples were weighed into 20 mL head space glass vials covered with Teflon capping and heated to 90 °C for 30′ to release dust bound volatiles. The conditioned fiber was then inserted 21 mm into the head space vial and incubated for 60′ unagitated at constant temperature of 90 °C for adsorption of volatiles. For desorption, the fiber was inserted for 2 min in the split less injector (250 °C, fiber penetration depth 57 mm).

The following SPME parameters were tested: equilibration time (0 and 30 min), extraction time (30 and 60 min), and equilibration and extraction temperature (30, 60 and 90 °C). For all subsequent experiments of parameter evaluation the best found SPME fiber CAR/PDMS, 85 μm was employed (fiber selection elaborated in Section 4).

3.1.4. Method evaluation: reproducibility, representative sampling and source recognition/apportionment studies

For all the above purposes systematic conserved component identification was done, using open source software http://spectconnect.mit.edu. The working principle, algorithm, data extraction procedures followed by Spectconnect are described elsewhere in detail [33]. This is the first report of application of Spectconnect for evaluation of both analytical method and comparability of samples without compound identification. Criterions for picking conserved components were kept stringent and are as follows: elution threshold of 0.5 min (high), support threshold occurrence in all samples (high) and similarity threshold with minimum spectral similarity of 90% (high). Statistical software Unscrambler® [34] and R package (R 2.12.0) [35] were used for multivariate statistics Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), respectively. The data was leverage corrected and centered before subjecting to PCA and for the hierarchical clustering "Euclidean" distance and "Ward" linkage methods (between groups) were used. The effect of several well established clustering methods as single linkage, complete linkage and centroid methods were tested before finalizing with ward linkage. The results from PCA and HCA were used in concurrence for drawing final conclusion.

3.1.5. Volatile data evaluation: identification and confirmation of components

The data acquisition software MSD Chemstation G1701EA E.01.00.237 (Agilent, Waldbronn, Germany) was used to compare mass spectra of chromatographic peaks found in combined Nist and Wiley 2008 databases/spectral libraries. For peak picking all chromatograms were queried to an empty/blank msl library prior querying against combined Nist and Wiley 2008 spectral library. This method was found beneficial avoiding omission of peaks. Addition to this in-house sub libraries were built deriving mass spectra from Nist 05a and Wiley 7n for all those volatiles with AMDIS mass

Table 2
List of identified volatile organic compounds originating from settled floor dust samples from Groups A, B, C and D.

CAS No.	Name	*m/z* (precursor ion)	RT-HP5 (min)	RI-HP5 literature	RI-HP5 experimental	RT-Optima® Wax (min)	RI-Optima® Wax literature	RI-Optima® Wax experimental	References
123-73-9	2-Butenal, (E)-	70.08	6.29	644.0	657.3	9.95	1046.0	1046.4	
79-09-4	Propanoic acid	74.07	8.02	740.0	732.7	23.37	1528.0	1560.1	
71-36-3	1-Butanol	74.12	6.52	668.9*	669.3	13.05	1140.0	1162.6	[45,47]
110-62-3	Pentanal	86.13	7.22	698.0	704.3	8.35	983.0	981.0	[45,47]
107-92-6	Butanoic acid	88.10	10.81	831.0	827.4	25.57	1637.0	1654.7	
71-41-0	1-Pentanol	88.14	9.13	764.0	771.8	15.89	1261.0	1266.6	[47]
108-88-3	Toluene	92.13	9.10	771.2*	770.9	9.78	1037.9*	1040.0	
108-95-2	Phenol	94.11	16.01	983.9*	985.6	33.32	2023.4*	2026.0	[48]
109-08-0	2-Methylpyrazine	94.11	10.99	832.0	832.7	16.45	1312.0	1287.0	
6728-26-3	2-Hexenal, (E)-	98.14	11.81	859.7*	857.9	14.90	1228.8*	1230.1	
66-25-1	Hexanal	100.15	10.07	804.8*	804.6	10.97	1086.3*	1086.0	[45,47]
109-52-4	Valeric acid	102.13	13.15	921.0	899.0	28.10	1744.0	1770.0	[47]
503-74-2	Isovaleric acid	102.13	11.74	876.0	855.8	26.55	1674.0	1698.0	[47]
111-27-3	1-Hexanol	102.17	12.28	873.3*	872.3	18.61	1370.2*	1369.0	[47]
100-42-5	Styrene	104.14	13.09	880.0	897.3	15.73	1260.0	1260.6	[45]
100-41-4	Ethylbenzene	106.16	12.35	869.6*	874.4	12.12	1128.1*	1128.9	
106-42-3	p-Xylene	106.16	12.36	875.0*	874.6	12.31	1119.0	1135.9	
108-38-3	1,3-Dimethyl-benzene	106.16	12.35	864.4	874.6	12.31	1140.0	1136.0	
95-47-6	o-Xylene	106.16	12.36	879.0*	874.8	13.69	1189.2*	1185.7	
100-51-6	Benzyl alcohol	108.13	17.83	1042.0*	1041.3	30.86	1905.2*	1902.15	
4313-03-5′	2,4-Heptadienal, (E,E)-	110.15	17.03	1015.2*	1016.7	22.28	1515.8*	1514.7	
57266-86-1	2-Heptenal, (Z)-	112.16	15.22	958.0	961.5	17.83	1331.0	1339.1	
111-71-7	Heptanal	114.18	13.36	904.4*	905.4	13.79	1186.25*	1189.4	[45,47]
53535-33-4	1-Heptanol	116.20	15.58	974.0	972.6	21.24	1458.0	1472.2	
104-87-0	4-Methyl-benzaldehyde	120.14	19.45	1079.0	1091.0	25.44	1653.0	1649.0	
108-67-8	1,3,5-Trimethyl-benzene	120.19	16.48	1006.0	999.6	14.70	1221.0	1222.8	
526-73-8	1,2,3-Trimethyl-benzene	120.19	16.48	1019.9	999.7	18.00	1344.0	1345.5	[45]
611-14-3	o-Ethylmethylbenzene	120.19	15.95	975.0	983.7	15.36	1248.0	1247.2	
620-14-4	m-Ethylmethylbenzene	120.19	15.37	958.5	966.2	14.80	1231.0	1226.4	
622-96-8	p-Ethylmethylbenzene	120.19	15.50	960.0	970.0	14.15	1181.0	1202.5	
95-63-6	ψ-Cumene	120.19	16.46	989.0	999.2	15.36	1252.0	1247.2	
589-18-4	4-Methyl-benzenemethanol	122.16	20.96	1135.0	1141.2	32.58	1977.0	1989.0	
2363-89-5	2-Octenal	126.19	18.48	1060.0	1061.2	20.61	1436.0	1447.1	
124-13-0	Octanal	128.21	16.69	1005.6*	1006.3	16.70	1294.33*	1296.4	[45,47]
3391-86-4	1-Octen-3-ol	128.21	15.92	981.9*	982.7	21.00	1462.6*	1462.7	
104-76-7	2-Ethyl-1-hexanol	130.22	17.46	1031.4*	1030.0	22.03	1504.0*	1503.8	
111-87-5	1-Octanol	130.22	18.81	1073.1*	1071.2	23.73	1574.67*	1575.0	[47]
1195-32-0	Dehydro-p-cymene	132.20	19.54	1087.0	1093.7	20.25	1415.0	1433.0	
874-41-9	1-Ethyl-2,4-dimethyl-benzene	134.21	19.06	1078.0	1079.0	18.27	1348.0	1356.0	
99-87-6	p-Cimene	134.21	17.46	1040.0*	1029.9	16.08	1250.0	1273.3	[11]
138-86-3	α-Limonene	136.23	17.61	1041.9*	1034.3	13.92	1190.6*	1194.1	
5989-54-8	L-Limonene	136.23	17.61	1031.0	1034.3	13.92	1199.0	1194.1	[45]
80-56-8	α-Pinene	136.23	14.47	939.0	939.0	9.28	1026.0	1020.3	[46]
3777-69-3	2-Pentylfuran	138.20	16.26	994.0*	992.9	14.84	1222.8*	1228.2	[45,47]
18829-56-6	2-Nonenal, (E)-	140.22	21.59	1164.0	1162.3	23.26	1524.0	1555.5	
90-12-0	1-Methyl-naphthalene	142.19	25.77	1306.8	1310.6	30.46	1875.0	1882.4	
124-19-6	Nonanal	142.23	19.90	1107.0*	1105.4	19.52	1403.0*	1403.6	[47]
4180-23-8	Anethole	148.20	25.31	1283.0	1293.7	29.76	1818.0	1848.4	
25152-84-5	2,4-Decadienal, (E,E)-	152.23	22.90	1314.0	1207	29.56	1800.0	1838.6	
3913-81-3	2-Decenal, (E)-	154.24	24.53	1261.0	1265.5	25.78	1630.0	1664.3	
112-31-2	Decanal	156.26	22.90	1207.7*	1207.1	22.185	1510.33*	1510.3	
112-05-0	Nonanoic acid	158.23	24.70	1273.7*	1271.8	36.51	2233.5*	2196.0	

Table 2 (*Continued*)

CAS No.	Name	*m/z* (precursor ion)	RT-HP5 (min)	RI-HP5 literature	RI-HP5 experimental	RT-Optima® Wax (min)	RI-Optima® Wax literature	RI-Optima® Wax experimental	References
644-08-6	p-Phenyltoluene	168.23	30.58	1492.0	1498.9	35.11	2117.0	2117.1	
2463-77-6	2-Undecenal	168.27	27.28	1350.0	1368.2	28.26	1755.0	1777.0	
334-48-5	n-Decanoic acid	172.26	27.72	1380.0	1384.9	38.35	2258.0	2303.4	[47]
112-53-8	1-Dodecanol	186.33	30.82	1472.0	1509.0	32.57	1920.0	1988.4	
143-07-7	Dodecanoic acid	200.31	32.55	1565.0*	1582.8	41.97	2564.0*	2526.5	[48]
112-70-9	n-Tridecan-1-ol	200.36	32.46	1577.0*	1578.7	34.47	2076.0	2083.5	[48]
96-76-4	2,4-Bis(1,1-dimethylethyl)-phenol	206.32	30.82	1519.0	1509.1	38.71	2323.3*	2324.75	
120-51-4	Benzyl benzoate	212.24	36.98	1765.0	1783.9	44.27	2613.0	2658.6	
128-37-0	Butylated hydroxytoluene	220.35	30.80	1513.6*	1508.3	31.28	1920.6*	1923.1	
629-80-1	Palmitaldehyde	240.42	37.72	1811.0	1819.7	35.76	2124.0	2153.5	
36653-82-4	Cethyl alcohol	242.44	38.99	1876.0	1882.8	39.97	2363.0	2400.6	
57-10-3	Palmitic acid	256.42	40.63	2010.0	1966.7	49.04	2865.0	2903.0	[48]
502-69-2	Hexahydrofarnesyl acetone	268.47	38.18	1846.0*	1842.6	35.55	2134.0	2141.7	
1921-70-6	2,6,10,14-Tetramethyl-pentadecane	268.52	35.29	1703.0	1704.6	25.93	1669.0	1670.7	
110-27-0	Isopropyl myristate	270.45	37.78	1824.0	1822.7	33.77	2023.0	2048.3	
112-39-0	Hexadecanoic acid methyl ester	270.45	39.81	1926.7*	1924.3	37.15	2229.5*	2233.0	
112-92-5	1-Octadecanol	270.49	42.87	2081.0	2084.4	43.26	2569.0	2607.0	
84-69-5	Isobutyl-o-phthalate	278.34	38.66	1868.0	1866.2	42.78	2526.0	2578.1	
84-74-2	Dibutyl phthalate	278.34	40.51	1969.0	1960.3	45.18	2726.0	2705.3	

* Retention index (RI) values determined by us with pure reference standards. Listed in the order of increasing precursor ion mass.

spectral match factor ≥90. This was done to minimize data evaluation time in addition to convenient automation. Moreover, the in-house sub libraries dedicated for individual column specifications were supplemented with linear temperature programmed retention indices (LTPRI, Van Den Dool and Kratz index values) corresponding to the stationary phases of the GC used in the study. The added LTPRI values were either experimentally determined by us using authentic standards or literature values retrieved from NIST Chemistry Web Book (2009). In case a putatively identified substance was reported with more than one LTPRI, the value most frequently stated was taken into account. Automated data evaluation was done by AMDIS software (automated mass spectral deconvolution and identification system, version 2.64) [36]. The optimization of AMDIS parameters for deconvolution and identification were done as described earlier by Meyer et al. [37]. The following parameters were found optimal and used for deconvolution and identification during the study; width, 20; adjacent peak subtraction, 1; sensitivity, high; resolution, high; shape requirement, high. Mixture of alkane standards (C_5–C_{10}), (C_8–C_{20}) and (C_{21}–C_{40}) were analysed separately and LTPRI values were determined [38]. Data presented in Table 2 are designated as "identified" when LTPRI value of a volatile compound was within relative deviation of ±2% from literature or from experimentally determined value (using pure standard) in addition to mass spectral match factor greater or equal to 90 on both columns of inverse polarities in triplicates. In cases where detection was on one of the columns meeting the other three criterions for identification, compounds were designated "annotated" (Supplementary Table 1). The set criterions in this study are based on our previous investigation [39] of fungal and other complex matrices and other reports for impact of matrix composition on RI [40,41].

3.2. Liquid chromatography/tandem mass spectrometry – non volatile microbial metabolites

3.2.1. Analytical reagents and supplies

Methanol, acetonitrile (both LC gradient grade) were purchased from J.T. Baker (Deventer, The Netherlands), ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma–Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France). Individual fungal and bacterial metabolites were from the same sources as mentioned in our earlier publication [7].

3.2.2. LC–MS/MS

Detection and quantification was done with a QTrap 4000 LC–MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C18-column, 150 mm × 4.6 mm i.d., 5 μm particle size, equipped with a C18 security guard cartridge, 4 mm × 3 mm i.d. (all from Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10:89:1 (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 100% within 12 min, followed by a hold-time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The flow rate was 1000 μL min^{-1}.

ESI-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and in negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The sMRM detection window of each analyte was set to the respective retention time ±24 s and the target scan time was set to 1 s. The settings of the ESI source

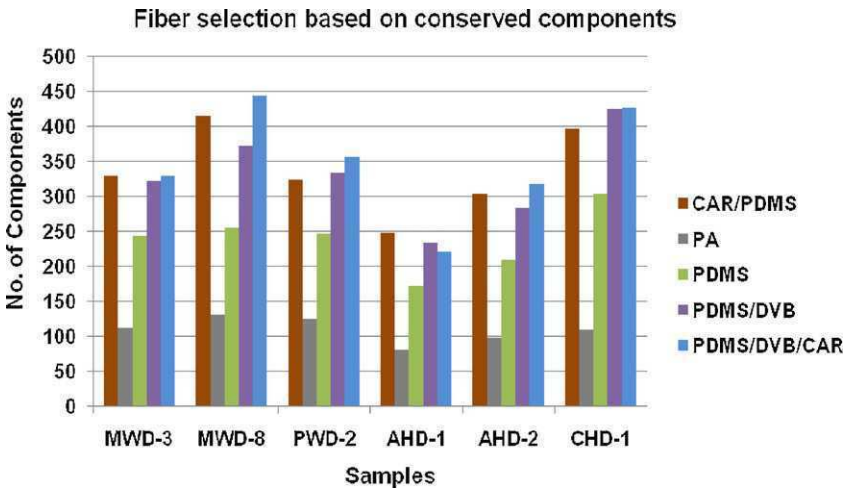

Fig. 1. Comparison of different fiber coatings and number of adsorbed components.

were as follows: source temperature 550 °C, curtain gas 10 psi (69 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 50 psi (345 kPa of nitrogen), ion source gas 2 (drying gas) 50 psi (345 kPa of nitrogen), ion-spray voltage −4000 V and +4000 V, respectively, collision gas (nitrogen) high. Confirmation of positive analyte identification is obtained by the acquisition of two sMRMs per analyte, which yields 4.0 identification points according to commission decision 2002/657/EC [42]. In addition, the LC retention time and the intensity ratio of the two sMRM transitions have to agree with values of corresponding standards.

3.2.3. Secondary metabolite data evaluation

LC–MS/MS data evaluation was done using the Analyst® 1.5 (AB SCIEX 2008). Identification of positive target analytes in samples was confirmed by comparing retention time (RT) and ratios of qualifier to quantifier to authentic standards, measured before and after a particular sample batch of 30 samples.

4. Results and discussion

4.1. SPME and GC–MS

The sensitivity of the HS-SPME-GC–MS technique depends mainly on the distribution constant of analytes partitioned between sample and stationary phase of fiber (K_{fs}) [43]. To generate comprehensive profile of volatiles with variable volatility, selection of optimal fiber becomes a crucial factor in qualitative but mainly in quantitative analysis, where limits of detection are related to amount of adsorbed compound on phase covering the fiber. Among the tested fibers the most polar fiber coatings of polyacrylate (PA) as well as non polar polydimethylosiloxane (PDMS) were found not suitable for our profiling study. Fiber performances of CAR/PDMS, PDMS/DVB and PDMS/DVB/CAR were similar concerning the number of conserved components derived from Spectconnect (Figs. 1 and 2a–e). Hence annotation was considered as an additional criterion for the selection. Among the three semipolar fibers CAR.PDMS was found superior for the purpose with 30% more identification or annotation (Fig. 3). CAR.PDMS is often a fiber of choice in food industries for sensory aroma evaluation [44]. Better suitability of this fiber for profiling purpose is also reported for fungal volatile profiling [6]. Comparison of different extraction temperatures showed that use of 90 °C was suitable for evaluation of substances with wide range of volatilities. Choice of extraction temperature becomes critical, since use of higher temperatures though promoting detection of low volatile substance can be inappropriate as causing premature desorption of other more volatile analytes from the fiber coating which is not suitable for profiling studies like ours [43]. Other factors that need to be considered selecting extraction temperature are nature of matrix and stationary phase. Sample volume and pre-incubation time and temperature were also evaluated in order not to overlook low abundant or moderately volatile substances. Optimization for these parameters resulted in values of 0.05 g sample mass/vial and extraction at 90 °C for 30 min, respectively (Supplementary Tables 2 and 3). Increasing the sample volume did not show any significant increase in number of conserved components indicating either fiber saturation or limitation of the fiber in terms of its potential to adsorb different compounds. The reduction of empirical sample amount (0.025 g) reduced number of components by 25% possibly due to inadequate ion current/intensity of low abundant substances. In our method pre incubation and extraction temperatures are kept constant to minimize temperature ramping. We assume that this in addition to reducing ramping time is also beneficial achieving equilibrium of low and semi volatile compounds in the mixture. Application of a pair of columns with inverse polarities for identification was compared to earlier reports based on single column [6,11,45–47], found advantageous in case of stereo isomers o- and p-xylenes and constitutional isomers ψ-cumene, mesitylene, hemimellitene. The compounds which were barely resolved on apolar column were well resolved on polar Optima® Wax with RT and RI of 13.69 min and 12.31 min and 1186 and 1136, respectively, for o and p-xylenes. Similarly mesitylene, ψ-cumene, hemimellitene had RT of 14.7, 15.4 and 18.0 min and LTPRI 1223, 1247, 1346, respectively.

Total of 71 volatile organic compounds were detected (Table 2) on both columns of different polarity. 20 of the compounds have been reported as microbial volatile organic compounds produced by either individual or mixtures of microbes under laboratory conditions on different matrices [5,6,45,48–51]. Additionally 85 substances designated as “annotated” (Supplementary table 1) were detected on only one of the two columns HP5(89%)/Optima® Wax (11%) with exact LTPRI match or LTPRI within relative deviation of ±2% from literature or from a value determined by measuring a pure standard. Spectral match factor for both identified and annotated compounds were above or equal to 90. Assigning the origin of a compound might not be straight forward as many of the detected volatiles are produced by microbes and plants, as well as they were known to be integral part of many solvents that are commonly used indoors. The best observed examples for

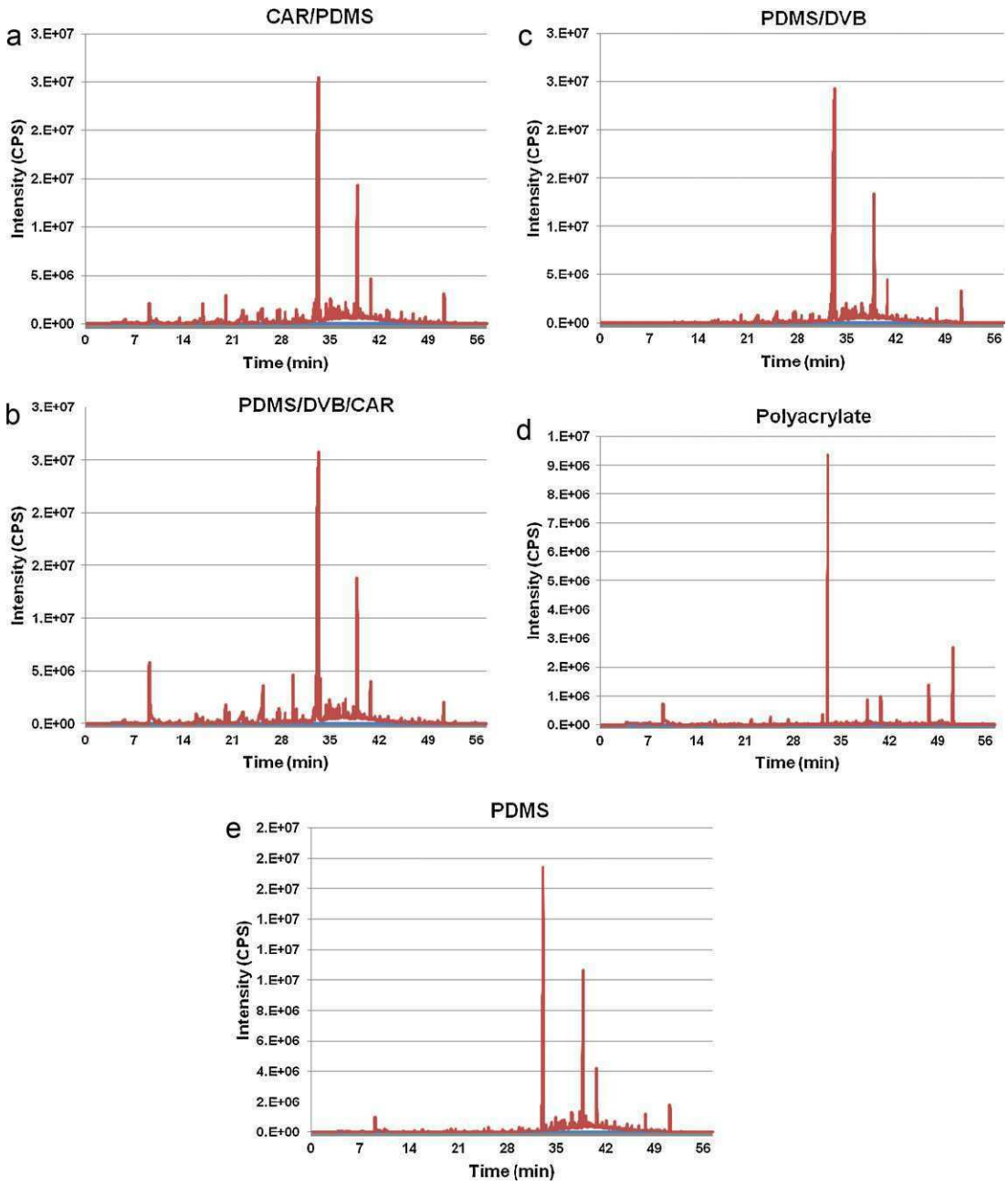

Fig. 2. Fiber selection-chromatographic profile of extracted volatile from sample AHD-1 on different fiber coatings.

this class of compounds were toluene, phenol, ethylbenzene, 1-butanol, limonene, styrene and α-pinene [51–53]. The compounds nonanal, toluene, butanoic acid, benzyl alcohol, phenol, 1-octanal, phthalic acid, and dibutyl phthalate were uniform in their occurrence across all four groups of samples. 1-Octanol and nonanal without any ambiguity could be related to microbial origin [51]. All other frequently found substances with exception of toluene and phenol could be traced back to either combustion by product of gasoline, adhesive or plasticizers [53]. Limonene is a constituent of many household consumer products such as deodorizers, polishes, fabric softeners, cigarettes and food beverages [52,54]. Limonene occurrence in samples derived exclusively from municipal waste management units and not in other can be an argument for its insignificant synthesis and release by microbes compared to non biological sources.

4.1.1. Statistical evaluation: GC–MS method performance and source identity recognition/apportionment based on volatile profile of samples

Multivariate statistics PCA and HCA were employed to identify probably existing differences and similarities in volatile profiles of different indoor environments. Our assumption for this was discrepancies in volatile profiles of different indoor environments also exist in dust samples (due to surface adsorption and particle accumulation), and arises as a result of dissimilar indoor purposes, climate and geographical location. For this purpose ISmatrix generated by Spectconnect was used. ISmatrix is a result output Microsoft Excel CSV file, consisting of complement peaks areas consistently detected in multiple chromatograms across sample groups or sample replicates. ISmatrix of the order 66*1993 representing conserved peak areas across 22 samples in replicates of

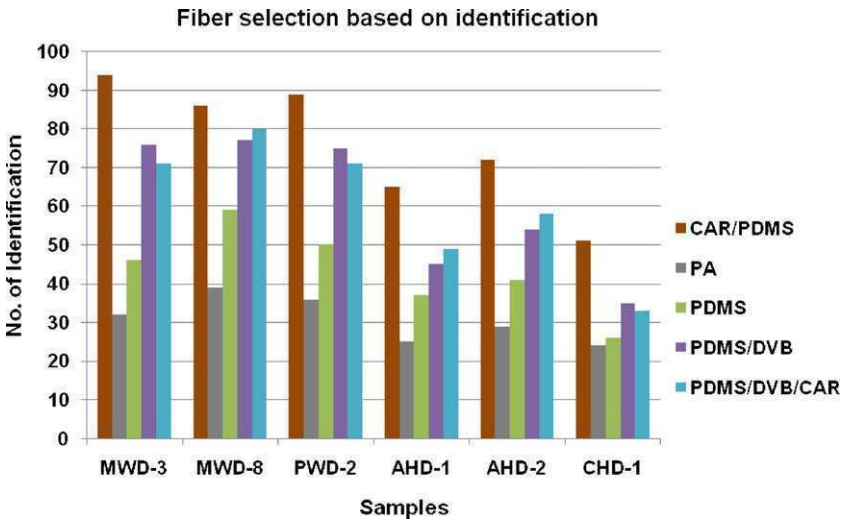

Fig. 3. Comparison of different fiber coatings and number of identified components.

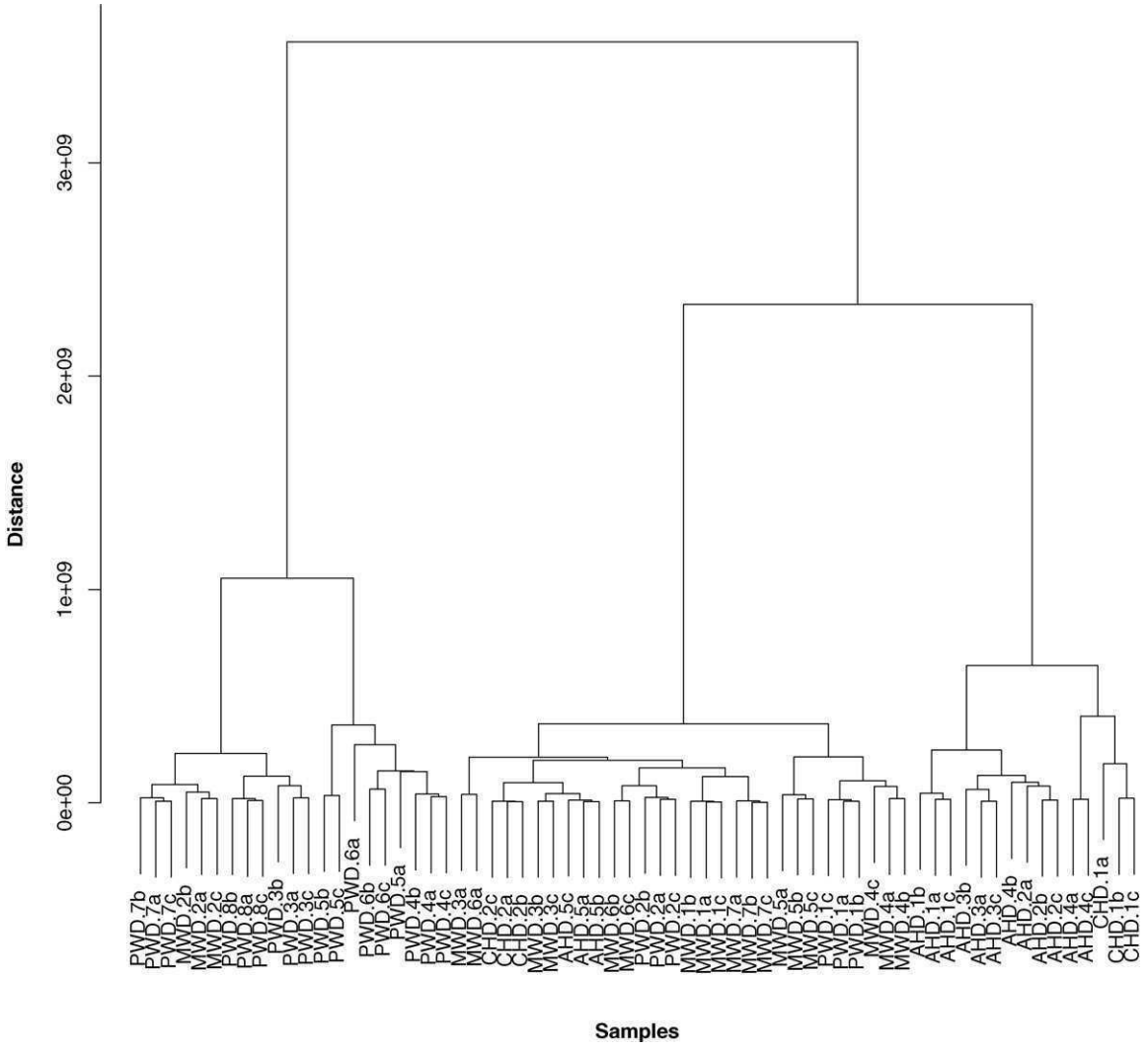

Fig. 4. Hierarchical clustering considering selected volatile components with loading score >0.1 with resultant matrix (66*7). Data clusters 1: PWD, Data clusters 2: AHD, CHD, MWD, PWD, Data clusters 3: AHD.

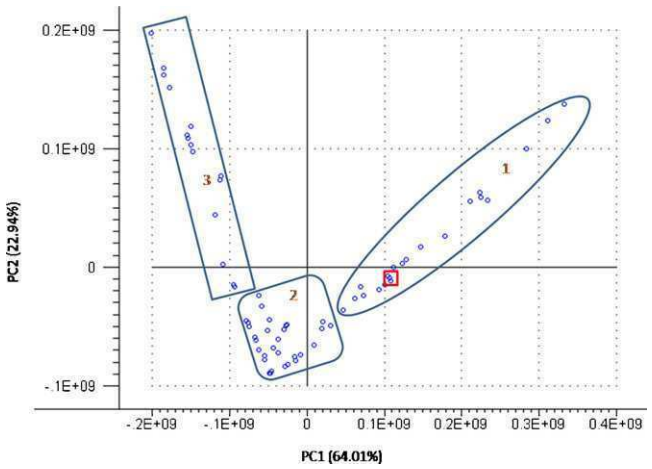

Fig. 5. PCA of selected volatile components with loading score >0.1 with resultant matrix (66*7). Data clusters 1: PWD, Data clusters 2: AHD, CHD, MWD, PWD, Data clusters 3: AHD.

three or at least conserved in triplicates of a single sample was used for hierarchical clustering to verify analytical aspects (sample homogeneity and component distribution) in addition to other mentioned purposes. Concerning homogeneity and component distribution 17 of the 22 samples showed good clustering among replicates. This resulted in two super-clusters with 2 sub clusters each (Supplementary Fig. S1). Replicates of individual house dust samples from distant geographical origins clustered along with the samples from municipal waste handling units forming one of the four major clusters consisting of 11 elements (Supplementary Fig. S1) with the exception one sample MWD-2. Four of the five Finnish house dust samples from moisture damaged houses (AHD 1–5) were equidistant and clustered together and were different to other groups in their volatile pattern. Contrary to anticipation samples from paper recycling units (exception of PWD 1–2) split into two sub clusters of four and three elements each, representing occurrence of probable intrinsic differences. This minor separation could be explained by the different waste handling methods and its influence on aerosols or deposited particles and ultimately leading to formation of dust with compositional irregularities. One or more sample replicates clustering sparsely or distant samples merging into a close knit cluster may not be amenable for an easy explanation (example Fig. 5, clustering of MWD-2 along with PWD samples highlighted with red square). Nevertheless clustering among the majority of sample replicates is good evidence for validity and applicability of our method and instrumental set up for indoor evaluation studies. The recommended procedure for PCA for differentiation of samples is to analyse all variables at the same time. But in cases where numbers of variable are higher than the number of cases this may not be feasible. The observation of loading scores from the matrix 66*1993 suggests that the majority of variables had typically low in the magnitude of <0.1 indicating insignificant influence on separation. Hence a cut off loading score of 0.1 was fixed for variable selection. The resulting new matrix (66*7) comprising both volatiles and reported microbial volatiles (two) accounted for 86.95% of the total variance in the data on PCA. The first principal component (PC1) explained

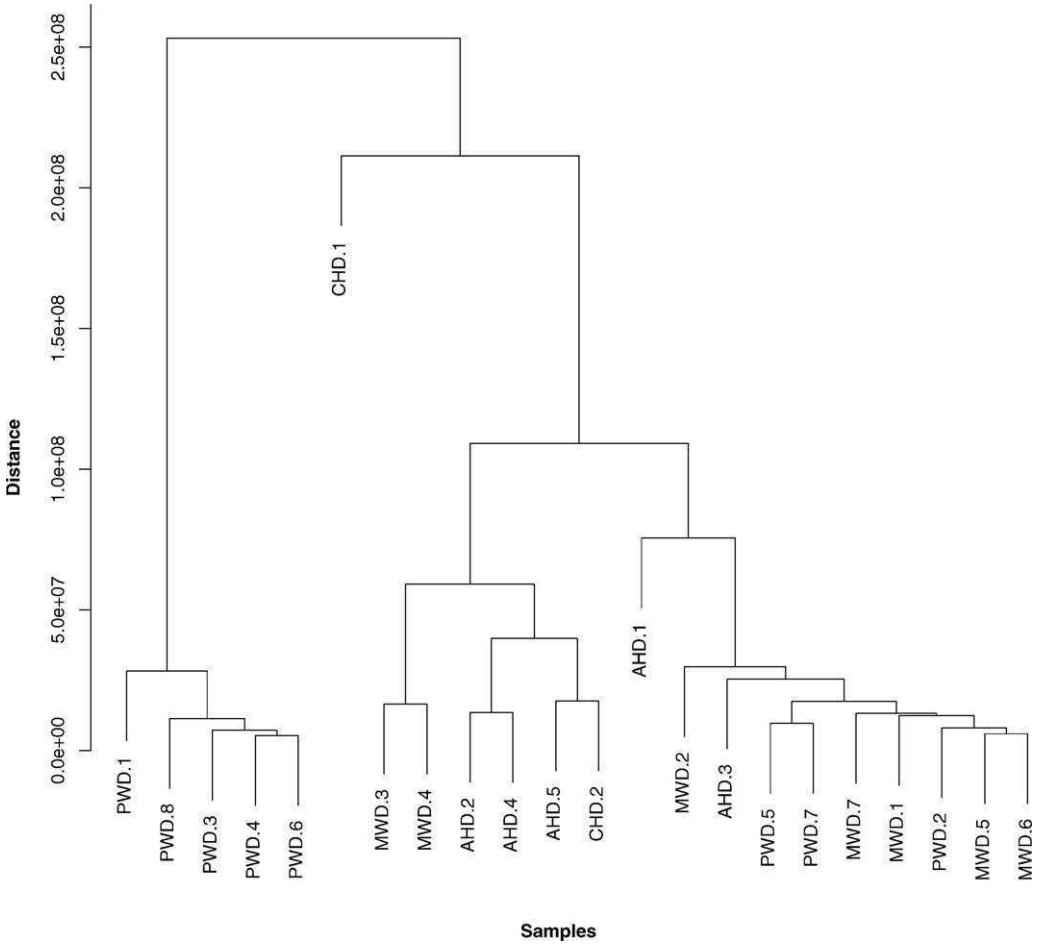

Fig. 6. Hierarchical clustering considering literature reported microbial volatile components with resultant matrix (22*18). Data cluster: 1: CHD1, Data cluster: 2: AHD, MWD, PWD, Data cluster: 3: AHD, MWD, CHD, Data cluster: 4: PWD.

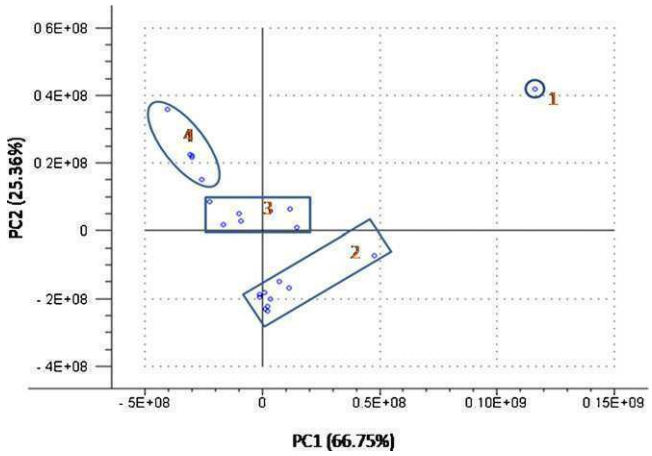

Fig. 7. PCA of selected microbial volatile components with loading score >0.1 with resultant matrix (22*18). Data cluster: 1: CHD1, Data cluster: 2: AHD, MWD, PWD, Data cluster: 3: AHD, MWD, CHD, Data cluster: 4: PWD.

64.01% of the variance separating samples and second principal component (PC2) with 22.94% of variance. The PC1 identified as linear combination of 2,6-diisopropylnaphtalene and 1-tetradecanol. The PC2 was mainly characterized by variables diethyl phthalate and minor influence by 1-butyl 2-isobutyl phthalate and Toluene. Scattered plot corresponding to this discrimination is illustrated in Fig. 5.The conclusion that can be drawn from the illustrated classification study is that 71% of affected house dust samples and 75% of waste management units dealing with paper were clearly separable on first principal component. Considering samples from a distinct cluster on negative PC1 and PC2 it can be concluded that overlap in volatile and semi volatile profiles are consistent with the statistical reports of the Parliament office of science and technology, England (statistics discussed at later part) for household contribution for municipal waste formation. Hierarchical clustering of the same matrix resulted in two major clusters each having two minor clusters (Figs. 4 and 5). Comparative evaluation of dendrograms showed grouping in Fig. 4 is similar to Supplementary Fig. S1, thus confirming the independent influence of chosen variables in separation for the group of samples. In parallel averaged replicates peak areas of identified MVOCs that are well documented in the scientific literature were considered for PCA. This was done to verify their probable role in separation of samples and differentiation of enclosures (22*18). The PCA of MVOCs indicated the extraction of two principal components representing total variance of 92.10% of the data set. PC1 represented 66.75% of variance and was strongly characterized by toluene, PC2 on the other hand accounted for 25.36% of variance due to nonanal. Due to ambiguity concerning the origin of toluene this compound may not be an ideal volatile marker for separation of samples and sources, based on microbial volatile profile. In this view nonanal underlying PC2 and explaining a variance of 25.36% could be an important microbial marker for separation of samples in the indoor environments. HCA of the same matrix (22*18) resulted in three super-clusters. Extreme right cluster (Fig. 6), where samples belonging to paper recycling units can be seen grouped together (PWD 1, 3, 4, 6, 8). This may explain similar purposes of indoor or occurrence of common abundant cellulose matrix promoting climate favorable for particular set microbes, in addition to influencing their physiology and volatile pattern. Similarly another main cluster consisting of house dust samples (AHD 2, 4, 5 and CHD 2) cluster at similar height which could be best reasoned as a consequence of comparable thermal and humidity comfort pattern practiced in homes leading to comparable/general micro climates in these indoor environments (Figs. 6 and 7).

4.2. Evidence for natural occurrence of microbial secondary metabolites in settled dust

The analysis of non volatile/secondary microbial metabolites in settled dust matrix was done using a validated method described earlier [7]. In cases of samples from waste management units containing paper and other matrices with absorptive consistency a larger sample to solvent ratio of 1:8 or 1:12 was used to ensure complete submersion of samples and optimum extraction. Dust samples were source of 38 different microbial metabolites to variable quantities. The concentrations of the investigated toxins in the positive samples are listed in Table 3. Their relative standard deviation between replicates was generally below 20% (e.g. 50% of samples), which we consider to be a reasonable value in view of the heterogeneity of the matrix (thus confirming the accuracy of the method). The microbial metabolite spectrum detected included following microbial taxa, *Penicillum* ($n=12$), *Aspergillus* ($n=5$), *Fusarium* ($n=7$), *Beauveria* ($n=1$),*Trichoderma* ($n=1$), *Claviceps* ($n=1$), *Alternaria* ($n=2$), *Stachybotrys* ($n=1$), *Metarrhizium* ($n=1$), *Chaetomium* ($n=1$) and Bacterial ($n=6$) [55]. A few of the metabolites could be attributed to more than one genus of indoor fungi and bacteria.

Samples from municipal and paper recycling waste management units were similar in terms of microbe-metabolite pattern and quantities. A post note released by the Parliament office of science and technology, England (http://www.parliament.uk/documents/post/postpn252.pdf) provides some insight concerning organic content of municipal waste and offers an additional indirect explanation for high prevalence of saprophytic/parasitic microbes in dust samples procured from municipal waste dealing units. The above mentioned report states that, the bulk of municipal waste generation is contributed by the households and consists of biodegradable material (41%, kitchen, garden, soil), biodegradable & recyclable material (18%, paper, cardboard), recyclable waste (17%, glass, plastic, metal) and other materials (20%, wood, non-combustibles, textiles). Municipal waste with complex organic content thus could be a potential matrix nurturing diverse set of fungi and bacteria. Each group of enclosures was unique having set of metabolites not found in the other sampling sites. The samples of WMUs dealing with municipal waste were distinct from samples of WMUs dealing with paper by the presence of griseofulvin, dechlorogriseofulvin, chlamydeosporal, malformin C, myriocin, patulin and puromycin. Presence of griseofulvin and its halogenated derivative, dechlorogirsofulvin in one and the same municipal waste management sample is confirmation for mutual occurrence addition to proving the greater relevance of our chosen indoor target metabolites in the course of method expansion [56,57]. Patulin, a mycotoxin on decomposing apples and a metabolite of species of *Pencillium* and *Aspergillus* is not frequently found in indoor dust makes its presence interesting. Presence of malformin C could indicate occurrence of the indoor mold. *Aspergillus niger* [58] known for causing skin diseases and ear infection [59]. Similarly samples from paper recycling units were different to those of municipal waste management units in their microbe-metabolite pattern due to the presence of deoxybrevinamide E and cytochalsin D. Cytochalasin D is a metabolite produced by filamentous saprophobic ascomycetes of the genus *Chaetomium* found in soil, air and plant debris [60]. Several species of *Chaetomium* are common in indoor environments such as *C. elatum*, *C. globosum*, *C. murorum* [61]. Similarities in the metabolite patterns of the two waste handling units might be a result of similarities in the fungal spectra present in such work places due to similar micro-climatic conditions, substrates and the hygiene principles applied.

As apprehended settled dust samples from inhabited houses showed lower metabolite diversity ($n=18$) compared to other groups. Metabolites enniatin B2 and alternariol were exclusively

Table 3
Non volatile/secondary microbial metabolites detected in settled floor dust samples from Groups A, B, C and D. Quantification values presented are average of replicates.

Metabolite	Precursor ion	Municipal waste (Gp A) management (μg/kg)	Household paper (Gp B) recycling (μg/kg)	Settled dust from (Gp C) house (μg/kg)	Settled dust from (Gp D) house (μg/kg)
3-Methylviridicatin	281.07$[M+H]^+$	5.8–10.0	8.5–9.0	–	–
Alamethicin F30	775.5 $[y7^d+H]^+$	4.0–35.0	14.0–15.0	–	–
Alternariol	257.0 $[M-H]^-$	–	–	39.7	34.6–41.0
AME	271.1 $[M-H]^-$	11.2–42.0	17.3–37.0	8.0	7.3–10.0
Apicidin	622.4 $[M-H]^-$	1.1–1.4	0.5–0.8	–	–
Beauvericin	801.5 $[M+NH_4]^+$806.5 $[M+Na]^+$	1.6–22.0	0.3–10.4	3.1	0.7–1.8
Chaetoglobosin A	695.0 $[M-H]^-$	193.0–258.0	83.0–242.0	–	–
Chanoclavine	257.1$[M+H]^+$	1.9–2.8	0.6–5.9	–	–
Chlamydosporol	245.2$[M+H]^+$	16.0–59.0	–	–	–
Chloramphenicol	320.9 $[M-H]^-$	39.5–108.3	2.7–22.6	–	3.6–4.6
Cyclopenin	295.1$[M+H]^+$	32.0–188.0	26.0–303.0	–	–
Cyclopeptine	281.07$[M+H]^+$	7.8–50.0	3.0–46.0		–
Cycloaspeptide A	642.3 $[M+H]^+$	9.6–29.0	10.8–11.7	155.4	–
Cytochalasin D	609.3 $[M+2H]^{2+}$	–	51.0–221.0	–	57.1
Dechlorogriseofulvin	319.1$[M+H]^+$	230.5	–	13.0	–
Deoxybrevianamid E	352.2$[M+H]^+$	–	221.3	–	–
Emodin	269.0 $[M-H]^-$	61.0–314.0	26.0–88.0	15.15	4.0–117.0
Enniatin A	699.4 $[M+NH_4]^+$	7.4–17.0	3.0–17.0	–	0.7–223.0
Enniatin A1	685.4 $[M+NH_4]^+$	2.0–30.0	1.3–14	–	1.4–185.0
Enniatin B	657.5 $[M+NH_4]^+$	2.2–23.0	0.2–8.1	–	0.9–10.3
Enniatin B1	671.4 $[M+NH_4]^+$	4.0–49.0	1.3–19.3	–	–
Enniatin B2	643.5 $[M+NH_4]^+$	–	–	1.9	0.6–2.0
Equisetin	372.2 $[M-H]^-$	21.2–422.4	12.5–185.3	–	19.0–20.0
Fumigaclavine	299.3 $[M+H]^+$	13.0–86.0	10.0–23.0	–	–
Griseofulvin	353.2 $[M+H]^+$	87.0–1598.0	–	210.0	–
Malformin C	530.3$[M+H]^+$	8.0–78.0	–	–	–
Meleagrin	434.3 $[M+H]^+$	14.0–52.0	29.0–67.0	–	–
Monactin	768.8 $[M+NH_4]^+$	4.0–101.0	0.4–2.3	–	0.65
Myriocin	402.4 $[M+H]^+$	52.0–1941.0	–	–	–
Nonactin	754.6 $[M+NH_4]^+$	0.2–50.0	0.5–1.2	0.8	0.2–0.3
Patulin	152.9 $[M-H]^-$	49144.0	–	–	–
Pentoxyfylline	279.2 $[M+H]^+$	11.0–186.0	2.0–101.0	–	–
Physcion	283.0 $[M-H]^-$	409.0–1034.0	231.0–1565.0	–	–
Puromycin	472.4 $[M+H]^+$	40.0–126.0	–	–	–
Roquefortine C	390.2 $[M+H]^+$	83.0–176.4	18.0–350.0	–	–
Stachybotrylactam	386.3 $[M+H]^+$	52.6–104.5	87.0–160.0	–	–
Sterigmatocystine	325.1 $[M+H]^+$	3.0–45.3	6.0–32.0	–	1.6–11.0
Valinomycin	1128.8 $[M+NH_4]^+$	0.05–8.0	0.3–2.0	0.4	0.04–0.6
Viridicatin	238.1 $[M+H]^+$	110.0–920.0	108.0–369.0	–	–

detected in settled floor dust (SFD) samples of control and houses with severe moisture damage (Table 3) along with some other *Fusarium* metabolites. However control and damaged houses had certain metabolite signature pattern. The control houses differed by the presence of metabolites griseofulvin, dechlorogriseofulvin and cyclosapeptide A. The moisture damaged houses were positive for the presence of alternariol monomethyl ether (AME) and monactin which are metabolites of tertiary colonizers *Alternaria alternata* and *Actinomycetes* [14] indicating for water damage. The presence of bacterial metabolites produced by *Streptomyces* and related genera in the damaged houses clearly links to conditions of excess moisture damage and dampness indoors, as this groups of bacteria has been described earlier as being indicative for such indoor conditions [62].

5. Conclusion

The detection and quantification of wider range of metabolites in concentration range of 0.04–49,144.0 μg/kg are evidences for the competency of our developed multi target LC–MS/MS method for the purpose. Occurrence of broader array of metabolites (primary, secondary and tertiary colonizing microbes) in samples of waste handling units (municipal waste (>50%) and paper recycling units (>33%)) is affirmation for microbial succession. Metabolites of *S. chartarum,* which is well known to be indoor specific fungi was found in waste management units in high concentrations (52–160 μg/kg). In the absence of moisture monitoring data of evaluated environments, origin (indoor or outdoor) of stachybotrylactam remains to be an open question. The presence of emodin, griseofulvin and dechlorogriseofulvin along with metabolites of tertiary colonizer are not unexpected findings, considering processes involved in waste handling (collection, transportation, storage) during which moisture accumulation is an obvious possibility. To the best of our knowledge 30 of the 71 identified volatiles are new report in the indoor context, particularly in settled floor dust matrix. Though as many as 20 previously reported MVOCs were found in our study, a direct correlation to secondary metabolite profiles could not be possible due to the known ambiguity in MVOC and the non-availability of authentic environmental control samples and volatile profiles corresponding to them. The applications of AMDIS and Spectconnect for volatile profiling are advantageous identifying major indoor pollutants clouded amidst extremely high number of variables/components. The wide range biogenic and anthropogenic pollutants in dust qualify it as an indicator matrix of indoor status, hence can be valuable for evaluation purposes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.07.043.

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Supplementary Table **(1); List of annotated volatile organic compounds found in settled floor dusts from Groups A, B , C and D, * Retention index(RI) values determined by us with pure reference standards. Listed in the order of precursor ion mass.**

CAS.No	Name	m/z(precursor ion)	RT-HP5 (min)	RI-HP5-Literature	RI-HP5-Experimental
64-19-7	Acetic acid	60.05	6.02	600.0	643.0
3102-33-8	3-Penten-2-one, (E)-	84.11	8.34	735.0	743.9
498-60-2	3-Furaldehyde	96.08	11.16	815.0	838.2
98-01-1	2-Furaldehyde	96.08	11.18	836.0	838.6
98-00-0	2-Furanmethanol	98.09	12.01	853.0	863.9
80-62-6	2-Methyl-2-propenoic acid methyl ester	100.11	7.50	732.0	714.1
100-52-7	Benzaldehyde	106.12	15.51	961.0	970.3
18829-55-5	2-Heptenal, (E)-	112.16	15.22	956.0	961.5
142-62-1	Hexanoic acid	116.15	17.61	981.0	1034.3
111-70-6	1-Heptanol	116.20	15.59	969.0	972.7
122-78-1	Benzeneacetaldehyde	120.14	18.14	1042.0	1050.8
529-20-4	2-methyl- Benzaldehyde	120.14	19.45	1067.0	1091.0
98-86-2	Acetophenone	120.14	18.85	1066.0	1072.5
65-85-0	Benzoic acid	122.12	22.49	1214.4*	1192.7
60-12-8	Benzyl Carbinol	122.16	20.32	1120.3*	1119.4
91-20-3	Naphthalene	128.17	22.62	1183.0	1197.1
111-13-7	2-Octanone	128.21	16.24	992.0	992.3
646-14-0	1-nitro- hexane	131.17	18.04	1046.5	1047.8
122-00-9	p-Acetyltoluene	134.17	22.50	1189.0	1193.1
585-74-0	1-(3-methylphenyl)- ethanone	134.17	22.50	1176.0	1193.1
488-23-3	1,2,3,4-tetramethyl- Benzene	134.21	20.46	1150.0	1124.3
95-93-2	1,2,4,5-tetramethyl- benzene	134.21	20.42	1131.0	1122.8
272-16-2	1,2-Benzisothiazole	135.18	23.81	1221.0	1239.8
95-16-9	Benzothiazole	135.18	23.81	1228.0	1239.9
5989-27-5	D-Limonene	136.23	17.61	1027.0	1034.3
122-99-6	2-Phenoxyethanol	138.16	23.43	1245.3	1226.1
5910-87-2	2,4-Nonadienal, (E,E)-	138.20	23.26	1219.0	1220.1
6750-03-4	2,4-Nonadienal	138.20	23.26	1219.0	1220.1
60784-31-8	2-Nonenal, (Z)-	140.22	21.59	1147.0	1162.3
91-57-6	Naphthalene, 2-methyl-	142.19	25.76	1290.5	1310.6
124-07-2	Octanoic Acid	144.21	21.95	1177.3*	1174.5
149-57-5	2-ethyl- Hexanoic acid	144.21	20.13	1116.7	1113.0
143-08-8	Nonan-1-ol	144.25	21.85	1173.4*	1171.3
106-46-7	p-Dichlorobenzene	147.00	17.24	1013.0	1023.0
541-73-1	1,3-dichloro- Benzene	147.00	17.24	1005.0	1022.9
104-46-1	Anethole	148.20	25.31	1289.3	1293.7
1197-01-9	p-Cymen-α-ol	150.21	22.28	1182.0	1185.8
2363-88-4	2,4-Decadienal	152.23	25.45	1317.0	1298.7
92-52-4	Phenylbenzene	154.20	27.94	1377.3	1393.4
2497-25-8	2-Decenal, (Z)-	154.24	24.53	1250.0	1265.5
571-61-9	1,5-Dimethylnaphthalene	156.22	29.00	1439.8	1435.4
575-37-1	1,7-dimethyl- Naphthalene	156.22	29.00	1418.7	1435.4
575-43-9	1,6-dimethyl- naphthalene	156.22	29.00	1419.6	1435.3
581-42-0	2,6-dimethyl- naphthalene	156.22	28.69	1401.0	1423.1
582-16-1	2,7-dimethyl- naphthalene	156.22	28.69	1402.2	1423.1
112-32-3	n-Octyl formate	158.23	20.56	1117.0	1127.7
112-30-1	1-Decanol	158.28	24.74	1272.0	1273.2
643-58-3	2-Methylbiphenyl	168.23	28.33	1402.3	1408.7
643-93-6	3-Methylbiphenyl	168.23	30.58	1487.8	1499.0
90-43-7	o-Xenol(fungicide)	170.20	31.35	1506.0	1531.6
112-12-9	2-Undecanone	170.29	25.30	1295.1*	1293.3
112-42-5	1-Undecanol	172.30	27.90	1370.0	1391.8
120-12-7	Anthracene	178.22	37.56	1786.4	1811.8
136-60-7	n-Butyl benzoate	178.22	27.63	1377.0	1381.4
85-01-8	Phenanthrene	178.22	37.56	1780.0	1811.7
78-40-0	Triethyl phosphate	182.15	20.32	1137.2	1119.5
119-61-9	Benzophenone	182.21	34.02	1621.0	1647.3
3796-70-1	trans-Geranylacetone	194.31	29.36	1454.6	1450.1
295-17-0	Cyclotetradecane	196.37	34.74	1673.0	1679.7
30021-74-0	1-Isopropyl-7-methyl-4-methylene-1,2,3,4,4a,5,6,8a-octahydronaphthalene	204.35	30.82	1477.0	1509.1
13360-61-7	1-Pentadecene	210.39	30.41	1492.5	1492.2
24157-81-1	2,6-Diisopropylnaphthalene	212.33	35.91	1728.0	1733.7
124-25-4	Tetradecanal	212.37	33.32	1611.0	1616.1
112-72-1	1-Tetradecanol	214.38	34.73	1672.0	1679.5
84-66-2	Diethyl phthalate	222.23	32.80	1597.0	1593.4
295-65-8	Cyclohexadecane	224.42	38.99	1883.0	1882.8
544-63-8	Tetradecanoic acid	228.37	36.62	1780.0	1767.2
84-15-1	o-Terphenyl	230.30	39.37	1903.0	1901.3
64437-47-4	9-hexadecenol (E)	240.42	39.00	1868.0	1883.3
18435-45-5	1-Nonadecene	266.50	39.01	1892.0	1883.5

Supplementary Table **(1)** continued

CAS.No	Name	m/z (precursor ion)	RT-Optima® Wax (min)	RI-Optima® Wax-Literature	RI-Optima® Wax-Experimental
17851-53-5	1-Butyl 2-isobutyl phthalate	278.34	38.66	1900.0	1866.3
117-82-8	2-Methoxyethyl phthalate	282.28	40.52	1965.0	1961.0
638-36-8	2,6,10,14-tetramethyl- Hexadecane	282.54	37.45	1810.7	1806.6
4170-30-3	Crotonaldehyde	70.08	9.95	1408.0	1046.4
123-72-8	Butanal	72.10	6.28	877.0	877.0
534-22-5	2-methyl- Furan	82.10	6.14	871.0	867.3
1003-29-8	α-Pyrrolaldehyde	95.09	33.91	2044.0	2055.5
142-83-6	2,4-Hexadienal, (E,E)-	96.12	20.01	1423.6*	1423.1
3658-80-8	Dimethyl trisulfide	126.26	18.81	1378.0	1376.8
629-33-4	n-Hexyl formate	130.18	18.61	1382.0	1369.0
104-61-0	γ-Nonalactone	156.22	34.19	2028.0	2069.1
112-34-5	O-Butyl diethylene glycol	162.22	29.36	1796.0	1829.0
1454-84-8	n-Nonadecanol-1	284.52	43.28	2637.0	2607.8

Supplementary Table **(2); Fiber selection for SPME :All samples used for the purpose are naturally contaminated real world samples•**

	CAR.PDMS		Polyacrylate		PDMS		PDMS.DVB		PDMS.DVB.CAR	
Samples	**Conserved**	**Identified**	**Conserved**	**Identified**	**Conserved**	**Identified**	**Conserved**	**Identified**	**Conserved**	**Identified**
MWD-3	329	94	112	32	244	46	322	76	329	71
MWD-6	415	86	131	39	255	59	372	77	443	80
PWD-2	323	89	126	36	247	50	334	75	356	71
AHD-1	248	65	81	25	173	37	233	45	221	49
AHD-2	303	72	98	29	210	41	284	54	318	58
CHD-1	396	51	110	24	303	26	425	35	426	33

Supplementary Table **(3); HS-SPME: The values shown above are averages of triplicates. Spectconnect (conserved components) and AMDIS (Identification).**

AHD-1 sample extraction and chromatography with CAR.PDMS and apolar HP5 column

Sample amount (grams)			Extraction/incubation temperature			Extraction/incubation time		
	Spectconnect	**AMDIS**		**Spectconnect**	**AMDIS**		**Spectconnect**	**AMDIS**
0.025	132	34	**30'60'30°C**	40	6	**0'30'90°C**	145	39
0.05	248	65	**30'60'60°C**	76	19	**0'60'90°C**	170	44
0.1	171	49	**30'60'90°C**	248	65	**30'30'90°C**	152	45
						30'60'90°C	248	65

Establishment and application of a metabolomics workflow for identification and profiling of volatiles from leaves of *Vitis vinifera* by HS-SPME-GC-MS

Georg Weingart[a], Bernhard Kluger[a], Astrid Forneck[b], Rudolf Krska[a], Rainer Schuhmacher[a]*

[a]Center for Analytical Chemistry, Department IFA-Tulln, University of Natural Resources and Life Sciences, Vienna, Konrad-Lorenz-Str. 20; 3430 Tulln, Austria

[b]Division of Viticulture and Pomology, Department of Crop Sciences, University of Natural Resources and Life Sciences, Vienna, Peter-Jordan-Str. 82, 1190 Vienna, Austria

* Corresponding author: rainer.schuhmacher@boku.ac.at

Abstract

Introduction - Volatile organic compounds (VOCs) occurring in leaves of plants carry information about the physiological state of the plant. Monitoring of VOCs assists in detecting plant stress before visible.

Objective - To establish and apply a simple workflow for the automated extraction, measurement and annotation/identification of *Vitis vinifera* cv. Pinot Noir leaf metabolites.

Methodology - Leaf samples were harvested, cooled with liquid nitrogen and homogenised under cooled conditions. VOCs were extracted and enriched by solid phase microextraction (SPME) and analysed by GC-MS. Samples were measured on two columns with different polarity of stationary phases. Mass spectral deconvolution and identification was done by AMDIS software. Strict identification criteria were applied: match factor ≥90; relative retention index deviation ≤2% from reference value on both columns. Data of two sampling dates were analysed with multivariate statistics.

Results - We found ~600 components in a single chromatogram. Applying the mentioned criteria resulted in annotation of 63 metabolites of which 47 were confirmed with authentic standards. For the majority of the compounds technical variability was <40% (RSD), biological variability among plants was 7 – 119%. Principal component analysis (PCA) scores plot of leaf samples from two different sampling dates showed two clearly separated clusters. The presented workflow enabled for the first time the detection and identification of 19 metabolites, which have so far not been described for *Vitis* spp.

Conclusion – The developed workflow enabled the identification of grapevine leaf metabolites, which allowed the separation of leaves from two sampling dates by PCA.

Introduction

Volatile organic compounds (VOCs) are emitted by nearly all plant organs including leaves, flowers or roots. Typical substance classes of VOCs are alkanes, alkenes, aldehydes, ketones, aromatic compounds and terpenes (including modified terpenes, terpenoides). All of them have been detected in grapevine leaves. The emission of volatiles by plants enables these sessile organisms to send signals over relatively

long distances. Moreover, VOCs are well known to be involved in defence related processes (e.g. Schulze et al., 2006; Choudhary et al., 2008) and can be used for signalling within the plant or to attract e.g. predators of an attacking aphid/insect. Hence, the synthesis of primary and secondary metabolites changes in response to biotic (e.g. Maes and Debergh, 2003; Batovska et al., 2008, 2009a) and abiotic stress (e.g. Ormeño et al., 2007; Vickers et al., 2009). Changes of secondary metabolites as a reaction to abiotic stress is not yet fully understood (Loreto and Schnitzler, 2010) but can serve as markers for stress, which often cannot be noticed visually. Detecting relevant volatile profiles and novel volatiles indicating plant stress requires unbiased and comprehensive approaches including stringent controls to a treated group.

An analytical concept, which has proven to have a high potential for the comprehensive determination of volatile metabolites is metabolomics or, if restricted to a number of predefined compounds, metabolite profiling (Fiehn, 2002). It aims to detect, identify and quantify (at least semi quantitatively or relatively) as many metabolites as possible within one single analysis. Recently, some studies using a metabolomics approach investigating wine (e.g. Son et al., 2009) and grapevine leaves (Figueiredo et al., 2008) have been published which used NMR, a well suited technique for fingerprint approaches. However, it is less suited for profiling of low level metabolites which often occur in plant samples (Hall, 2006). An adequate technique for this purpose is gas chromatography – mass spectrometry (GC-MS). It enables metabolite identification due to the high separation power of GC in combination with the large mass spectra libraries available. The number of false positive identifications can be reduced by the use of retention time indices (RIs) which are also available through databases.

Different sample preparation techniques for the determination of VOCs produced by plants are available: liquid extraction with organic solvents, often followed by a solid phase extraction (SPE) cleanup or concentration or derivatisation step or a combination of all (Dunn and Ellis, 2005; Kim and Verpoorte, 2010). Wildenradt et al. (1975) and Hebash et al. (1991) for example, investigated volatiles of grapevine leaves by steam distillation extraction followed by GC–MS and found 32 and 27 metabolites, respectively. Wirth et al. (2001) described 73 substances for leaves and berries after liquid extraction followed by enzymatic hydrolysis and GC-MS. Most substances were identified by comparison of mass spectra and RI of authentic standards. Batovska et al. (2008, 2009a,b) analysed liquid extracts of grapevine leaves resulting in more than 125 detected metabolites.

In contrast to these time consuming and hardly automatable sample extraction techniques solid phase microextraction (SPME, Arthur and Pawliszyn, 1990) offers minimal sample treatment and the possibility to fully automate the extraction and measurement process. Additionally, the analytes are enriched on the fibre during the extraction process so that very low concentrations can be detected. SPME has been successfully employed to extract volatiles from wine (e.g. Rebière et al. 2010, Robinson et al. 2011) and from grapevine leaves. E.g. Tasin et al. (2005) identified 24 volatiles in leaves whereas Cha et al. (2008) investigated grape shoot volatiles and identified 11 volatiles.

A key step in comprehensive metabolite profiling is data analysis. To handle large amounts of data and to receive as much information from the samples as possible, efficient software tools for peak picking, peak deconvolution, mass spectra comparison and retention index calculation are necessary (Dunn and Ellis, 2005; Boccard et al., 2010). For this purpose, various platform independent software tools are available, e.g. AMDIS (Automated Mass Spectral Deconvolution and Identification System, Stein, 1999), Tagfinder (Luedemann et al., 2008) or MetaboliteDetector (Hiller et al., 2009).

The second step of data processing is the detection of differentially expressed metabolites. This can be done with uni- or multivariate statistics like for example, simple t-test or principal component analysis (PCA).

The objectives of this study were (i) to create a workflow for the annotation of metabolites based on mass spectra and RIs, (ii) to apply the workflow to Pinot Noir leaf samples, (iii) to confirm the identity of the annotated substances with authentic standards, (iv) to check the technical and biological variability of the developed method with field samples and (v) apply multivariate statistics. The resulting workflow should provide extended knowledge of the grapevine leaf metabolome and function as a basis to detect differentially expressed volatile metabolites indicating for example stress in grapevine plants in future studies.

Experimental

Chemicals and standards. All Standards listed in Table 2, the alkane calibrants C8-C20 (40 mg/L each in hexane, Fluka) and C21-C40 (40 mg/L each in toluene, Fluka) were purchased from Sigma-Aldrich (Vienna, Austria). A C5-C10 alkane calibrant was prepared using the pure substances in a ratio resulting in narrow and symmetric peak shapes (pentane 99% Sigma-Aldrich, hexane SupraSolv Merck, heptane 99.5% J.T.Baker, octane 99% Sigma-Aldrich, nonane 99% Sigma-Aldrich, decane p.a. Promochem). Methanol (LiChrosolv) was from Merck (Darmstadt, Germany), acetonitrile (HPLC gradient grade) from VWR (Vienna, Austria) and MilliQ-water from an in-house equipment (Millipore, Molsheim, France).

Standard preparation and RI determination. From the original pure standard compounds (liquids and solids) individual stock solutions with a concentration of 100 mg/L in acetonitrile were prepared and stored at 4 °C. Standards and dilutions were always handled with gastight Hamilton syringes.

The standards from stock solutions were combined in 4 mixtures resulting at a concentration of 100 µg/L in MilliQ-water (mix 1, 2, 3) or acetonitrile (mix 4) for each component (see Table 1 for details). For GC-MS measurement, 20 µL of a standard mix were transferred to a 20 mL HS-vial. Measurements were done within several weeks on both columns (see GC-MS section) at least 4 times. Some standards that could not be detected in the mixes were measured individually at a higher concentration (20 µL of 100 mg/L stock) in duplicate. The RI was based on a series of n-alkanes (C5–C40) and automatically calculated by AMDIS software (see below) using the formula of Van den Dool and Kratz (1963), calculating the linear temperature programmed retention time index (LTPRI).

Plant material. Grapevine leaves of healthy *Vitis vinifera* cv. Pinot Noir clone 18 Gm, (3 years old in 2009, vertical shoot positioning- (VSP) trellis) were sampled from the field in an experimental vineyard (Jedlersdorf, Vienna, Austria). Vineyard management was done to provide optimum plant growth including fertilisation, plant protection treatments, irrigation and canopy management according to standards in commercial viticulture of the region (Schoedl et al., unpublished data).

From each of nine plants, four leaves of the outer layer were sampled: two leaves from two shoots, opposite the primary cluster (insertion level 3) and the leaf below (insertion level 2). Leaves were sampled at two dates between 10 and 12 am on June, 3rd and July, 30th in 2009 and inspected visually before sampling and only intact and healthy leaves were sampled. A control sample for determining technical variability and to check instrument performance was obtained by pooling four leaves of one grapevine plant. All other leaves (n = 32) were measured individually.

Sample preparation. The leaves were cooled with liquid nitrogen immediately after harvest. In the lab they were subsequently ground using a ball mill (MM301 Retsch, Haan, Germany) and pre-cooled (liquid nitrogen) 10 mL stainless steel-beakers (Retsch) with a 9 mm stainless steel ball (Retsch) for 3 min at 30 Hz resulting to a fine powder. Leaf powder was transferred into plastic tubes and stored at -80 °C until analysis. 20 mL headspace (HS) vials (Supelco, Gerstel, Mühlheim a.d. Ruhr, Germany) were rinsed with MeOH/H_2O (50/50 v/v) and vials, caps and septa (1.3 mm silicone/PTFE, Supelco, Gerstel) were baked out in an oven for at least 60 min at 120 °C before usage. 105±5 mg of homogenized leaf sample were weighed in and the vials were tightly sealed with screw caps.

HS-SPME. For the extraction of volatiles from leaf samples and standards we used a 2 cm 50/30 µm CAR/DVB/PDMS fibre (Supelco, Gerstel). The fibre was conditioned before first use at 270 °C for 1 hour according to the supplier's recommendation. Sample extraction was done with an autosampler (MPS2XL, Gerstel, Mühlheim a.d. Ruhr, Germany) equipped with a coolable sample tray holder, a needle heater and an agitator/stirrer (all parts from Gerstel) for heating the vials. Gerstel Maestro software was used for autosampler control. The fibre was beaked out in the needle heater for 10 min at 270 °C before each extraction. The HS vial was placed in the cooled tray (10 °C) of the autosampler. After 30 min of equilibration in the agitator at 90 °C the fibre was inserted into the HS of the vial for 60 min at 90 °C and the volatiles were extracted and enriched on the fibre. After extraction, the SPME needle was removed

from the HS vial and inserted into the GC-MS inlet, where the analytes were desorbed for 2 min in splitless mode at 250 °C.

Optimization of SPME parameters was carried out in triplicate using bulked grapevine leaf powder obtained after homogenisation (see above). Equilibration times of 0, 10, 20 and 30 min were tested as well as extraction times of 20, 40 and 60 min with the aim to maximise the overall peak area of the total ion current (TIC) chromatogram determined manually (from beginning of the first peak to the end of the last peak) by Agilent MSD ChemStation (G1701EA E.02.00.493). Additionally the data was automatically processed by AMDIS program for evaluation of the number of derived components.

For alkane RI-calibrants different SPME conditions were necessary to achieve narrow peak shapes. C5-C10: 1 µL in 20 mL HS vial, sampling out of tray (10 °C) for 0.01 min, C8-C20: 10 µL in 20 mL HS vial, extraction for 10 min at 90 °C, C21-C40: 30 min equilibration, 60 min extraction both at 120 °C.

GC-MS of extracted leaf samples and VOC standards. For separation and detection of the volatiles an Agilent 6890N coupled to a 5975B MSD detector (Agilent, Waldbronn, Germany) was used. The apolar column was a DB-5MS (95% dimethyl-diphenyl polysiloxane, Agilent J&W), length 30 m, inner diameter 0.25 mm and film thickness 0.25 µm. The polar column was an Optima-WAX (100% polyethylene glycol, Machery-Nagel, Düren, Germany) with identical dimensions. To facilitate column exchange we used a restriction capillary connected to the column with SilTite ferrules (Agilent). Inlet septa were prepierced low bleed septa (Agilent), extra pierced with an old SPME needle before first use. The liner was a 1.5 mm HS-liner (Gerstel) and inlet temperature was 250 °C. Oven program started with 35 °C (hold 2 min), increase of 5 °C/min to 260 °C (hold 5 min) and the transfer line was set at 270 °C. The carrier gas was helium 5.0 (Messer, Gumpoldskirchen, Austria) with a flow rate of 1 mL/min (constant flow mode).

The MS was operated in electron ionisation mode (EI 70 eV); source temperature: 230 °C; quadrupole temperature: 150 °C; scan range: m/z 35 – 500; scan speed: 3 scans/s; tuning: weekly with PFTBA.

Data evaluation and annotation of metabolites. For peak picking, RI calculation, determination of a number of components in a sample and substance annotation we used AMDIS software (version 2.65, www.amdis.net, Stein, 1999). The default settings for deconvolution were: component width: 12; adjacent peak subtraction: one; resolution, sensitivity, shape requirements: medium; level: infinite; maximum penalty and “no RI in library”: 100.

For substance annotation deconvoluted mass spectra were compared to the Wiley/NIST 08 MS library (McLafferty, 2008) using the “Search NIST library” function of AMDIS which uses the NIST MS Search software (version 2.0, delivered with Wiley/NIST spectra library).

Reference RI values were obtained from NIST Chemistry WebBook (NCWB, Stein, 2010, http://webbook.nist.gov/chemistry/). In case of multiple literature RI values for an individual substance, the median of the reported values (corresponding to the same column diameter and film thickness as well as a comparable stationary phase material) was used for comparision (Stoppacher et al., 2010).

Evaluation of method performance

Trueness of RI values. RI trueness was determined by comparing the arithmetic mean values (n = 2-11) of the experimentally measured RIs of the standard substances with the respective median values of NCWB entries. The relative bias in percent was calculated.

Formation of artefacts. One mixture containing four alcohols (1-hexanol, 1-tridecanol, benzenemethanol, E-geraniol) and one containing four aldehydes (E-2-hexenal, benzaldehyde, hexanal, tridecanal) were measured with the same SPME-GC-MS method as the leaf samples. The chromatograms were evaluated by AMDIS software with the aim to detect the substances added to the mixture and their potential oxidation products (hexanal, tridecanal, benzaldehyde, E-geranial and hexenoic acid, benzoic acid, hexanoic acid, tridecanoic acid). The same identification criteria as described above were applied.

Technical and biological variability. Peak areas of the annotated and identified metabolites were derived by AMDIS software. The setting for minimum match factor was 60 and the RI deviation was ±5 RI units from the value obtained from the annotation/identification step. Technical variability was determined for the annotated/identified substances by calculating the relative standard deviation (RSD) of AMDIS derived peak areas obtained from the control samples (pooled leaf sample). The control samples were analysed at the beginning of a measuring sequence and after every fifth sample. This resulted in eight replicate measurements.

Biological variability (including the technical variability) within the plants was calculated as the root mean square (RMS) of RSD of metabolite peak areas obtained for each of eight grapevine plants (four leaves per plant). The average variability of a substance between the plants was calculated as the RSD of the arithmetic means of peak areas derived from the leaves of each plant.

Statistics

Multivariate statistics (PCA) was applied to differentiate between the two sampling dates using R software (2.13.0). Only substances detected in at least 50% of the leaf samples of (at least) one sampling date were taken into account. Peak areas derived by AMDIS were scaled and mean centred prior to PCA using the 'scale' function of R.

Results and Discussion

Different strategies have been applied in so far published plant metabolomics studies searching for differentially expressed peaks (biomarkers). This is often done via a so-called top down approach (e.g. Jonsson et al., 2004). Thereby the differentially expressed peaks are searched first and then only these peaks are considered for further identification of the corresponding metabolite. This is a very elegant way and avoids the identification of many compounds without biological significance but requires appropriate software, which often works as a black box.

The presented study uses a bottom up metabolomics approach (e.g. Kanani et al., 2008). First step is the identification of *Vitis* leaf metabolites followed by multivariate statistics applied to the data obtained from two sampling dates to detect differentially expressed metabolites. Together with sampling and measurement, the developed workflow is composed of four steps: sampling and sample pre-treatment, measurement, metabolite annotation/identification and data processing/statistics for detection of differentially expressed metabolites (Fig. 1). We did not further evaluate the sampling and sample preparation steps as the procedure is already described in literature to be sufficient for efficient quenching of metabolism (e.g. Fiehn, 2002; Álvarez-Sánchez et al., 2010). Freeze-drying of samples was omitted to avoid the loss of volatiles (e.g. Aprea et al., 2011). Our study aimed to develop a highly automatable workflow for the detection of a high number of volatile leaf metabolites as well as minimum sample manipulation to avoid the generation of artefacts. Therefore, we decided to use SPME, which is a well-established extraction and enrichment technique (Wang et al., 2008).

HS-SPME Optimisation

All SPME experiments were carried out using a Gerstel MPS2XL autosampler in standard configuration in combination with Gerstel Maestro software for programming instrument sequence parameters. In a pre-study (data not shown) we tested 5 fibre coatings (PA, PDMS, CAR/PDMS, DVB/PDMS, CAR/DVB/PDMS) by using a mix of 10 standards (3-methyl-1-butanol, hexanal, 6-methyl-5-hepten-2-one, limonene, E-geranial, E-geraniol, nonanoic acid, beta-caryophyllene, nerolidol, 6,10,14-trimethyl-2-pentadecanone). The PA fibre showed lowest abundance for the substances followed by the PDMS fibre. Between the three fibres with mixed coating there was no obvious difference in terms of abundance and repeatability of GC-MS peak intensities so we decided to use the CAR/DVB/PDMS fibre which is also recommended by the supplier to extract the widest range of substances. As we aimed to maximise the number of detected substances in a single run our goal was to explore conditions resulting in high peak

abundances and high peak numbers. The number of peaks was determined by AMDIS, which was used to evaluate the number of components. The overall peak area derived by manual integration (Agilent ChemStation) functioned as parameter for overall abundance of all peaks in the chromatogram.

Higher extraction temperature lead to an increase in the number of volatiles in the headspace above the leaf sample. Thus, we chose a temperature of 90 °C, which is slightly below the boiling point of water and therefore minimises the risk of damage of the vials and the agitator. The test of different extraction times (20, 40, 60 min) showed increasing overall peak area ($1.82x10^7$ at 20 min up to $2.74x10^7$ at 60 min, mean of three replicates, 30 min equilibration time) and greater number of components (414 at 20 min up to 523 at 60 min, mean of three replicates, 30 min equilibration time) with increasing extraction time. The increase of equilibration time from 0 to 30 min only resulted in a small increase in both total peak area and number of components (461 components without equilibration, Fig. 2). Nevertheless, we kept the longer equilibration time of 30 min to ensure that we obtain as many substances as possible and to standardise conditions at the beginning of extraction. This resulted in an overall measuring duration of 90 min per leaf, which corresponds to a measurement capacity of 16 samples per day.

GC-MS of extracted leaf samples and annotation of metabolites

A preliminary study (data not shown) showed that a decreasing number of components was detected with increasing rate of temperature gradient by the use of the AMDIS programme. Therefore, we chose a flat ramp of 5 °C/min. Additionally, this kind of GC settings is typically described in literature for the separation of a large number of analytes (e.g. Fiehn et al., 2000; Broeckling et al., 2005; Batovska et al., 2008). When measuring leaf samples the subsequent annotation of metabolites from all AMDIS deconvoluted spectra resulted in an average number of 150 hits per chromatogram. The results of all files from both columns were combined and only substances found on both columns were kept which led to a list of 313 substances (including multiple hits, Fig. 1). The RIs for those substances were searched within NCWB and compared with the experimentally determined RIs. For 113 substances we found a RI for both types of columns. A maximum relative deviation of measured RI of ±2% from literature value was accepted reducing the list of annotated metabolites to 71. Among them were seven alkanes, which were also part of the alkane RI calibrant, which was measured within the same sequence. Therefore only a small deviation of the RI of maximum 5 RI units was accepted which was only met by one alkane, nonadecane. Two of the annotated substances are known as plasticizers, and therefore were removed from the list resulting in 63 annotated metabolites.

Confirmation of annotated metabolites

For these 63 metabolites, 47 authentic standards were available at our lab. To experimentally determine the RI of the standards they were measured in four different mixtures (mix 1 – 4) with the same method as the leaf samples at least in duplicate. The mean of the RI obtained by the replicate measurements was compared with the RI of the annotated metabolites. The choice of appropriate identification criteria, i.e. mass spectral match factor and RI deviation from a given reference value, are crucial for the correct assignment/annotation of metabolites. Different studies use various settings for the identification (e.g. Schauer et al., 2005; Stoppacher et al., 2010) but no standardised rules are available at the date. Again, we accepted a maximum deviation of the RI of the annotated metabolite from the reference RI value of ±2% which led to the falsification of two and the confirmation of 45 annotated metabolites (Table 1).

Some of the metabolites for which spectral match factor was ≥ 90 but which were rejected because their RI deviated more than ±2% from literature RI value, were available as pure standard compounds. In additional measurements their RI was also determined. Thus, the identity of two substances could be confirmed and they were added to the result list (Table 1) of finally 63 metabolites, of which 47 were confirmed with authentic standards. Table 1 lists the metabolites found with this workflow in Pinot Noir clone 18 Gm leaf samples. They cover the substance classes of terpenes, alcohols, aldehydes, ketones and aromatic compounds. To the best of our knowledge 19 of them have never been reported to occur in *Vitis* plants so far. Of the remaining 44 metabolites, 10 were detected in *Vitis* leaves for the first time. The references for the above mentioned 44 substances which are already described in the literature to occur in *Vitis* plants (leaves, flowers, roots, berries) are given exemplarily in Table 1 (for more information see supplementary material). Hexanal, (2E)-hexenal, 1-hexanol and (3Z)-hexen-1-ol acetate belong to the group of green leaf odour compounds and were also reported for example by Tasin et al. (2005), Loughrin

et al. (1997) and Wirth et al. (2001). From the group of terpenes and terpenoids several have been detected in other studies, e.g. E-geraniol, 4-ethenyl-2-methoxy-phenol and eugenol by Wirth et al. (2001), beta-limonene, beta-ionone and methyl salicylate by Tasin et al. (2005), beta-myrcene, beta-cyclocitral, E-citral and allo-ocimene by Rocha et al. (2007). Batovska et al. (2008, 2009a,b) also found nonadecane, hexahydrofarnesylacetone, phytol, dihydroactinoline, (2E)-undecenal, nonanal and decanal in grapevine leaves. From the substance class of alcohols 2-ethyl-1-hexanol, 1-octanol and 1-nonanol are reported for example by Canuti et al. (2009), benzenemethanol and benzeneethanol by Wirth et al. (2001). The annotated/identified volatile metabolites include several substance classes, which have been described to be directly associated to biotic or abiotic plant stress. Isoprenoids for example have been described to have antioxidant properties (Vickers et al., 2009). Further, Ormeño et al. (2007) report a change in the mono- and sesquiterpene emission due to water stress. Increased emission of green leaf volatiles (GLV, mainly C6-compounds, saturated or monounsaturated aldehydes, alcohols, esters, e.g. hexanol) is also a symptom of plant stress (Holopainen, 2004). Thus, the presented method appears to be well suited to investigate metabolic response to different types of biotic and abiotic plant stress in the future.

Evaluation of method performance

Determination of RI from standards, RI trueness

Table 2 shows the RIs derived from the authentic standards compared with NCWB entries. For all standards measured on the DB-5 column, the measured RIs were within ±1.7% of the corresponding NCWB median, implying satisfying trueness. The measurements with the polar column resulted in a maximum relative deviation from NCWB of 3.1%. This is in accordance with the findings of d' Acampora Zellner et al. (2008), who reported the accuracy of RIs on polar columns to be worse compared to apolar columns. Nevertheless, we used the RI value of our standards for deciding whether a metabolite is included in the results list or not. Isidorov and Szczepaniak (2009) suggested the ageing of columns to be an explanation for the broad range found for some RIs reported in NCWB. Regarding our own replicate measurements, we observed a difference in the obtained RI between the beginning and the end of the measurements (some 100 injections in between) of max. 16 RI values for early eluting compounds (2-ethyl-furan) on DB-5 column. For the majority of the compounds the difference was less than five RI units. Strehmel et al. (2008) reported similar observations. On the PEG column, the RI showed a similar behaviour. These findings support the acceptance of a deviation of ±2% of the experimentally found RI from the reference value even when using the reference RI from a standard. The observed standard deviation (SD) of RIs within a measuring sequence was maximum 1.2 RI units. Comparing the RI or retention time of a particular substance within a sequence can serve as a technical control for the correct peak detection of the data processing software AMDIS.

Formation of Artefacts

To check whether the SPME conditions lead to the production of artefacts we measured two standard mixes (one with alcohols, one with aldehydes) with the same SPME conditions used for the leaf samples. For the alcohols some of the oxidation products (aldehydes) were detected in small amounts (< 5% of the peak area of the corresponding alcohol). Oxidation products of the aldehydes could not be observed. This indicates that oxidation products have to be rejected from the result list if both, the oxidation product and its educt are present in a similar ratio as observed for the standards. Additionally, artefacts could origin from the column, the fibre, the septa or anything else that was in contact with the sample. Therefore, all substances containing silicium were excluded from the results list as well as all substances reported to be used as plasticizers. We identified for example diisobutylphthalate and 2,4-bis(1,1-dimethylethyl)-phenol which are also reported in literature as plant metabolites (e.g. Du et al., 2009; Roy et al., 2010). Due to the ubiquity of these substances, it is possible that they are really found in plant samples. We also found them after measurement of empty vials without plant material and therefore excluded them from the resulting leaf metabolites list.

Technical and biological variability

It is our intention to use the presented method in the future with the aim to detect differentially expressed metabolites of differentially treated samples. Therefore, knowledge is required on the extent of the

variability of both, the analytical method and the plants in the field. For these measurements a sublibrary (leaf-lib) containing only mass spectra and RI of the annotated and identified substances present in leaf samples (Table 1) was created. Hence, AMDIS was used to process all raw data files in a batch job. Now the RI criterion was set more stringent (maximum 5 RI values deviation) but a less strict mass spectral match factor (≥ 60) was applied to include low abundant substances or substances where the deconvolution process did not lead to a complete elimination of interfering mass signals. Table 3 shows the technical variability which was in the range of 13-86% (RSD). The majority of metabolites (35) showed a RSD < 40%. Other studies which deal with SPME and metabolomics found lower levels for the analytical variability, for example Aprea et al. (2011) who report a CV% of < 30% for the majority of investigated substances and Tikunov et al. (2005) who report an analytical variation (%SD) of 3-23% (16 substances, n = 4). Stashenko et al. (2004) report a variation up to 83% RSD (n = 5). Using a standard mixture (10 substances) for quality control in another study, we achieved also a lower technical variability of 7-58% (RSD, n = 17, data not shown). Table 3 also shows the biological variability (including the technical variability) within the plants and between the plants. The within plant variability, given as root mean square (RMS), ranged from 4-30% for 19 substances, 31-50% for 23 and 51-106% for 18 substances. The variability between the plants was 7-30% for 32 substances 31-50% for 21 and 51-119% for 8 substances. Tikunov et al. (2005) report a biological variability of 8-35% within one tomato genotype for 16 substances. Another study dealing with open field samples (Ossipov et al., 2008) reported a biological variation of 10-50% for the majority of the metabolites but for a few ranging up to 140%. Our findings are in agreement with the results reported in these studies.

Literature study and compilation of an in-house *Vitis* metabolites database (vitis-db)

For the leaf metabolites listed in Table 1, literature references for their occurrence in *Vitis* were searched. This literature study resulted in a database (vitis-db) which lists the substance name as used in the article, the CAS-number if available (source SciFinder Scholar 2007 or NCWB), the part of the plant where it was detected (leaf, flower, root, berry) and the corresponding reference. At present, the database contains 1619 entries referring to *Vitis* volatiles from 39 studies and is available as supplementary material.

Statistics

The last step of the workflow provides the differentially expressed metabolites by means of uni- or multivariate statistics. 61 substances, occurring in more than 50% of the leaves of (at least) one sampling date, were used for PCA (Fig. 3). Each of the points in the scores-plot represents a single leaf sample. There are two clusters, which correspond to the two sampling dates. The first principal component (PC1) explains 50% of the variance in the data. The loadings-plot shows the contribution of each substance to the variance in the data. In the present study, we did not investigate this further. Our aim here was to demonstrate that the established workflow leads to annotation/identification of metabolites, which are suited to separate leaves originating from different sample dates.

To summarise, this study presents a comprehensive metabolomics workflow including steps for sampling, sample preparation, measurement, metabolite annotation/identification and statistical data analysis. We intended to use only established devices and open source software to provide the opportunity for a broad application of the method. SPME parameters were optimised to be able to detect a large number of metabolites and to achieve sufficient method precision. Additionally, the presented study provides extended knowledge of the grapevine leaf metabolome by describing 63 grapevine leaf metabolites whereof 19 are reported for the first time in plants of *Vitis* spp. Moreover, multivariate statistics (PCA) were applied and showed two clearly separated clusters for leaves from two different sampling dates. The workflow will be applied in further studies for the detection of differentially expressed metabolites indicating for example stress in grapevine plants. It can also easily be applied to leaves of various plant species or other plant tissues.

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Table 1. Substances found in *Vitis vinifera* cv. Pinot Noir (clone 18 Gm) leaves sorted by ascending RI on DB-5 column.

Number	CAS-number[a]	Name (trivial name in parenthesis)	Described in *Vitis* by (e.g.)[b]	Measured[c] RI DB-5	Reference[d] RI	% dev. from ref.	Measured[c] RI WAX	Reference[d] RI	% dev. from ref.
1	616251	1-Penten-3-ol	-	710	708	-0.3	1172	1164	-0.7
2	3208160	Furan, 2-ethyl-	-	718	714	-0.6	952	948	-0.4
3	1576870	2-Pentenal, (2E)-	-	764	763	-0.1	1138	1141	0.3
4	1576961	2-Penten-1-ol, (2E)-	-	778	775	-0.4	1329	1328	-0.1
5	66251	Hexanal	Tasin	801	805	0.5	1087	1087	0.0
6	98011	2-Furancarboxaldehyde (2-Furfural)	Lamorte	838	837	-0.1	1473	1479	0.4
7	6728263	2-Hexenal, (2E)-	Tasin	860	858	-0.2	1226	1229	0.2
8	111273	1-Hexanol	Wirth	872	873	0.1	1362	1370	0.6
9	111717	Heptanal	Hebash	904	904	0.0	1183	1186	0.3
10	142836	2,4-Hexadienal, (2E,4E)-	Tasin	914	916	0.2	1407	1424	1.2
11	100527	Benzaldehyde	Tasin	965	967	0.2	1540	1546	0.4
12	110930	5-Hepten-2-one, 6-methyl-	Buchbauer	989	990	0.1	1340	1349	0.7
13	123353	1,6-Octadiene, 7-methyl-3-methylene- (β-Myrcene)	Rocha	992	993	0.1	1143	1135	-0.7
14	3777693	Furan, 2-pentyl-	-	993	994	0.1	1215	1223	0.7
15	124130	Octanal	Hebash	1005	1005	0.0	1286	1294	0.6
16	3681718	3-Hexen-1-ol, 1-acetate, (3Z)-	Tasin	1008	1007[N]	-0.1	1313	1321[N]	0.6
17	111900	Ethanol, 2-(2-ethoxyethoxy)-	-	1012	1001[N]	-1.1	1655	1628[N]	-1.7
18	4313035	2,4-Heptadienal, (2E,4E)-	-	1013	1015	0.2	1510	1516	0.4
19	99865	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- (alpha-Terpinen)	-	1019	1021	0.2	1143	1160	1.5
20	38514135	1-Pentanol, 3-ethyl-4-methyl-	-	1023	1020[N]	-0.3	1521	1507[N, e]	-0.9
21	104767	1-Hexanol, 2-ethyl-	Lamorte	1031	1031	0.0	1496	1504	0.5
22	5989548	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (4S)- (beta-Limonene)	Tasin	1033	1032	-0.1	1183	1191	0.7
23	2408379	Cyclohexanone, 2,2,6-trimethyl-	Kalua 2010	1038	1039	0.1	1325	1333	0.6
24	100516	Benzenemethanol (Benzyl Alcohol)	Tasin	1043	1042	-0.1	1907	1905	-0.1
25	122781	Benzeneacetaldehyde (Phenylacetaldehyde)	Hernandez	1047	1044[N]	-0.3	1666	1648[N]	-1.1
26	2548870	2-Octenal, (2E)-	Yang	1060	1062	0.2	1439	1450	0.8
27	111875	1-Octanol	Hebash	1071	1073	0.2	1569	1575	0.4
28	529204	Benzaldehyde, 2-methyl-	-	1073	1073	0.0	1647	1646	-0.1
29	78706	1,6-Octadien-3-ol, 3,7-dimethyl- (beta-Linalool)	Tasin	1100	1102	0.2	1554	1560	0.4
30	124196	Nonanal	Tasin	1104	1106	0.2	1393	1403	0.7

Table 1. continued

Number	CAS-number[a]	Name (trivial name in parenthesis)	Described in Vitis by (e.g.)[b]	Measured[c] RI DB-5	Reference[d] RI	% dev. from ref.	Measured[c] RI WAX	Referenced RI	% dev. from ref.
31	60128	Benzeneethanol (2-Phenylethanol)	Wirth	1118	1120	0.2	1946	1944	-0.1
32	118718	4H-Pyran-4-one, 3-hydroxy-2-methyl- (Maltol)	-	1119	1121	0.2	2015	2011	-0.2
33	3016191	2,4,6-Octatriene, 2,6-dimethyl-, (2E,6E)- ((E)-Allo-ocimene)	Rocha	1129	1130[N]	0.1	1367	1367[N]	0.0
34	4748781	Benzaldehyde, 4-ethyl-	-	1167	1182[N]	1.3	1735	1714[N]	-1.2
35	143088	1-Nonanol	Fan	1170	1173	0.3	1674	1678	0.2
36	124072	Octanoic acid	Fan	1175	1177	0.2	2105	2117	0.6
37	585740	Ethanone, 1-(3-methylphenyl)- (m-Methylacetophenone)	-	1193	1176[N]	-1.5	1814	1804[N, e]	-0.6
38	112312	Decanal	Cha	1203	1208	0.4	1501	1510	0.6
39	119368	Benzoic acid, 2-hydroxy-, methyl ester (Methyl salicylate)	Tasin	1203	1202	-0.1	1805	1805	0.0
40	432257	1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl- (beta-Cyclocitral)	Rocha	1224	1229	0.4	1646	1650	0.2
41	67470	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	Batovska08	1232	1224[N]	-0.7	2486	2513[N]	1.1
42	106241	2,6-Octadien-1-ol, 3,7-dimethyl-, (E)- (E-Geraniol)	Tasin	1259	1258	-0.1	1869	1869	0.0
43	141275	2,6-Octadienal, 3,7-dimethyl-, (2E)- (E-Geranial)	Rocha	1273	1275	0.2	1757	1758	0.1
44	7786610	Phenol, 4-ethenyl-2-methoxy- (4-Vinylguaiacol)	Wirth	1319	1322	0.2	2214	2221	0.3
45	97530	Phenol, 2-methoxy-4-(2-propen-1-yl)- (Eugenol)	Wirth	1360	1365	0.4	2186	2195	0.4
46	53448070	2-Undecenal, (2E)-	Batovska09a	1367	1367	0.0	1775	1776	0.1
47	121335	Benzaldehyde, 4-hydroxy-3-methoxy- (Vanillin)	Wirth	1410	1400[N]	-0.7	2542	2577[N]	1.4
48	3796701	5,9-Undecadien-2-one, 6,10-dimethyl-, (5E)- (E-Geranyl acetone)	Buchbauer	1452	1456	0.3	1875	1876	0.1
49	79776	3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, (3E)- (trans-beta-Ionone)	Tasin	1488	1495	0.5	1977	1976	-0.1
50	10486198	Tridecanal	-	1506	1513	0.5	1833	1834	0.1
51	15356748	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl- (Dihydroactinolide)	Batovska08	1542	1537[N]	-0.3	2384	2372[N]	-0.5
52	143077	Dodecanoic acid (Lauric acid)	Batovska08	1558	1568[N]	0.6	2478	2495[N]	0.7
53	112709	1-Tridecanol	-	1574	1577	0.2	2088	2086	-0.1
54	124254	Tetradecanal	-	1609	1611[N]	0.1	1940	1927[N]	-0.7
55	544638	Tetradecanoic acid (Myristic acid)	Batovska08	1760	1771[N]	0.6	2648	2694[N]	1.7
56	629801	Hexadecanal	-	1816	1817[N]	0.1	2148	2135[N]	-0.6
57	502692	2-Pentadecanone, 6,10,14-trimethyl- (Hexahydrofarnesylacetone)	Batovska08	1844	1846	0.1	2136	2140	0.2
58	629925	Nonadecane	Batovska09a	1899	1900	0.1	1897	1900	0.2
59	1117528	5,9,13-Pentadecatrien-2-one, 6,10,14-trimethyl-, (5E,9E)- (E-Farnesylaceton)	-	1924	1917[N]	-0.4	2360	2385[N, e]	1.1

Table 1. continued

Number	CAS-number[a]	Name (trivial name in parenthesis)	Described in Vitis by (e.g.)b	Measured[c] RI DB-5	Reference[d] RI	% dev. from ref.	Measured[c] RI WAX	Referenced RI	% dev. from ref.
60	112390	Hexadecanoic acid, methyl ester	Buchbauer	1928	1927	-0.1	2217	2230	0.6
61	505328	1-Hexadecen-3-ol, 3,7,11,15-tetramethyl- (Isophytol)	-	1948	1950	0.1	2276	2297	0.9
62	57103	Hexadecanoic acid	Batovska08	1962	1972[N]	0.5	2858	2909[N]	1.8
63	150867	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, (2E,7R,11R)-(E-Phytol)	Batovska08	2111	2106	-0.2	2548	2589	1.6

[a] source: SciFinder Scholar 2007
[b] (Hebash et al., 1991; Buchbauer et al., 1994; Wirth et al., 2001; Tasin et al., 2005; Rocha et al., 2007; Batovska et al., 2008, 2009a; Cha et al., 2008; Hernández-Orte et al., 2008; Lamorte et al., 2008; Yang et al., 2009; Kalua and Boss, 2010; Fan et al., 2010)
[c] refers to RI in leaf samples
[d] refers to RI from authentic standard, except those indicated with "N" which refer to RI values from NIST Chemistry WebBook
[e] column dimensions for this RI value do not exactly correspond to the one used in this study
[N] reference RI value was taken from NIST Chemistry WebBook

Table 2. Retention indices of standard substances measured on DB-5 and Optima-WAX column compared to corresponding median RIs of NIST Chemistry WebBook. Substances sorted by increasing RI on DB-5.

CAS-number[a]	Standard brand, minimum purity	Mix	Name (trivial name in parenthesis)	Measured[b] RI Std	RI in NCWB (median)	% dev. from NCWB	Measured RI Std	RI in NCWB (median)	% dev. from NCWB
				DB-5	5% phenyl		PEG	polar	
616251	A 99%	2	1-Penten-3-ol	708	704	0.6	1164	1158	0.5
3208160	SAFC 99%	4	Furan, 2-ethyl-	714	702	1.7	948	955	-0.7
1576870	SAFC 95%	1	2-Pentenal, (2E)-	763	754	1.2	1141	1143	-0.2
1576961	A 95%	2	2-Penten-1-ol, (2E)-	775	769	0.8	1328	1306	1.7
66251	Fluka 97%	4	Hexanal	805	802	0.4	1087	1085	0.2
98011	A 98%	3	2-Furancarboxaldehyde (2-Furfural)	837	832	0.6	1479	1465	1.0
6728263	A 98%	3	2-Hexenal, (2E)-	858	854	0.5	1229	1212	1.4
111273	SA 98%	2	1-Hexanol	873	867	0.7	1370	1355	1.1
111717	A 95%	3	Heptanal	904	900	0.4	1186	1186	0.0
142836	A 95%	3	2,4-Hexadienal, (2E,4E)-	916	911	0.6	1424	1408	1.1
100527	SA 99%	3	Benzaldehyde	967	961	0.6	1546	1523	1.5
110930	SAFC 98%	4	5-Hepten-2-one, 6-methyl-	990	985	0.5	1349	1324	1.9
123353	Fluka 95%	4	1,6-Octadiene, 7-methyl-3-methylene- (β-Myrcene)	993	991	0.2	1135	1157	-1.9
3777693	SAFC 97%	4	Furan, 2-pentyl-	994	993	0.1	1223	1236	-1.1

Table 2. continued

124130	SAFC 92%	3	Octanal	1005	1002	0.3	1294	1279	1.2
4313035	A 90%	3	2,4-Heptadienal, (2E,4E)-	1015	1009	0.6	1516	1491	1.7
99865	A 95%	4	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- (alpha-Terpinen)	1021	1017	0.4	1160	1165	-0.4
104767	Fluka 99%	2	1-Hexanol, 2-ethyl-	1031	1029	0.2	1504	1484	1.4
5989548	A 98%	4	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (4S)- (beta-Limonene)	1032	1030	0.2	1191	1195	-0.3
2408379	SAFC 98%	4	Cyclohexanone, 2,2,6-trimethyl-	1039	1029	1.0	1333	1312	1.6
100516	SA 99%	2	Benzenemethanol; (Benzyl Alcohol)	1042	1034	0.8	1905	1870	1.9
2548870	A 94%	1	2-Octenal, (2E)-	1062	1058	0.4	1450	1430	1.4
111875	Su >99%	2	1-Octanol	1073	1070	0.3	1575	1553	1.4
529204	Fluka 98%	3	Benzaldehyde, 2-methyl-	1073	1067	0.6	1646	1644	0.1
78706	A 97%	2	1,6-Octadien-3-ol, 3,7-dimethyl-; (beta-Linalool)	1102	1099	0.3	1560	1544	1.0
124196	Aldrich 95%	3	Nonanal	1106	1103	0.3	1403	1390	0.9
60128	Fluka 99%	2	Benzeneethanol (2-Phenylethanol)	1120	1114	0.5	1944	1899	2.4
118718	A 99%	4	4H-Pyran-4-one, 3-hydroxy-2-methyl-; (Maltol)	1121	1111	0.9	2011	1969	2.1
143088	SAFC 98%	2	1-Nonanol	1173	1172	0.1	1678	1658	1.2
124072	RdH 99%	4	Octanoic acid	1177	1187	-0.8	2117	2055	2.9
119368	Fluka 99%	4	Benzoic acid, 2-hydroxy-, methyl ester (Methyl salicylate)	1202	1192	0.8	1805	1754	2.9
112312	Sigma 98%	3	Decanal	1208	1205	0.3	1510	1485	1.7
432257	SAFC 90%	1	1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl- (beta-Cyclocitral)	1229	1218	0.9	1650	1601	3.1
106241	SAFC 97%	2	2,6-Octadien-1-ol, 3,7-dimethyl-, (E)- (E-Geraniol)	1258	1255	0.2	1869	1838	1.7
141275	Fluka 95%	3	2,6-Octadienal, 3,7-dimethyl-, (2E)- (E-Geranial)	1275	1270	0.4	1758	1714	2.6
7786610	SAFC 98%	1	Phenol, 4-ethenyl-2-methoxy- (4-Vinylguaiacol)	1322	1315	0.5	2221	2181	1.8
97530	Fluka 99%	2	Phenol, 2-methoxy-4-(2-propen-1-yl)- (Eugenol)	1365	1357	0.6	2195	2155	1.9
53448070	SAFC 90%	1	2-Undecenal, (2E)-	1367	1365	0.2	1776	1734	2.4
3796701	SAFC 97%	1	5,9-Undecadien-2-one, 6,10-dimethyl-, (5E)- (E-Geranyl acetone)	1456	1454	0.1	1876	1859	0.9
79776	Fluka 95%	1	3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, (3E)- (trans-beta-Ionone)	1495	1485	0.7	1976	1936	2.1
10486198	A 90%	3	Tridecanal	1513	1511	0.1	1834	1812	1.2
112709	A 97%	2	1-Tridecanol	1577	1575	0.1	2086	2076	0.5
502692	SA	1	2-Pentadecanone, 6,10,14-trimethyl- (Hexahydrofarnesylacetone)	1846	1846	0.0	2140	2118	1.0
112390	Fluka 99%	1	Hexadecanoic acid, methyl ester	1927	1926	0.1	2230	2218	0.5
505328	A 97%	1	1-Hexadecen-3-ol, 3,7,11,15-tetramethyl- (Isophytol)	1950	1949	0.4	2297	2282	0.7
150867	SAFC 97%	1	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, (2E,7R,11R)- (E-Phytol)	2106	2114	-0.4	2589	2588	0.0

[a] source: SciFinder Scholar 2007 [b] refers to RI from authentic standard A Aldrich, SA Sigma-Aldrich, RdH Riedel de Haën, Su Supelco

Table 3. Technical and biological variability of grapevine leaf samples obtained from measurements within the same sequence. Technical variability is given as arithmetic mean and % RSD of peak areas [counts x s] of metabolites detected in ≥ 50% (n = 5-8) of control samples are shown. Biological (including the technical) variability was calculated as root mean square (% RMS) of eight plants (four leaves each) and shows the variability within the plants in the vineyard. The last column reports the variability between the plants.

Variability	**Technical**		**Biological**	
			within	between
	control samples		plants	
Metabolite	Area	RSD	RMS	RSD
1-Penten-3-ol	$1.97x10^3$	68.9	30.0	22.3
Furan, 2-ethyl-	$6.01x10^3$	53.5	37.8	37.1
2-Pentenal, (2E)-	$8.62x10^3$	36.9	23.8	14.8
2-Penten-1-ol, (2E)-	$5.56x10^3$	55.6	80.6	32.8
Hexanal	$5.54x10^4$	40.1	23.8	17.2
2-Furancarboxaldehyde	$1.55x10^4$	85.9	69.4	36.1
2-Hexenal, (2E)-	$1.26x10^6$	28.6	15.7	8.5
1-Hexanol	$3.40x10^3$	44.6	83.6	33.8
Heptanal	$1.26x10^4$	34.7	56.6	47.5
2,4-Hexadienal, (2E,4E)-	$1.64x10^5$	39.3	20.1	11.0
Benzaldehyde	$7.07x10^4$	24.9	21.5	10.2
5-Hepten-2-one, 6-methyl-	$8.94x10^3$	24.8	39.8	27.2
beta-Myrcene	$3.01x10^4$	41.2	45.3	52.6
Furan, 2-pentyl	$1.86x10^3$	27.8	30.1	9.9
Octanal	-	-	44.5	39.5
3-Hexen-1-ol, acetate, (3Z)-	$1.61x10^3$	50.8	93.7	114.5
Ethanol, 2-(2-ethoxyethoxy)-	-	-	-	-
2,4-Heptadienal, (2E,4E)-	$6.45x10^4$	29.5	19.7	14.7
alpha-Terpinene	$1.06x10^3$	34.6	36.5	39.0
1-Pentanol, 3-ethyl-4-methyl-	$4.22x10^3$	51.5	56.4	59.3
1-Hexanol, 2-ethyl-	$3.30x10^3$	32.2	74.0	65.7
beta-Limonene	$4.16x10^3$	55.0	52.8	35.7
Cyclohexanone, 2,2,6-trimethyl-	-	-	33.0	59.6
Benzyl alcohol	$2.08x10^4$	77.0	70.0	40.5
Benzeneacetaldehyde	$7.05x10^4$	29.7	39.7	32.3
2-Octenal, (2E)-	$3.68x10^3$	29.7	31.8	16.4
1-Octanol	$2.37x10^4$	31.5	46.3	29.5
Benzaldehyde, 2-methyl-	$2.50x10^3$	45.5	29.0	21.6
beta-Linalool	$4.65x10^4$	25.9	32.3	41.6
Nonanal	$2.16x10^5$	31.4	51.0	43.3
Benzeneethanol	$2.23x10^4$	66.7	70.3	44.6
Maltol	$4.16x10^3$	39.9	36.8	24.9
Allo-Ocimene, (E)-	$1.61x10^3$	49.7	56.6	69.7
Benzaldehyde, 4-ethyl-	$6.69x10^3$	43.4	20.7	14.6
1-Nonanol	$3.03x10^4$	27.4	35.4	23.3
Octanoic acid	$3.99x10^3$	32.3	61.5	38.7
Ethanone, 1-(3-methylphenyl)-	$2.67x10^3$	36.4	19.1	14.4
Benzoic acid, 2-hydroxy-, methyl ester	$3.78x10^3$	30.0	74.0	45.0
Decanal	$5.32x10^4$	24.8	25.5	10.2
beta-Cyclocitral	$4.82x10^4$	36.3	24.1	12.9
2-Furancarboxaldehyde, 5-(hydroxymethyl)-	$1.92x10^4$	58.4	43.3	29.7
Geraniol, (E)-	$3.93x10^5$	27.0	34.4	46.8

Table 3. continued

Variability	Technical		Biological	
			within plants	between plants
	control samples			
Metabolite	Area	RSD	RMS	RSD
Citral, (E)-	$1.66x10^4$	50.6	36.0	30.7
Phenol, 4-ethenyl-2-methoxy-	$3.74x10^3$	56.7	30.5	25.4
Eugenol	$3.36x10^4$	17.8	51.5	40.7
2-Undecenal, (E)-	-	-	-	51.5
Vanillin	-	-	-	-
Geranyl acetone, (E)-	$8.67x10^4$	23.2	21.6	15.2
beta-Ionone	$2.13x10^5$	35.7	15.0	14.9
Tridecanal	$5.48x10^4$	21.4	18.9	7.2
2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	$9.11x10^4$	34.4	12.9	13.3
1-Tridecanol	$3.91x10^3$	12.6	34.8	27.0
Dodecanoic acid	$2.20x10^4$	49.6	3.6	19.3
Tetradecanal	$5.46x10^4$	21.8	56.1	33.4
Tetradecanoic acid	$6.74x10^3$	64.3	36.1	18.7
Hexadecanal	$1.06x10^4$	14.6	43.8	21.4
Hexahydrofarnesyl acetone	$9.90x10^4$	25.0	22.7	7.9
Nonadecane	$3.37x10^3$	24.2	46.3	28.7
Farnesylacetone, (E,E)-	$5.17x10^4$	23.0	24.1	16.3
Hexadecanoic acid, methyl ester	$6.33x10^3$	34.1	105.8	118.6
Isophytol	$3.50x10^5$	42.4	49.1	26.1
Hexadecanoic acid	$4.97x10^4$	69.8	39.7	36.4
Phytol	$3.58x10^5$	46.1	57.0	37.8

- insufficient number of data points for calculation.

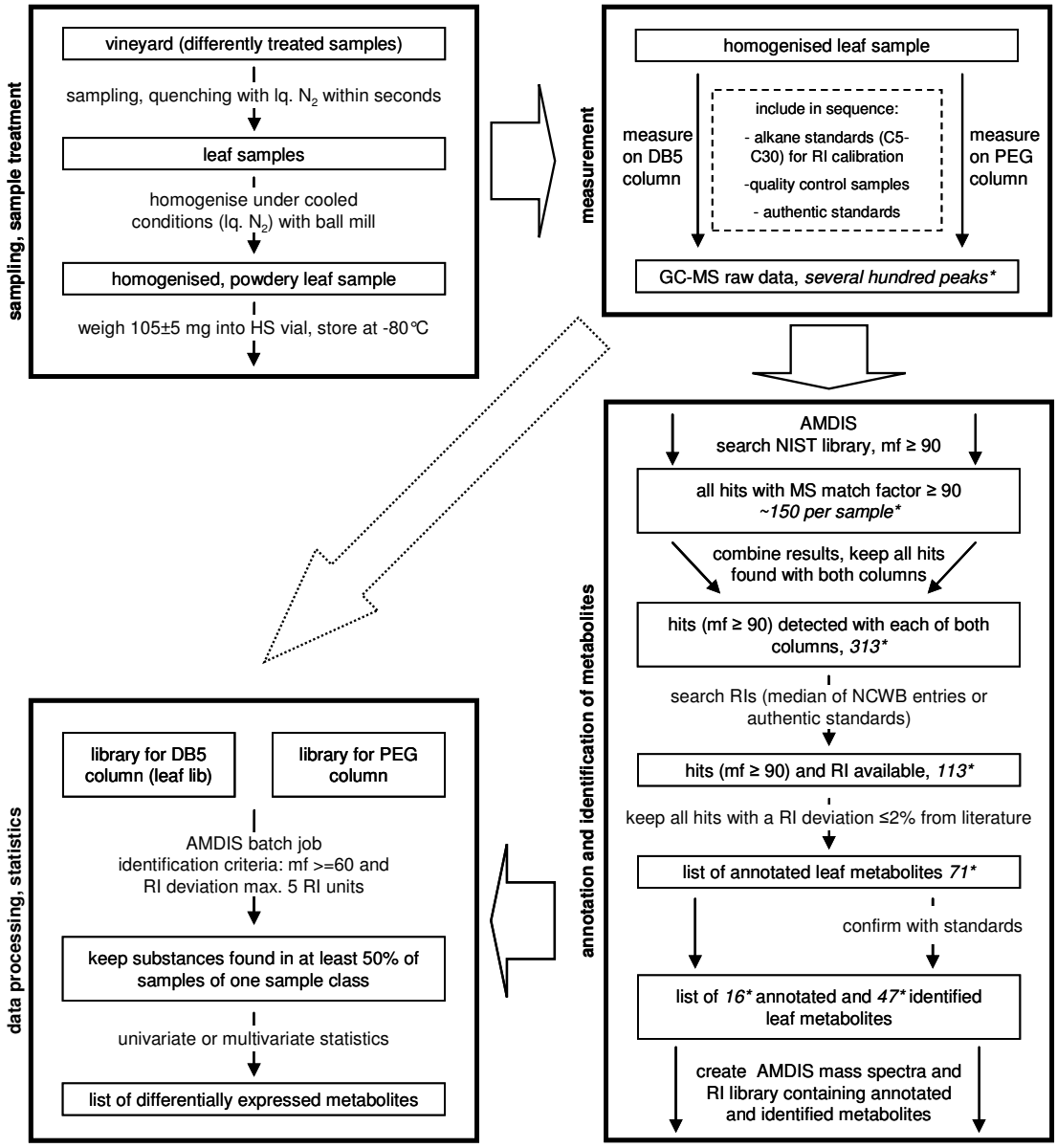

Figure 1. Workflow for the annotation / identification of leaf metabolites and the detection of differentially expressed peaks. Once the metabolites are annotated/identified this step can be skipped and the data processing step follows directly the measurements (dotted arrow).
RI: retention index, NCWB: NIST Chemistry WebBok, *: refers to numbers of metabolites detected in this study

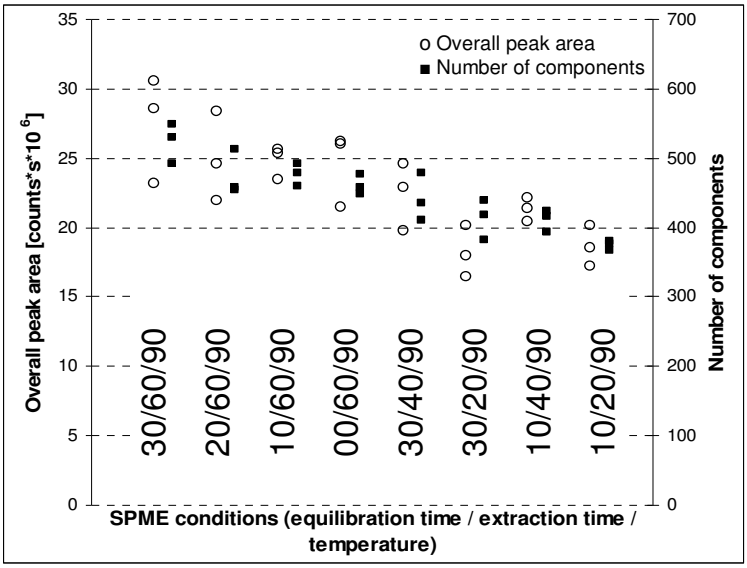

Figure 2. Overall peak area and number of AMDIS derived components obtained for different SPME conditions regarding equilibration time, extraction time and temperature. Results of three replicates are depicted.

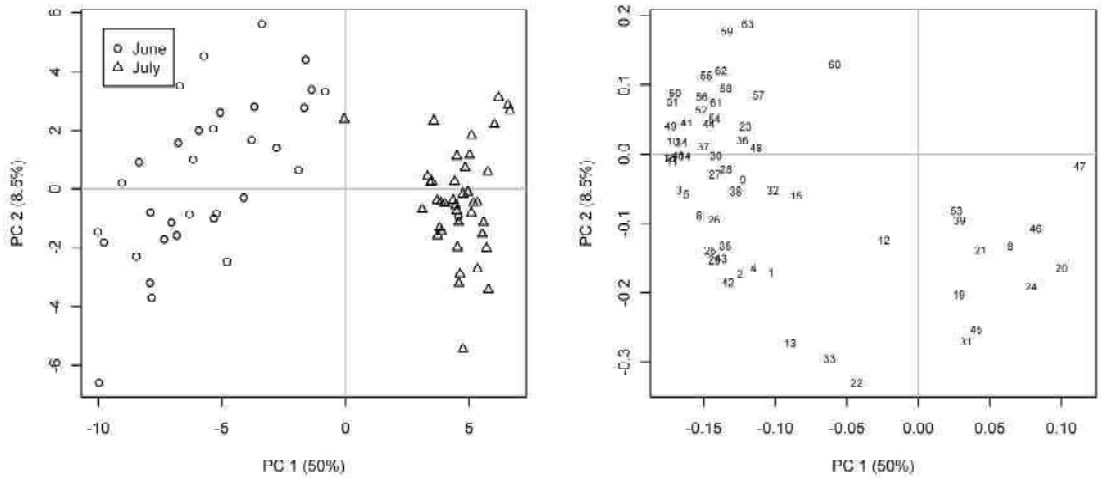

Figure 3. Left: Scores-plot of PCA. The leaves of two sampling dates cluster in two groups. 50% of the variance in the data result from the different sampling dates. Right: Loadings plot of PCA. Numbers correspond to numbers of metabolites listed in Table 1.

Supplementary data

The following pages show the *Vitis* metabolites database, which is added to this paper. The database was compiled by the author in the years 2008-2011.

Abbreviations:
l leaf
b berry, grape
f flower
s shoot
nf not found

References in *Vitis* database:

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Batovska DI, Todorova IT, Nedelcheva DV, Parushev SP, Atanassov AI, Hvarleva TD, Djakova GJ, Bankova VS, Popov SS. 2008. Preliminary study on biomarkers for the fungal resistance in *Vitis vinifera* leaves. J Plant Physiol 165: 791-795.

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Nr.	Name	CAS	l	b	f	s	other	reference
1	Alanine	56417	x					Batovska et al., 2009a
2	Glutamine	56859	x					Batovska et al., 2009a
3	Hexadecanoic acid	57103	x					Batovska et al., 2009a
4	Ethylamine	75047	x					Batovska et al., 2009a
5	Hydroxyacetic acid	79141	x					Batovska et al., 2009a
6	Sitosterol	83465	x					Batovska et al., 2009a
7	Myoinositol	87898	x					Batovska et al., 2009a
8	Inositol	87898	x					Batovska et al., 2009a
9	Nonanal	124196	x					Batovska et al., 2009a
10	Ethanedioic acid	144627	x					Batovska et al., 2009a
11	3-Hydroxybutanoic acid	300856	x					Batovska et al., 2009a
12	beta-Amyrine	559706	x					Batovska et al., 2009a
13	Hydroxybutanedioic acid	6915157	x					Batovska et al., 2009a
14	Fructose	7660255	x					Batovska et al., 2009a
15	Arabinoic acid	13752835	x					Batovska et al., 2009a
16	14,16-Hentriacontanedione	24724843	x					Batovska et al., 2009a
17	2,3-Dihydroxypropanoic acid	473814	x					Batovska et al., 2009a
18	2-Butanedioic acid	nf	x					Batovska et al., 2009a
19	2,3,4-Trihydroxybutanoic acid	10191352	x					Batovska et al., 2009a
20	2,3,4-Trihydroxybutanoic acid isomer	nf	x					Batovska et al., 2009a
21	5-(Hydroxymethyl)-2-furancarboxaldehyde	67470	x					Batovska et al., 2009a
22	Aldohexoses	nf	x					Batovska et al., 2009a
23	Monoethyl esther of phophoric acid	nf	x					Batovska et al., 2009a
24	3-Hydroxyhexanoic acid	10191249	x					Batovska et al., 2009a
25	3-(hydroxyphenyl)-2-propenoic acid	588307	x					Batovska et al., 2009a
26	1-(4-Methoxyphenyl)-2-propanone	122849	x					Batovska et al., 2009a, suppl. info
27	14,16-Hentriacontanedione	24724843	x					Batovska et al., 2009a, suppl. info
28	2,3,4-Trihydroxybutanoic acid	10191352	x					Batovska et al., 2009a, suppl. info
29	2,3,4-Trihydroxybutanoic acid (isomer)	nf	x					Batovska et al., 2009a, suppl. info
30	2,3-Dihydro-1,1,3-trimethyl-3-phenyl-1H-indene	3910358	x					Batovska et al., 2009a, suppl. info
31	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	28564832	x					Batovska et al., 2009a, suppl. info
32	2,3-Dihydroxybutanedioic acid	87694	x					Batovska et al., 2009a, suppl. info
33	2,3-Dihydroxypropanoic acid	473814	x					Batovska et al., 2009a, suppl. info
34	2,4-Diphenyl-4-methyl-1-pentene	6258737	x					Batovska et al., 2009a, suppl. info
35	2-Butenedioic acid	6915180	x					Batovska et al., 2009a, suppl. info
36	2-Ethylhexanoic acid	149575	x					Batovska et al., 2009a, suppl. info
37	2-Hydroxybutanoic acid	600157	x					Batovska et al., 2009a, suppl. info
38	2-Hydroxypentanoic acid	617312	x					Batovska et al., 2009a, suppl. info
39	2-Hydroxypropane- 1,2,3-tricarboxylic acid	77929	x					Batovska et al., 2009a, suppl. info
40	2-Hydroxypropanoic acid	50215	x					Batovska et al., 2009a, suppl. info
41	2-Methyl-butanoic acid	116530	x					Batovska et al., 2009a, suppl. info

Nr.	Name	CAS	l	b	f	s	other	reference
42	3-(3,4-Dihydroxyphenyl)-2-propenoic acid	331395	x					Batovska et al., 2009a, suppl. info
43	3-(4-Hydroxyphenyl)-2-propenoic acid	7400080	x					Batovska et al., 2009a, suppl. info
44	3-(Hydroxyphenyl)-2-propenoic acid	588307	x					Batovska et al., 2009a, suppl. info
45	3,4,5-Trihydroxybenzoic acid	149917	x					Batovska et al., 2009a, suppl. info
46	3,4-Dihydroxybenzoic acid	99503	x					Batovska et al., 2009a, suppl. info
47	3-Hydroxybutanoic acid	300856	x					Batovska et al., 2009a, suppl. info
48	3-Hydroxyhexanoic acid	10191249	x					Batovska et al., 2009a, suppl. info
49	3-Methyl-4-oxo-2-pentenoic acid	nf	x					Batovska et al., 2009a, suppl. info
50	4,14-Dimethyl-ergosta-8,24(28)-dien-3-ol	nf	x					Batovska et al., 2009a, suppl. info
51	5-(Hydroxymethyl)-2-furancarboxaldehyde	67470	x					Batovska et al., 2009a, suppl. info
52	9,19-Cyclolanost-24-en-3-ol	13639388	x					Batovska et al., 2009a, suppl. info
53	Alanine	56417	x					Batovska et al., 2009a, suppl. info
54	Aldohexoses	nf	x					Batovska et al., 2009a, suppl. info
55	alpha-Amyrin	638959	x					Batovska et al., 2009a, suppl. info
56	alpha-Tocopherol	1406184	x					Batovska et al., 2009a, suppl. info
57	Arabinoic acid	13752835	x					Batovska et al., 2009a, suppl. info
58	Arabinoic acid (isomer)	nf	x					Batovska et al., 2009a, suppl. info
59	beta-Amyrine	559706	x					Batovska et al., 2009a, suppl. info
60	Branched hydrocarbon	nf	x					Batovska et al., 2009a, suppl. info
61	Butanedioic acid	110156	x					Batovska et al., 2009a, suppl. info
62	Butyl ester of hydroxybutanedioic acid	nf	x					Batovska et al., 2009a, suppl. info
63	Campesterol	474624	x					Batovska et al., 2009a, suppl. info
64	Cholesterol	57885	x					Batovska et al., 2009a, suppl. info
65	delta-Tocopherol	119131	x					Batovska et al., 2009a, suppl. info
66	Dihydro-3,4-hydroxy-2(3H)-furanone	nf	x					Batovska et al., 2009a, suppl. info
67	Dihydro-3,4-hydroxy-2(3H)-furanone (isomer)	nf	x					Batovska et al., 2009a, suppl. info
68	Docosane	629970	x					Batovska et al., 2009a, suppl. info
69	Ethane-1,2,3-triol	nf	x					Batovska et al., 2009a, suppl. info
70	Ethane-1,2-diol	107211	x					Batovska et al., 2009a, suppl. info
71	Ethanedioic acid	144627	x					Batovska et al., 2009a, suppl. info
72	Ethyl ester of 2,3-dihydroxybutanedioic acid	nf	x					Batovska et al., 2009a, suppl. info
73	Ethylamine	75047	x					Batovska et al., 2009a, suppl. info
74	Fructose	7660255	x					Batovska et al., 2009a, suppl. info
75	Fucosterol	17605673	x					Batovska et al., 2009a, suppl. info
76	gamma-Tocopherol	7616220	x					Batovska et al., 2009a, suppl. info
77	Glucofuranose	18549401	x					Batovska et al., 2009a, suppl. info
78	Glutamine	56859	x					Batovska et al., 2009a, suppl. info
79	Glycerophosphate	927208	x					Batovska et al., 2009a, suppl. info
80	Heneicosane	629947	x					Batovska et al., 2009a, suppl. info
81	Hentriacontane	630046	x					Batovska et al., 2009a, suppl. info
82	Heptacosane	593497	x					Batovska et al., 2009a, suppl. info

Nr.	Name	CAS	l	b	f	s	other	reference
83	Hexacosane	630013	x					Batovska et al., 2009a, suppl. info
84	Hexadecanoic acid	57103	x					Batovska et al., 2009a, suppl. info
85	Hexanoic acid	142621	x					Batovska et al., 2009a, suppl. info
86	Hexenoic acid	1289403	x					Batovska et al., 2009a, suppl. info
87	Hydroxyacetic acid	79141	x					Batovska et al., 2009a, suppl. info
88	Hydroxybutanedioic acid	6915157	x					Batovska et al., 2009a, suppl. info
89	Hydroxyproline	51354	x					Batovska et al., 2009a, suppl. info
90	Indole	120729	x					Batovska et al., 2009a, suppl. info
91	Inositol	87898	x					Batovska et al., 2009a, suppl. info
92	Isopropyl hexadecanoate	142916	x					Batovska et al., 2009a, suppl. info
93	Isopropyl tetradecanoate	110270	x					Batovska et al., 2009a, suppl. info
94	Lanost-7-en-3-one	5985808	x					Batovska et al., 2009a, suppl. info
95	Lanosterol	79630	x					Batovska et al., 2009a, suppl. info
96	Levoglucosan	498077	x					Batovska et al., 2009a, suppl. info
97	Lupeol	545471	x					Batovska et al., 2009a, suppl. info
98	Methyl-2-hydroxybutanoic acid	nf	x					Batovska et al., 2009a, suppl. info
99	Monoethyl ester of phosphoric acid	nf	x					Batovska et al., 2009a, suppl. info
100	Myo-inositol	87898	x					Batovska et al., 2009a, suppl. info
101	Nonadecane	629925	x					Batovska et al., 2009a, suppl. info
102	Nonanal	124196	x					Batovska et al., 2009a, suppl. info
103	Nonanoic acid	112050	x					Batovska et al., 2009a, suppl. info
104	Octacosane	630024	x					Batovska et al., 2009a, suppl. info
105	Octadecanoic acid	57114	x					Batovska et al., 2009a, suppl. info
106	Pentacosane	629992	x					Batovska et al., 2009a, suppl. info
107	Phosphoric acid	78400	x					Batovska et al., 2009a, suppl. info
108	Proline	147853	x					Batovska et al., 2009a, suppl. info
109	Sitosterol	83465	x					Batovska et al., 2009a, suppl. info
110	Squalene	94016350	x					Batovska et al., 2009a, suppl. info
111	Stigmasterol	83487	x					Batovska et al., 2009a, suppl. info
112	Stigmasterol	83487	x					Batovska et al., 2009a, suppl. info
113	Tetracosane	646311	x					Batovska et al., 2009a, suppl. info
114	Tetradecanoic acid	544638	x					Batovska et al., 2009a, suppl. info
115	Triacontane	638686	x					Batovska et al., 2009a, suppl. info
116	Tricosane	638675	x					Batovska et al., 2009a, suppl. info
117	Glycolic acid	79141	x					Batovska et al., 2008
118	Tartaric acid	87694	x					Batovska et al., 2008
119	Isopropyl myristate	110270	x					Batovska et al., 2008
120	Squalen	94016350	x					Batovska et al., 2008
121	3-Hydroxybutanoic acid	300856	x					Batovska et al., 2008
122	Hexahydrofarnesylacetone	502692	x					Batovska et al., 2008
123	Lupeol	545471	x					Batovska et al., 2008

Nr.	Name	CAS	l	b	f	s	other	reference
124	beta-Amyrine	559706	x					Batovska et al., 2008
125	alpha-Amyrine	638959	x					Batovska et al., 2008
126	alpha-Amyrines	638959	x					Batovska et al., 2008
127	alpha-Tocopherol	1406184	x					Batovska et al., 2008
128	Malic acid	6915157	x					Batovska et al., 2008
129	gamma-Tocopherol	7616220	x					Batovska et al., 2008
130	Arabinoic acid	13752835	x					Batovska et al., 2008
131	3-Hydroxycaproic acid	10191249	x					Batovska et al., 2008
132	Erythronic acid	nf	x					Batovska et al., 2008
133	Monoethyl phosphate	1623149	x					Batovska et al., 2008
134	Undecyl laurate	3658444	x					Batovska et al., 2008
135	Stigmata-3,5-dien-7-one	nf	x					Batovska et al., 2008
136	Hexadecanoic acid	57103			x			Buchbauer et al., 1994
137	2-Phenylethanol	60128			x			Buchbauer et al., 1994
138	2-Phenylethanol	60128			x			Buchbauer et al., 1994
139	Ethanol	64175			x			Buchbauer et al., 1994
140	Linalool	78706			x			Buchbauer et al., 1994
141	Linalool	78706			x			Buchbauer et al., 1994
142	(E)-3,7-Dimethyl-2,6-octadien-1-ol	106241			x			Buchbauer et al., 1994
143	6-Methyl-5-hepten-2-one	110930			x			Buchbauer et al., 1994
144	Hexan-1-ol	111273			x			Buchbauer et al., 1994
145	Hexan-1-ol	111273			x			Buchbauer et al., 1994
146	Octan-1-ol	111875			x			Buchbauer et al., 1994
147	Octan-1-ol	111875			x			Buchbauer et al., 1994
148	Methyl hexadecanoate	112390			x			Buchbauer et al., 1994
149	Methyl hexadecanoate	112390			x			Buchbauer et al., 1994
150	Benzyl benzoate	120514			x			Buchbauer et al., 1994
151	Nonanal	124196			x			Buchbauer et al., 1994
152	Nonanal	124196			x			Buchbauer et al., 1994
153	1-Methyl-4-(1-methylethenyl)-cyclohexene	138863			x			Buchbauer et al., 1994
154	Ethyl acetate	141786			x			Buchbauer et al., 1994
155	Ethyl acetate	141786			x			Buchbauer et al., 1994
156	Ethyl acetate	141786			x			Buchbauer et al., 1994
157	1-Methyl-4-(6-methylhepta-1,5-dien-2-yl)-cyclohex-1-ene	495614			x			Buchbauer et al., 1994
158	1-Hexene	592416			x			Buchbauer et al., 1994
159	Nonadecane	629925			x			Buchbauer et al., 1994
160	1-Tridecene	2437561			x			Buchbauer et al., 1994
161	1-Tridecene	2437561			x			Buchbauer et al., 1994
162	(Z)-1,2,3,5,6,7,8,8alpha-Octahydro-1,4-dimethyl-7-(prop-1-en-2-yl)azulene	3691110			x			Buchbauer et al., 1994
163	(Z)-1,2,3,5,6,7,8,8alpha-Octahydro-1,4-dimethyl-7-(prop-1-en-2-yl)azulene	3691110			x			Buchbauer et al., 1994
164	7-Isopropenyl-1,4-dimethyl-1,2,3,4,5,6,7,8-octahydroazulene	3691121			x			Buchbauer et al., 1994

Nr.	Name	CAS	l	b	f	s	other	reference
165	(E)-6,10-Dimethyl-5,9-undecadien-2-one	3796701			x			Buchbauer et al., 1994
166	1-Nonadecene	18435455			x			Buchbauer et al., 1994
167	2-Phenylethanol	60128		x				Canuti et al, 2009
168	beta-Linalool	78706		x				Canuti et al, 2009
169	beta-Ionone	79776		x				Canuti et al, 2009
170	beta-Citronellol	106229		x				Canuti et al, 2009
171	3-Octanone	106683		x				Canuti et al, 2009
172	Isovalerone	108838		x				Canuti et al, 2009
173	2-Octanone	111137		x				Canuti et al, 2009
174	Nonanal	124196		x				Canuti et al, 2009
175	1-Nonanol	143088		x				Canuti et al, 2009
176	(E)-3-Hexen-1-ol	928972		x				Canuti et al, 2009
177	(E,Z)-2,6-Nonadienal	557482		x				Canuti et al, 2009
178	3-Octanol	589980		x				Canuti et al, 2009
179	(Z)-2-Hexen-1-ol	2305217		x				Canuti et al, 2009
180	1-Octen-3-ol	3391864		x				Canuti et al, 2009
181	Geranyl/neryl acetone	nf		x				Canuti et al, 2009
182	(E)-2-Nonenal	18829566		x				Canuti et al, 2009
183	Nerol	106252		x				Canuti et al, 2009
184	1-Hexanol	111273		x				Canuti et al, 2009
185	1-Octanol	111875		x				Canuti et al, 2009
186	(Z)-2-Hexenal	505577		x				Canuti et al, 2009
187	Hexanal	66251		x				Canuti et al, 2009
188	(E)-2-Hexenal	6728263		x				Canuti et al, 2009
189	4-Methyl-2-heptanone	6137060		x				Canuti et al, 2009
190	2-Ethyl-1-hexanol	104767		x				Canuti et al, 2009
191	Dihydroedulan I	63335660		x				Canuti et al, 2009
192	(Z)-3-Nonen-1-ol	10340235		x				Canuti et al, 2009
193	beta-Damascenon	36649635		x				Canuti et al, 2009
194	Linalool	78706					whole plant	Cha et al., 2008
195	Decanal	112312	x				whole plant	Cha et al., 2008
196	Nonanal	124196	x				whole plant	Cha et al., 2008
197	Z-3-Hexen-1-yl acetate	3681718	x				whole plant	Cha et al., 2008
198	Z-Linalool Oxide	5989333	x				whole plant	Cha et al., 2008
199	E-Linalool Oxide	34995772	x				whole plant	Cha et al., 2008
200	Methyl Salicylate	119368					whole plant	Cha et al., 2008
201	Germacrene-D	23986745					whole plant	Cha et al., 2008
202	alpha-Farnesene	502614					whole plant	Cha et al., 2008
203	beta-Caryophyllene	87445					whole plant	Cha et al., 2008
204	E-4,8-Dimethyl 1,3,7-nonatriene	19945610	x				whole plant	Cha et al., 2008
205	2-Phenylethanol	60128		x				Coelho et al., 2006

Nr.	Name	CAS	l	b	f	s	other	reference
206	Linalool	78706		x				Coelho et al., 2006
207	beta-Ionone	79776		x				Coelho et al., 2006
208	Naphthalene	91203		x				Coelho et al., 2006
209	E-Geraniol	106241		x				Coelho et al., 2006
210	beta-Cyclocitral	432257		x				Coelho et al., 2006
211	alpha-Amorphene	483750		x				Coelho et al., 2006
212	Cadalene	483783		x				Coelho et al., 2006
213	beta-Cadinene	523477		x				Coelho et al., 2006
214	alpha-Guaiene	3691121		x				Coelho et al., 2006
215	Valencene	4630073		x				Coelho et al., 2006
216	gamma-Isogeraniol	5944207		x				Coelho et al., 2006
217	delta-Elemene	20307840		x				Coelho et al., 2006
218	E-beta-Damascenone	23726934		x				Coelho et al., 2006
219	gamma-Elemene	30824670		x				Coelho et al., 2006
220	gamma-Elemene	30824670		x				Coelho et al., 2006
221	(-)-Isoledene	95910364		x				Coelho et al., 2006
222	2-Isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-en	150320528		x				Coelho et al., 2006
223	Benzyl alcohol	100516		x				Coelho et al., 2006
224	Citronellol	106229		x				Coelho et al., 2006
225	Limonene	138863		x				Coelho et al., 2006
226	alpha-Ylangene	14912448		x				Coelho et al., 2006
227	Germacrene B	15423571		x				Coelho et al., 2006
228	Hotrienol	20053887		x				Coelho et al., 2006
229	alpha-Calacorene	21391991		x				Coelho et al., 2006
230	alpha-Calacorene	21391991		x				Coelho et al., 2006
231	Germacrene D	23986745		x				Coelho et al., 2006
232	alpha-Muurolene	31983229		x				Coelho et al., 2006
233	Geranyl acetone	3796701		x				Coelho et al., 2006
234	gamma-Cadinene	39029419		x				Coelho et al., 2006
235	gamma-Cadinene	39029419		x				Coelho et al., 2006
236	Epizonarene	41702630		x				Coelho et al., 2006
237	Geranic acid	459803		x				Coelho et al., 2006
238	delta-Cadinene	483761		x				Coelho et al., 2006
239	(+)-Aromadendrene	489394		x				Coelho et al., 2006
240	Manoyl oxide	596849		x				Coelho et al., 2006
241	3,7-Guaiadiene	6754047		x				Coelho et al., 2006
242	beta-Caryophyllene	87445		x				Coelho et al., 2006
243	alpha-Terpineol	98555		x				Coelho et al., 2006
244	1,1,6-Trimethyl-1,2-dihydronaphthalene	30364386		x				Coelho et al., 2006
245	(+)-Cycloisosativene	406485436		x				Coelho et al., 2006
246	beta-Bourbonene (iosmer 1)	nf		x				Coelho et al., 2006

Nr.	Name	CAS	l	b	f	s	other	reference
247	beta-Bourbonene (iosmer 2)	nf		x				Coelho et al., 2006
248	beta-Bourbonene (iosmer 3)	nf		x				Coelho et al., 2006
249	beta-Cubebene	13744155		x				Coelho et al., 2006
250	Epi-bicyclosesquiphellandrene	54274736		x				Coelho et al., 2006
251	(-)-delta-Selinene	28624239		x				Coelho et al., 2006
252	Calamenene	483772		x				Coelho et al., 2006
253	Theaspirane A (2R,5R)	43126223		x				Coelho et al., 2006
254	Dihydroedulan I	63335660		x				Coelho et al., 2006
255	Vitispiran isomer 1	nf		x				Coelho et al., 2006
256	Vitispiran isomer 2	nf		x				Coelho et al., 2006
257	Theaspirane B (2R,5S)	66537404		x				Coelho et al., 2006
258	2-Phenylethanol	60128		x				Coelho et al., 2007
259	n-Hexanal	66251		x				Coelho et al., 2007
260	Linalool	78706		x				Coelho et al., 2007
261	beta-Ionone	79776		x				Coelho et al., 2007
262	Z-Citral	106263		x				Coelho et al., 2007
263	E-2-Hexenol	928950		x				Coelho et al., 2007
264	gamma-Isogeraniol	5944207		x				Coelho et al., 2007
265	E-beta-Damascenone	23726934		x				Coelho et al., 2007
266	Linalool E-pyranic oxide	41720621		x				Coelho et al., 2007
267	Benzyl alcohol	100516		x				Coelho et al., 2007
268	Citronellol	106229		x				Coelho et al., 2007
269	Geraniol	1		x				Coelho et al., 2007
270	Nerol	106252		x				Coelho et al., 2007
271	1-Hexanol	111273		x				Coelho et al., 2007
272	Limonene	138863		x				Coelho et al., 2007
273	E-Citral	141275		x				Coelho et al., 2007
274	Hotrienol	20053887		x				Coelho et al., 2007
275	Geranic acid	459803		x				Coelho et al., 2007
276	alpha-Terpinolene	586629		x				Coelho et al., 2007
277	E-2-Hexenal	6728263		x				Coelho et al., 2007
278	Z-3-Hexenol	928961		x				Coelho et al., 2007
279	alpha-Terpineol	98555		x				Coelho et al., 2007
280	Linalool Z-pyranic oxide	nf		x				Coelho et al., 2007
281	Linalool Z-furanic oxide	nf		x				Coelho et al., 2007
282	Terpendiol I	nf		x				Coelho et al., 2007
283	beta-Ocimene	13877913					stem	Hampel et al., 2005
284	4,8-Dimethylnona-1,3,7-triene	51911821					stem	Hampel et al., 2005
285	E,E-alpha-Farnesene	502614					stem	Hampel et al., 2005
286	Z-beta-Ocimene	3338554					stem	Hampel et al., 2005
287	S-Linalool	126909					stem	Hampel et al., 2005

Nr.	Name	CAS	l	b	f	s	other	reference
288	beta-Caryophyllene	87445				stem		Hampel et al., 2005
289	alpha-Humulene	6753986				stem		Hampel et al., 2005
290	Germacrene D	23986745				stem		Hampel et al., 2005
291	Linalool	78706		x				Ji and Dami, 2008
292	Undecanal	112447		x				Ji and Dami, 2008
293	Phenylacetaldehyde	122781		x				Ji and Dami, 2008
294	Nonanal	124196		x				Ji and Dami, 2008
295	trans Rose Oxide	876186		x				Ji and Dami, 2008
296	cis-3-Hexen-1-ol	928961		x				Ji and Dami, 2008
297	cis-Ocimene	3338554		x				Ji and Dami, 2008
298	trans-Ocimene	3779611		x				Ji and Dami, 2008
299	trans-2-Hexenal	6728263		x				Ji and Dami, 2008
300	Geraniol	106241		x				Ji and Dami, 2008
301	Nerol	106252		x				Ji and Dami, 2008
302	Neral	106263		x				Ji and Dami, 2008
303	1-Hexanol	111273		x				Ji and Dami, 2008
304	Decanal	112312		x				Ji and Dami, 2008
305	Dodecanal	112549		x				Ji and Dami, 2008
306	Limonene	138863		x				Ji and Dami, 2008
307	Geranial	141275		x				Ji and Dami, 2008
308	Nerol Oxide	1786089		x				Ji and Dami, 2008
309	Geranyl acetone	3796701		x				Ji and Dami, 2008
310	Hexanal	66251		x				Ji and Dami, 2008
311	alpha-Terpineol	98555		x				Ji and Dami, 2008
312	cis Rose Oxide	3033236		x				Ji and Dami, 2008
313	Hexadecanoic acid	57103	x					Kawaguchi et al. 2000
314	Octadecanoic acid	57114	x					Kawaguchi et al. 2000
315	Eicosanoic acid	506309	x					Kawaguchi et al. 2000
316	Uncosanoic acid	nf	x					Kawaguchi et al. 2000
317	Docosanoic acid	112856	x					Kawaguchi et al. 2000
318	Tricosanoic acid	2433967	x					Kawaguchi et al. 2000
319	Tetracosanoic acid	557595	x					Kawaguchi et al. 2000
320	Pentacosanoic acid	506387	x					Kawaguchi et al. 2000
321	Hexacosanoic acid	506467	x					Kawaguchi et al. 2000
322	4-Hydroxysphinganine	13552119	x					Kawaguchi et al. 2000
323	4-Hydroxy-8-E-sphingenine	nf	x					Kawaguchi et al. 2000
324	4-Hydroxy-8-Z-sphingenine	nf	x					Kawaguchi et al. 2000
325	Sphinganine	764227	x					Kawaguchi et al. 2000
326	8-E-sphingenine	nf	x					Kawaguchi et al. 2000
327	8-Z-Sphingenine	nf	x					Kawaguchi et al. 2000
328	4-E,8-E-Sphingadienine	nf	x					Kawaguchi et al. 2000

Nr.	Name	CAS	l	b	f	s	other	reference
329	4-E,8-Z-Sphingadienine	41679338	x					Kawaguchi et al. 2000
330	2-Phenylethanol	60128				x		Lamorte et al., 2008
331	1-Pentanol	71410				x		Lamorte et al., 2008
332	Linalool	78706				x		Lamorte et al., 2008
333	Eugenol	97530				x		Lamorte et al., 2008
334	Furfural	98011				x		Lamorte et al., 2008
335	Benzaldehyde	100527				x		Lamorte et al., 2008
336	2-Ethyl-1-hexanol	104767				x		Lamorte et al., 2008
337	1-Heptanol	111706				x		Lamorte et al., 2008
338	4-Methyl-3-penten-1-ol	763893				x		Lamorte et al., 2008
339	1H-Pirrole-2-carboxaldehyde	1003298				x		Lamorte et al., 2008
340	1-Phenylethanol	1445916				x		Lamorte et al., 2008
341	1-Octen-3-ol	3391864				x		Lamorte et al., 2008
342	Z-Linalool oxide	5989333				x		Lamorte et al., 2008
343	Exo-2-hydroxy-1,8-cineole	92999785				x		Lamorte et al., 2008
344	Benzyl alcohol	100516				x		Lamorte et al., 2008
345	Citronellol	106229				x		Lamorte et al., 2008
346	Geraniol	106241				x		Lamorte et al., 2008
347	Nerol	106252				x		Lamorte et al., 2008
348	1-Hexanol	111273				x		Lamorte et al., 2008
349	3-Methyl-1-butanol	123513				x		Lamorte et al., 2008
350	Geranic acid	459803				x		Lamorte et al., 2008
351	Hexanal	66251				x		Lamorte et al., 2008
352	E-2-Hexenal	6728263				x		Lamorte et al., 2008
353	E-2-Hexen-1-ol	928950				x		Lamorte et al., 2008
354	Z-3-Hexen-1-ol	928961				x		Lamorte et al., 2008
355	E-3-Hexen-1-ol	928972				x		Lamorte et al., 2008
356	alpha-Terpineol	98555				x		Lamorte et al., 2008
357	Pyranic linalool oxide	nf				x		Lamorte et al., 2008
358	3-Oxo-alpha-Ionol	34318213				x		Lamorte et al., 2008
359	Hexanal	66251	x					Loughrin et al., 1997
360	Linalool	78706	x					Loughrin et al., 1997
361	Hexanol	111273	x					Loughrin et al., 1997
362	Methyl Salicylate	119368	x					Loughrin et al., 1997
363	Indole	120729	x					Loughrin et al., 1997
364	Limonene	138863	x					Loughrin et al., 1997
365	E,E-alpha-Farnesene	502614	x					Loughrin et al., 1997
366	E-beta-Ocimene	3779611	x					Loughrin et al., 1997
367	E-2-Hexenal	6728263	x					Loughrin et al., 1997
368	Z-3-Hexenal	6789806	x					Loughrin et al., 1997
369	Nerolidol	7212444	x					Loughrin et al., 1997

Nr.	Name	CAS	l	b	f	s	other	reference
370	Z-3-Hexenyl benzoate	25152856	x					Loughrin et al., 1997
371	E-4,8-Dimethyl 1,3,7-nonatriene	19945610	x					Loughrin et al., 1997
372	Z-3-Hexenyl Acetate	3681718	x					Loughrin et al., 1997
373	2-Phenylethanol	60128		x				Masa and Vilanova, 2008
374	1-Propanol	71238		x				Masa and Vilanova, 2008
375	Linalool	78706		x				Masa and Vilanova, 2008
376	beta-Ionone	79776		x				Masa and Vilanova, 2008
377	Methanol	89781		x				Masa and Vilanova, 2008
378	Eugenol	97530		x				Masa and Vilanova, 2008
379	Ethyl lactate	97643		x				Masa and Vilanova, 2008
380	Ethyl butyrate	105544		x				Masa and Vilanova, 2008
381	Ethyl octanoate	106321		x				Masa and Vilanova, 2008
382	Ethyl decanoate	110383		x				Masa and Vilanova, 2008
383	Diethyl succinate	123251		x				Masa and Vilanova, 2008
384	Isoamyl alcohol	123513		x				Masa and Vilanova, 2008
385	Ethyl hexanoate	123660		x				Masa and Vilanova, 2008
386	Isoamyl acetate	123922		x				Masa and Vilanova, 2008
387	Ethyl myristate	124061		x				Masa and Vilanova, 2008
388	Ethyl acetate	141786		x				Masa and Vilanova, 2008
389	Hexyl acetate	142927		x				Masa and Vilanova, 2008
390	Benzyl alcohol	100516		x				Masa and Vilanova, 2008
391	Citronellol	106229		x				Masa and Vilanova, 2008
392	Geraniol	106241		x				Masa and Vilanova, 2008
393	Nerol	106252		x				Masa and Vilanova, 2008
394	1-Butanol	71363		x				Masa and Vilanova, 2008
395	2-Methyl-1-propanol	78831		x				Masa and Vilanova, 2008
396	alpha-Terpineol	98555		x				Masa and Vilanova, 2008
397	Theaspirane-b	43126212		x				Masa and Vilanova, 2008
398	Terminem-4-ol	nf		x				Masa and Vilanova, 2008
399	4-Terpinenol	562743		x				Rocha et al., 2007
400	Z-Herboxide	13679862		x				Rocha et al., 2007
401	beta-Myrcene	123353		x				Rocha et al., 2007
402	1S-alpha-Pinene	7785264		x				Rocha et al., 2007
403	E-Herboxide	13679862		x				Rocha et al., 2007
404	1,8-Cineole	470826		x				Rocha et al., 2007
405	2-Carene	554610		x				Rocha et al., 2007
406	Linalool Z-furanic oxide	nf		x				Rocha et al., 2007
407	Z-Rose oxide (cis)	16409431		x				Rocha et al., 2007
408	alpha-Phellandrene+B1055	nf		x				Rocha et al., 2007
409	E-Rose oxide	876186		x				Rocha et al., 2007
410	4-Carene	29050337		x				Rocha et al., 2007

Nr.	Name	CAS	l	b	f	s	other	reference
411	alpha-Pinene oxide	1686142		x				Rocha et al., 2007
412	1R-alpha-Pinene	7785708		x				Rocha et al., 2007
413	Limonene	138863		x				Rocha et al., 2007
414	Nerol oxide	1786089		x				Rocha et al., 2007
415	beta-Phellandrene	555102		x				Rocha et al., 2007
416	E-2,3-Epoxycarane	35071295		x				Rocha et al., 2007
417	Linalool E-pyranic oxide	41720621		x				Rocha et al., 2007
418	beta-Ocimene	13877913		x				Rocha et al., 2007
419	2,6-Dimethyl-2,6-octadiene	2792394		x				Rocha et al., 2007
420	Dihydromyrcenol	53219219		x				Rocha et al., 2007
421	Linalool Z-pyranic oxide	nf		x				Rocha et al., 2007
422	Linalool	78706		x				Rocha et al., 2007
423	2,6-Dimethyl-1,7-octadien-3-ol	22460599		x				Rocha et al., 2007
424	gamma-Terpinene	99854		x				Rocha et al., 2007
425	alpha-Terpinolene	586629		x				Rocha et al., 2007
426	neo-allo-Ocimene	673847		x				Rocha et al., 2007
427	Hotrienol	20053887		x				Rocha et al., 2007
428	Plinol C	4028608		x				Rocha et al., 2007
429	Ocimenol	5986389		x				Rocha et al., 2007
430	p-Menthan-1-ol	21129271		x				Rocha et al., 2007
431	Borneol	507700		x				Rocha et al., 2007
432	p-Cymen-8-ol	1197019		x				Rocha et al., 2007
433	(+)-alpha-Terpineol	98555		x				Rocha et al., 2007
434	gamma-Isogeraniol	5944207		x				Rocha et al., 2007
435	Citronellol	106229		x				Rocha et al., 2007
436	Lilac alcohol D	33081377		x				Rocha et al., 2007
437	Myrtenol (alpha-pinene-10-ol)	515004		x				Rocha et al., 2007
438	Nerol	106252		x				Rocha et al., 2007
439	3,7-Dimethyl-1,5-octadien-3,7-diol	13741214		x				Rocha et al., 2007
440	Geraniol	106241		x				Rocha et al., 2007
441	3,7-Dimethyl-1,7-octadien-3,6-diol	51276336		x				Rocha et al., 2007
442	3,7-Dimethyl-1-octen-3,7-diol	nf		x				Rocha et al., 2007
443	Lilac aldehyde B	53447464		x				Rocha et al., 2007
444	Z-Citral	106263		x				Rocha et al., 2007
445	Safranal	116267		x				Rocha et al., 2007
446	p-Menth-1-en-9-al	29548149		x				Rocha et al., 2007
447	beta-Cyclocitral	432257		x				Rocha et al., 2007
448	E-Citral	141275		x				Rocha et al., 2007
449	Geranyl formate	105862		x				Rocha et al., 2007
450	Isobornyl acetate	125122		x				Rocha et al., 2007
451	E-Ethyl geranate	32659215		x				Rocha et al., 2007

Nr.	Name	CAS	l	b	f	s	other	reference
452	Neryl acetate	141128		x				Rocha et al., 2007
453	1R-(+)-Norinone	38651659		x				Rocha et al., 2007
454	Carvone	99490		x				Rocha et al., 2007
455	Geranic acid	459803		x				Rocha et al., 2007
456	Palmitic acid	57103					stem	Ruberto et al., 2008
457	Linalool	78706			x		stem	Ruberto et al., 2008
458	Biphenyl oxide	101848					stem	Ruberto et al., 2008
459	beta-Phenyl-ethyl-acetate	103457					stem	Ruberto et al., 2008
460	Ethyl propionate	105373					stem	Ruberto et al., 2008
461	Ethyl octanoate	106321					stem	Ruberto et al., 2008
462	Ethyl octanoate	106321					pomace	Ruberto et al., 2008
463	Ethyl dodecanoate	106332					stem	Ruberto et al., 2008
464	Ethyl dodecanoate	106332					pomace	Ruberto et al., 2008
465	Ethyl decanoate	110383					stem	Ruberto et al., 2008
466	Ethyl decanoate	110383					pomace	Ruberto et al., 2008
467	Ethyl octadecanoate	111625					pomace	Ruberto et al., 2008
468	3-Methyl-butan-1-ol	123513					stem	Ruberto et al., 2008
469	Ethyl hexanoate	123660					pomace	Ruberto et al., 2008
470	Ethyl tetradecanoate	124061					pomace	Ruberto et al., 2008
471	2-Methylbutan-1-ol	137326					stem	Ruberto et al., 2008
472	2-Methylbutan-1-ol	137326					pomace	Ruberto et al., 2008
473	Hexyl acetate	142927					stem	Ruberto et al., 2008
474	Eudesm-7(11)-en-4-ol	473041					stem	Ruberto et al., 2008
475	6,10,14-Trimethyl-pentadecan-2-one	502692					stem	Ruberto et al., 2008
476	1,2-Dimethylcyclohexane	583573					stem	Ruberto et al., 2008
477	1,3-Dimethylcyclohexane	591219					stem	Ruberto et al., 2008
478	Ethyl hexadecanoate	628977					stem	Ruberto et al., 2008
479	Ethyl hexadecanoate	628977					pomace	Ruberto et al., 2008
480	Palmitic aldehyde	629992					stem	Ruberto et al., 2008
481	3-E-Hexen-1-ol	928972					stem	Ruberto et al., 2008
482	Ethyl linolenate	1191419					stem	Ruberto et al., 2008
483	3-Methyl butyl octanoate	2035996					pomace	Ruberto et al., 2008
484	3-Methyl butyl decanoate	2306914					pomace	Ruberto et al., 2008
485	3-Z-Hexenyl acetate	3681718					stem	Ruberto et al., 2008
486	cis-Linalool oxide (Z)	5989333					stem	Ruberto et al., 2008
487	gamma-Amorphene	6980467					pomace	Ruberto et al., 2008
488	delta-Cadinol	19435973					stem	Ruberto et al., 2008
489	Cyclosativene	22469529					stem	Ruberto et al., 2008
490	alpha-Cadinene	24406051					stem	Ruberto et al., 2008
491	9-Z-Tricosene	27519024					stem	Ruberto et al., 2008
492	alpha-Muurolene	31983229					stem	Ruberto et al., 2008

Nr.	Name	CAS	l	b	f	s	other	reference
493	trans-Linalool oxide	34995772	x				stem	Ruberto et al., 2008
494	1-Butoxy-1-ethoxy ethane	57006878					pomace	Ruberto et al., 2008
495	Vitispirane	65416593					stem	Ruberto et al., 2008
496	2-Methyl butyl decanoate	68067334					pomace	Ruberto et al., 2008
497	1,1-Diethoxy ethane	105577					stem	Ruberto et al., 2008
498	1,1-Diethoxy ethane	105577					pomace	Ruberto et al., 2008
499	Geraniol	106241					stem	Ruberto et al., 2008
500	Propyl acetate	109604					stem	Ruberto et al., 2008
501	Cyclohexane	110827					pomace, stem	Ruberto et al., 2008
502	Cyclohexane	110827					pomace	Ruberto et al., 2008
503	Hexanol	111273					stem	Ruberto et al., 2008
504	Octane	111659					pomace	Ruberto et al., 2008
505	Heptanal	111717					stem	Ruberto et al., 2008
506	Nonane	111842					pomace	Ruberto et al., 2008
507	Decanal	112312					stem	Ruberto et al., 2008
508	Phenylacetaldehyd	122781					stem	Ruberto et al., 2008
509	1-Butanol, 3-methyl acetate	123922					pomace	Ruberto et al., 2008
510	Nonanal	124196					stem	Ruberto et al., 2008
511	alpha-trans-Bergamotene	13474594					stem	Ruberto et al., 2008
512	Limonene	138863					stem	Ruberto et al., 2008
513	Limonene	138863					pomace	Ruberto et al., 2008
514	Heptane	142825					stem	Ruberto et al., 2008
515	Heptane	142825					pomace	Ruberto et al., 2008
516	alpha-Ylangene	14912448					stem	Ruberto et al., 2008
517	alpha-Ylangene	14912448					pomace	Ruberto et al., 2008
518	Germacrene B	15423571					stem	Ruberto et al., 2008
519	2-E-Nonenal	18829566					stem	Ruberto et al., 2008
520	alpha-Calacorene	21391991					stem	Ruberto et al., 2008
521	Germacrene D	23986745					stem	Ruberto et al., 2008
522	2-E-4-Z-Decadienal	25152834					stem	Ruberto et al., 2008
523	delta-Selinene	28624239					stem	Ruberto et al., 2008
524	gamma-Muurolene	30021740					stem	Ruberto et al., 2008
525	gamma-Muurolene	30021740					pomace	Ruberto et al., 2008
526	1,1-Diethoxy-2-methyl butane	3658944					pomace	Ruberto et al., 2008
527	2-Pentylfuran	3777693					stem	Ruberto et al., 2008
528	2-Pentyl-furan	3777693					pomace	Ruberto et al., 2008
529	alpha-Copaene	3856255					stem	Ruberto et al., 2008
530	gamma-Cadinene	39029419					stem	Ruberto et al., 2008
531	gamma-Cadinene	39029419					pomace	Ruberto et al., 2008
532	2-E-Decenal	3913813					stem	Ruberto et al., 2008
533	Epizonarene	41702630					stem	Ruberto et al., 2008

Nr.	Name	CAS	l	b	f	s	other	reference
534	1,8-Cineole	470826					stem	Ruberto et al., 2008
535	Epi-alpha-Cadinol	481345					stem	Ruberto et al., 2008
536	delta-Cadinene	483761					stem	Ruberto et al., 2008
537	Aromadendrene	489394					stem	Ruberto et al., 2008
538	Aromadendrene	489394					pomace	Ruberto et al., 2008
539	beta-Bourbonene	5208593					stem	Ruberto et al., 2008
540	Ethyl linoleate	544354					stem	Ruberto et al., 2008
541	Ethyl linoleate	544354					pomace	Ruberto et al., 2008
542	Ethyl 9-hexadecenoate	54546224					stem	Ruberto et al., 2008
543	Ethyl 9-hexadecenoate	54546224					pomace	Ruberto et al., 2008
544	3,3-Dimethylpentane	562492					stem	Ruberto et al., 2008
545	Terpinolene	586629					pomace	Ruberto et al., 2008
546	3-Methylhexane	589344					pomace	Ruberto et al., 2008
547	3-Methylhexane	589344					pomace	Ruberto et al., 2008
548	2-Methylheptane	592278					stem	Ruberto et al., 2008
549	Manoyl oxide	596849					stem	Ruberto et al., 2008
550	Manoyl oxide	596849					pomace	Ruberto et al., 2008
551	1-Butanol, 2-methyl acetate	624419					pomace	Ruberto et al., 2008
552	Tetradecane	629594					stem	Ruberto et al., 2008
553	Pentadecane	629629					stem	Ruberto et al., 2008
554	3-Methylbutyl dodecanoate	6309519					pomace	Ruberto et al., 2008
555	Tricosane	638675					stem	Ruberto et al., 2008
556	Ethyl 9-octadecenoate	6512998					pomace	Ruberto et al., 2008
557	Hexanal	66251					stem	Ruberto et al., 2008
558	Ethyl 9-decenoate	67233914					pomace	Ruberto et al., 2008
559	3,7-Guaiadiene	6754047					stem	Ruberto et al., 2008
560	3,7-Guaiadiene	6754047					pomace	Ruberto et al., 2008
561	Nerolidol	7212444					stem	Ruberto et al., 2008
562	E-Muurola-4(14),5-diene	262352874					stem	Ruberto et al., 2008
563	Neophytadiene	504961					stem	Ruberto et al., 2008
564	2-Methyl butyl octanoate	nf					pomace	Ruberto et al., 2008
565	3-Ethyl-4-methyl-pentan-1-ol	nf					stem	Ruberto et al., 2008
566	1-Ethoxy-1-pentoxy ethane	13442905					pomace	Ruberto et al., 2008
567	2-E-Hexenal	6728263					stem	Ruberto et al., 2008
568	2-E-Heptenal	18829555					stem	Ruberto et al., 2008
569	2-Hexyl-3-methyl maleic anhydride	75052754					stem	Ruberto et al., 2008
570	3,4-Dihydroxy-7,8-dihydro-beta-ionone-3-O-beta-D-glucopyranoside	nf	x					Skouroumounis et al., 1994
571	3-Oxomegastigman-9-yl-tetra-O-acetyl-beta-D-glucopyranoside	nf	x					Skouroumounis et al., 1994
572	4,5-Dihydrovomifoliol-9-O-beta-D-glucopyranoside	nf	x					Skouroumounis et al., 1994
573	3-Hydroxy-5,6-epoxy-beta-ionone-3-O-beta-D-glucopyranoside	nf	x					Skouroumounis et al., 1994
574	Grasshopper ketone -3-O-beta-D-glucopyranoside	nf	x					Skouroumounis et al., 1994

Nr.	Name	CAS	l	b	f	s	other	reference
575	Benzyl-tetra-O-acetyl-beta-D-glucopyranoside	nf	x					Skouroumounis et al., 1994
576	8-Hydroxytheaspiranes (2 isomers)	nf	x					Skouroumounis et al., 1994
577	3-Oxo-4,5-dihydro-alpha-ionol	nf	x					Skouroumounis et al., 1994
578	3-Oxoactinidols (4 isomers)	nf	x					Skouroumounis et al., 1994
579	3-Hydroxy-7,8-dehydro-beta-ionol	58023726	x					Skouroumounis et al., 1994
580	3-Hydroxy-beta-ionol	27185804	x					Skouroumounis et al., 1994
581	3-Oxomegastigman-9-ol	nf	x					Skouroumounis et al., 1994
582	3-Oxo-alpha-ionol	34318213	x					Skouroumounis et al., 1994
583	3-Hydroxy-7,8-dihydro-beta-ionol	113110024	x					Skouroumounis et al., 1994
584	3-Hydroxyactinidol	nf	x					Skouroumounis et al., 1994
585	4-Oxo-beta-ionol	80945239	x					Skouroumounis et al., 1994
586	3-Hydroxy-5,6-epoxy-beta-ionone-3-O-beta-D-glucopyranoside	nf	x					Skouroumounis et al., 1994
587	3-Hydroxy-beta-ionone	14398346	x					Skouroumounis et al., 1994
588	3-Oxo-retro-alpha-ionol (2 isomers)	nf	x					Skouroumounis et al., 1994
589	3-Oxo-7,8-dihydro-alpha-ionol	nf	x					Skouroumounis et al., 1994
590	4-Oxo-7,8-dihydro-beta-ionol	nf	x					Skouroumounis et al., 1994
591	4,5-Dihydrovomifoliol	155418976	x					Skouroumounis et al., 1994
592	Grasshopper ketone	41703382	x					Skouroumounis et al., 1994
593	Vomifoliol	23526456	x					Skouroumounis et al., 1994
594	Dehydrovomifoliol	39763332	x					Skouroumounis et al., 1994
595	3,4-Dihydroxy-7,8-dihydro-beta-ionone	nf	x					Skouroumounis et al., 1994
596	3,4-Dihydroxy-7,8-dihydro-beta-ionol	nf	x					Skouroumounis et al., 1994
597	3,4-Dihydroxy-beta-ionone	28494340	x					Skouroumounis et al., 1994
598	7,8-Dihydrovomifoliol	nf	x					Skouroumounis et al., 1994
599	beta-Carotene	7235407	x				berry skin	Skouroumounis et al., 1994
600	Lutein	127402	x				berry skin	Skouroumounis et al., 1994
601	5,6-Epoxylutein	28368083	x				berry skin	Skouroumounis et al., 1994
602	Neoxanthin	14660914	x				berry skin	Skouroumounis et al., 1994
603	Dihydroderivative of vomifoliol	nf	x					Skouroumounis et al., 1994
604	Epoxyionone	nf	x					Skouroumounis et al., 1994
605	Glycosides of 3-oxonorisoprenoids	nf	x					Skouroumounis et al., 1994
606	Tetraacetyl-beta-D-glucopyranoside	nf	x					Skouroumounis et al., 1994
607	beta-D-Glucopyranoside of 4,5-dihydrovomifoliol	nf	x					Skouroumounis et al., 1994
608	beta-D-Glucopyranoside of vomifoliol (=roseoside)	nf	x					Skouroumounis et al., 1994
609	4,5-Dihydro-roseoside	nf	x					Skouroumounis et al., 1994
610	beta-D-Glucose	28905126	x					Skouroumounis et al., 1994
611	Benzyl-beta-D-glucopyranoside	nf	x					Skouroumounis et al., 1994
612	3-Hydroxy-5,6-epoxy-beta-ionone	38274010	x					Skouroumounis et al., 1994
613	Raspberry ketone -beta-D-glucopyranoside	38963949	x					Skouroumounis et al., 1994
614	Raspberry ketone	5471512	x					Skouroumounis et al., 1994
615	Raspberry ketone	5471512	x					Skouroumounis et al., 1994

Nr.	Name	CAS	l	b	f	s	other	reference
616	Benzyl glucoside	nf	x					Skouroumounis et al., 1994
617	2-Phenylethyl glycosides	nf	x					Skouroumounis et al., 1994
618	2-Phenylethanol	60128	x					Tesniere et al., 2006
619	Benzyl alkohol	100516	x					Tesniere et al., 2006
620	Benzaldehyde	100527	x					Tesniere et al., 2006
621	Methyl Salicylate	119368	x					Tesniere et al., 2006
622	Zingerone	122485	x	x				Tesniere et al., 2006
623	Isopentanol	123513	x					Tesniere et al., 2006
624	2-Hexenal	505577	x					Tesniere et al., 2006
625	cis-Furan Linalool Oxide	5989333	x					Tesniere et al., 2006
626	Vomifoliol	23526456	x					Tesniere et al., 2006
627	trans-Furan Linalool Oxide	34995772	x					Tesniere et al., 2006
628	4-Methyl-1-phenylethanol	589184 ?	x					Tesniere et al., 2006
629	3,4-Dihydro-3-oxoactinidol	nf	x					Tesniere et al., 2006
630	Methyl benzyl methanol isomer 1	nf	x					Tesniere et al., 2006
631	Methyl benzyl methanol isomer 2	nf	x					Tesniere et al., 2006
632	Methyl benzyl methanol iosmer 3	nf	x					Tesniere et al., 2006
633	Dimethyl-benzyl alcohol iosmer 1	nf	x					Tesniere et al., 2006
634	Dimethyl-benzyl alcohol iosmer 2	nf	x					Tesniere et al., 2006
635	Hexenol	17102646	x					Tesniere et al., 2006
636	3-Hydroxy-beta-ionone	116296754	x					Tesniere et al., 2006
637	Megastigmane-3,9-diol	nf	x					Tesniere et al., 2006
638	3-Oxo-7,8-dihydro-alpha-ionol	nf	x					Tesniere et al., 2006
639	3-Oxo-5,6-epoxy-beta-ionone	nf	x					Tesniere et al., 2006
640	4,5-Dihydrovomifoliol isomer 1	nf	x					Tesniere et al., 2006
641	4,5-Dihydrovomifoliol isomer 2	nf	x					Tesniere et al., 2006
642	3,6-Dihydroxy-megastig-7-ene-9-one	nf	x					Tesniere et al., 2006
643	7,8-Dihydrovomifoliol	nf	x					Tesniere et al., 2006
644	Geraniol hydrate	856943549	x					Tesniere et al., 2006
645	2-Phenylethanol	60128	x					Wirth et al., 2001
646	Pentanol	71410	x					Wirth et al., 2001
647	alpha-Terpineol	98555	x	x				Wirth et al., 2001
648	Benzyl alcohol	100516	x					Wirth et al., 2001
649	Geraniol	106241	x					Wirth et al., 2001
650	Phenol	108952	x	x				Wirth et al., 2001
651	Hexan-1-ol	111273	x	x				Wirth et al., 2001
652	Methyl Salicylate	119368	x					Wirth et al., 2001
653	Vanillin	121335	x	x				Wirth et al., 2001
654	Zingerone	122485	x	x				Wirth et al., 2001
655	e-Methylheptanol	123966	x					Wirth et al., 2001
656	Geranic acid	459803	x	x				Wirth et al., 2001

Nr.	Name	CAS	l	b	f	s	other	reference
657	Acetovanillone	498022	x	x				Wirth et al., 2001
658	Tyrosol	501940	x					Wirth et al., 2001
659	3,4,5-Trimethoxyphenol	642717	x	x				Wirth et al., 2001
660	3-Methylbut-3-en-1-ol	763326	x					Wirth et al., 2001
661	E-Hex-2-en-1-ol	928950	x	x				Wirth et al., 2001
662	Z-Hex-3-en-1-ol	928961	x	x				Wirth et al., 2001
663	Methyl 2,6-Dihydroxybenzoate	2150450	x	x				Wirth et al., 2001
664	2-(4-Guaiacyl)-Ethanol	2380781	x	x				Wirth et al., 2001
665	4-(4-Hydroxyphenyl)-butan-2-ol	3681718	x					Wirth et al., 2001
666	2-Methylbut-2-en-1-ol	4675870	x	x				Wirth et al., 2001
667	Raspberry ketone	5471512	x					Wirth et al., 2001
668	p-Menthan-7-ol	13674196	x					Wirth et al., 2001
669	cis-Pyran Linalool Oxide	14009713	x	x				Wirth et al., 2001
670	trans-Furan Linalool Oxide	34995772	x	x				Wirth et al., 2001
671	Zingerol + Norisoprenoidic unknown	nf	x					Wirth et al., 2001
672	trans-Pyran Linalool Oxide	39028585	x	x				Wirth et al., 2001
673	Tyrosol Isomer	nf	x	x				Wirth et al., 2001
674	Syringealdehyde + Methyl 4-Hydroxybenzoate	nf	x	x				Wirth et al., 2001
675	Furan Linolool Oxide + 6-Methylhept-5-en-2-ol	nf	x					Wirth et al., 2001
676	cis-Furan Linalool Oxide + 6-Methylhept-5-en-2-ol	nf	x	x				Wirth et al., 2001
677	Nerol + 1-Phenylethanol	nf	x	x				Wirth et al., 2001
678	Nerol hydrate + E-8-Hydroxylinalool	nf	x	x				Wirth et al., 2001
679	E-8-Hydroxynerol	nf	x					Wirth et al., 2001
680	Z-8-Hydroxygeraniol	nf	x	x				Wirth et al., 2001
681	E-8-Hydroxygeraniol	156155532	x	x				Wirth et al., 2001
682	Nerol + 1-Phenylethanol	nf	x	x				Wirth et al., 2001
683	Geraniol hydrate + Z-8-Hydroxylinalool	nf	x	x				Wirth et al., 2001
684	p-Menth-1-ene-7,8-diol	5502749	x	x				Wirth et al., 2001
685	Geranic acid hydrate	nf	x					Wirth et al., 2001
686	Acetic acid	64197	x	x	x			Tasin et al., 2005
687	Hexanoic acid	142621	x	x				Tasin et al., 2005
688	Ethylhexanoic acid	149575	x	x				Tasin et al., 2005
689	Tetradecane	629594			x			Tasin et al., 2005
690	1-Tetradecene	1120361	x		x			Tasin et al., 2005
691	Pentadecane	629629	x	x	x			Tasin et al., 2005
692	1-Pentadecene	13360617	x		x			Tasin et al., 2005
693	Heptadecane	629787	x	x	x			Tasin et al., 2005
694	1-Heptadecene	6765395		x	x			Tasin et al., 2005
695	Octadecane	593453	x		x			Tasin et al., 2005
696	Nonadecane	629925	x		x			Tasin et al., 2005
697	1-Nonadecene	18435455			x			Tasin et al., 2005

Nr.	Name	CAS	l	b	f	s	other	reference
698	Eicosane	112958			x			Tasin et al., 2005
699	Heneicosane	629947			x			Tasin et al., 2005
700	Ethanol	64175		x				Tasin et al., 2005
701	1-butanol	71363		x				Tasin et al., 2005
702	(Z)-3-Hexen-1-ol	928961		x				Tasin et al., 2005
703	(E)-3-Hexen-1-ol	928972		x				Tasin et al., 2005
704	3-Methylbutanol	6423069		x				Tasin et al., 2005
705	1-Hexadecanol	36653824			x			Tasin et al., 2005
706	1-Heptadecanol	1454859			x			Tasin et al., 2005
707	1-Octadecanol	112925			x			Tasin et al., 2005
708	Hexanal	66251	x	x	x			Tasin et al., 2005
709	(E)-2-Hexenal	6728263	x	x				Tasin et al., 2005
710	(Z)-3-Hexenal	6789806	x		x			Tasin et al., 2005
711	(E,E)-2,4-Hexadienal	142836		x				Tasin et al., 2005
712	Nonanal	124196	x	x	x			Tasin et al., 2005
713	(E)-2-Nonenal	18829566	x	x	x			Tasin et al., 2005
714	(E,Z)-2,6-Nonadienal	557482			x			Tasin et al., 2005
715	2-Undecanone	112129			x			Tasin et al., 2005
716	2-Dodecanone	6175491			x			Tasin et al., 2005
717	2-Tridecanone	593088			x			Tasin et al., 2005
718	2-Tetradecanone	2345279			x			Tasin et al., 2005
719	Methyl acetate	79209		x	x			Tasin et al., 2005
720	Ethyl acetate	141786		x				Tasin et al., 2005
721	Butyl acetate	123864		x				Tasin et al., 2005
722	(Z)-3-Hexenyl acetate	3681718		x	x			Tasin et al., 2005
723	Methyl hexadecanoate	112390		x	x			Tasin et al., 2005
724	1-Methylethyl benzene	nf			x			Tasin et al., 2005
725	Propyl benzene	103651		x	x			Tasin et al., 2005
726	Methyl benzoate	93583		x				Tasin et al., 2005
727	Benzyl alcohol	100516	x	x	x			Tasin et al., 2005
728	Benzaldehyde	100527		x				Tasin et al., 2005
729	Methyl salicylate	119368	x		x			Tasin et al., 2005
730	beta-Ionone	79776	x					Tasin et al., 2005
731	4,8-Dimethyl-1,(E)3,7-nonatriene	19945610	x		x			Tasin et al., 2005
732	Limonene	138863			x			Tasin et al., 2005
733	(Z)-beta-Ocimene	3338554			x			Tasin et al., 2005
734	(E)-beta-Ocimene	3779611	x	x	x			Tasin et al., 2005
735	alpha-Phellandrene	99832			x			Tasin et al., 2005
736	alpha-terpineol	98555	x		x			Tasin et al., 2005
737	Geraniol	106241	x					Tasin et al., 2005
738	Linalool	78706		x				Tasin et al., 2005

Nr.	Name	CAS	l	b	f	s	other	reference
739	beta-caryophyllene	87445			x			Tasin et al., 2005
740	Humulene	6753986			x			Tasin et al., 2005
741	Germacrene-D	23986745	x					Tasin et al., 2005
742	Methyl farnesoate	10485708			x			Tasin et al., 2005
743	(E)-beta-Farnesene	18794848			x			Tasin et al., 2005
744	(Z,E)-alpha-Farnesene	26560145	x	x	x			Tasin et al., 2005
745	(E,E)-alpha-Farnesene	502614	x	x	x			Tasin et al., 2005
746	(Z)-3-Hexenyl acetate	3681718		x				Kalua and Boss, 2009
747	(Z)-3-Hexenyl butanoate	16491364		x				Kalua and Boss, 2009
748	(E)-2-Hexenal	6728263		x				Kalua and Boss, 2009
749	Hexanal	66251		x				Kalua and Boss, 2009
750	Heptanal	111717		x				Kalua and Boss, 2009
751	Pentanal	110623		x				Kalua and Boss, 2009
752	Hexan-1-ol	111273		x				Kalua and Boss, 2009
753	(Z)-3-Hexen-1-ol	928961		x				Kalua and Boss, 2009
754	Eucalyptol (1,8-cineole)	470826		x				Kalua and Boss, 2009
755	beta-caryophyllene	87445		x				Kalua and Boss, 2009
756	beta-caryophyllene	87445		x				Kalua and Boss, 2009
757	2-Phenylethanol	60128		x				Kalua and Boss, 2009
758	trans-Furan linalool oxide	34995772		x				Tamborra and Esti, 2010
759	cis-Furan linalool oxide	5989333		x				Tamborra and Esti, 2010
760	Linalool	78706		x				Tamborra and Esti, 2010
761	trans-Pyran linalool oxide	39028585		x				Tamborra and Esti, 2010
762	cis-Pyran linalool oxide	14009713		x				Tamborra and Esti, 2010
763	2,6-Dimethyl-3,7-octadien-2,6-diol	13741214		x				Tamborra and Esti, 2010
764	2,6-Dimethyl-7-octen-2,6-diol	nf		x				Tamborra and Esti, 2010
765	8-Hydroxydihydrolinalool	nf		x				Tamborra and Esti, 2010
766	trans-8-Hydroxylinalool	75991616		x				Tamborra and Esti, 2010
767	cis-8-Hydroxylinalool	103619063		x				Tamborra and Esti, 2010
768	Nerol	106252		x				Tamborra and Esti, 2010
769	Geraniol	106241		x				Tamborra and Esti, 2010
770	Hydroxycitronellol	107744		x				Tamborra and Esti, 2010
771	Hydroxynerol	nf		x				Tamborra and Esti, 2010
772	Hydroxygeraniol	nf		x				Tamborra and Esti, 2010
773	Geranic acid	459803		x				Tamborra and Esti, 2010
774	alpha-Terpineol	98555		x				Tamborra and Esti, 2010
775	2-Hydroxy-1,8-cineole	nf		x				Tamborra and Esti, 2010
776	Terpine 1	nf		x				Tamborra and Esti, 2010
777	p-menth-1-ene-7,8-diol	5502749		x				Tamborra and Esti, 2010
778	5,6-Epoxy-beta-ionon	23267574		x				Tamborra and Esti, 2010
779	3-Hydroxy-beta-damascone	35734613		x				Tamborra and Esti, 2010

Nr.	Name	CAS	l	b	f	s	other	reference
780	3-Oxo-alpha-ionol	34318213		x				Tamborra and Esti, 2010
781	3,9-Dihydroxymegastigma-5-ene	nf		x				Tamborra and Esti, 2010
782	3-Hydroxy-beta-ionon	116296754		x				Tamborra and Esti, 2010
783	Vomifoliol	23526456		x				Tamborra and Esti, 2010
784	Benzoic aldehyde	100527		x				Tamborra and Esti, 2010
785	Methyl salicylate	119368		x				Tamborra and Esti, 2010
786	alpha-Methyl benzenmethanol	nf		x				Tamborra and Esti, 2010
787	Benzyl alcohol	100516		x				Tamborra and Esti, 2010
788	2-Phenyl ethanol	60128		x				Tamborra and Esti, 2010
789	Eugenol	97530		x				Tamborra and Esti, 2010
790	Vanillin	121335		x				Tamborra and Esti, 2010
791	Methyl vanillate	3943746		x				Tamborra and Esti, 2010
792	Acetovanillone	498022		x				Tamborra and Esti, 2010
793	Zingerone	122485		x				Tamborra and Esti, 2010
794	Vanillic alcohol	498000		x				Tamborra and Esti, 2010
795	Butirrovanillone	nf		x				Tamborra and Esti, 2010
796	Homovanillic alcohol	2380781		x				Tamborra and Esti, 2010
797	Syringaldehyde	134963		x				Tamborra and Esti, 2010
798	Methyl syringate	884355		x				Tamborra and Esti, 2010
799	Dihydroconiferyl alcohol	2305137		x				Tamborra and Esti, 2010
800	Hexanol	111273		x				Tamborra and Esti, 2010
801	cis-3-Hexenol	928961		x				Tamborra and Esti, 2010
802	trans-2-Hexenol	928950		x				Tamborra and Esti, 2010
803	Myrcene	123353		x				Fenoll et al., 2009
804	Limonene	138863		x				Fenoll et al., 2009
805	Rose oxide I (trans)	5258117		x				Fenoll et al., 2009
806	Rose oxide II (cis)	3033236		x				Fenoll et al., 2009
807	Linaloloxide I	nf		x				Fenoll et al., 2009
808	Linaloloxide II	nf		x				Fenoll et al., 2009
809	Benzaldehyde	100527		x				Fenoll et al., 2009
810	Linalool	78706		x				Fenoll et al., 2009
811	Methyl benzoate	93583		x				Fenoll et al., 2009
812	Acetophenone	98862		x				Fenoll et al., 2009
813	alpha-Terpineol	98555		x				Fenoll et al., 2009
814	Citral	5392405		x				Fenoll et al., 2009
815	Benzyl acetate	140114		x				Fenoll et al., 2009
816	Citronellol	106229		x				Fenoll et al., 2009
817	Methylsalicylate	119368		x				Fenoll et al., 2009
818	Nerol	106252		x				Fenoll et al., 2009
819	Geraniol	106241		x				Fenoll et al., 2009
820	Benzyl alcohol	100516		x				Fenoll et al., 2009

Nr.	Name	CAS	l	b	f	s	other	reference
821	2-Phenylethanol	60128		x				Fenoll et al., 2009
822	Diendiol I	nf		x				Fenoll et al., 2009
823	Isopropyl myristinate	nf		x				Fenoll et al., 2009
824	o-Cresol	95487		x				Fenoll et al., 2009
825	Diphenylether	32575517		x				Fenoll et al., 2009
826	p-Cresol	106445		x				Fenoll et al., 2009
827	m-Cresol	108394		x				Fenoll et al., 2009
828	Diendiol II	nf		x				Fenoll et al., 2009
829	Eugenol	97530		x				Fenoll et al., 2009
830	2,6-Dimethoxyphenol	91101		x				Fenoll et al., 2009
831	Vanillin	121335		x				Fenoll et al., 2009
832	Benzyl Benzoate	120514		x				Fenoll et al., 2009
833	Hexanal	66251		x				Yang et al., 2009
834	trans-2-Hexenal	6728263		x				Yang et al., 2009
835	cis-3-Hexenol	928961		x				Yang et al., 2009
836	trans2-Hexenol	928950		x				Yang et al., 2009
837	1-Hexanol	111273		x				Yang et al., 2009
838	1-Octen-3-ol	3391864		x				Yang et al., 2009
839	2-Ethylhexanol	104767		x				Yang et al., 2009
840	1-Octanol	111875		x				Yang et al., 2009
841	Phenylethyl alcohol	60128		x				Yang et al., 2009
842	Heptanal	111717		x				Yang et al., 2009
843	Benzeneacetaldehyde	122781		x				Yang et al., 2009
844	trans-2-Octenal	2548870		x				Yang et al., 2009
845	Mesifuran	4077478		x				Yang et al., 2009
846	Nonanal	124196		x				Yang et al., 2009
847	Ethyl acetate	141786		x				Yang et al., 2009
848	Isopropyl acetate	108214		x				Yang et al., 2009
849	Ethyl propanoate	105373		x				Yang et al., 2009
850	Propyl acetate	109604		x				Yang et al., 2009
851	Methyl butanoate	623427		x				Yang et al., 2009
852	Ethyl isobutanoate	97621		x				Yang et al., 2009
853	Ethyl butanoate	105544		x				Yang et al., 2009
854	Ethyl 2-butenoate	10544635		x				Yang et al., 2009
855	Ethyl pentanoate	539822		x				Yang et al., 2009
856	Methyl hexanoate	106707		x				Yang et al., 2009
857	Ethyl 3-hydroxybutanoate	5405414		x				Yang et al., 2009
858	Ethyl tiglate	5837785		x				Yang et al., 2009
859	Ethyl trans-2-pentenoate	24410842		x				Yang et al., 2009
860	Ethyl hexanoate	123660		x				Yang et al., 2009
861	Hexyl acetate	142927		x				Yang et al., 2009

Nr.	Name	CAS	l	b	f	s	other	reference
862	trans-2-Hexenyl acetate	2497189		x				Yang et al., 2009
863	Isopropyl hexanoate	2311468		x				Yang et al., 2009
864	Ethyl 2-hexenoate	1552676		x				Yang et al., 2009
865	Ethyl heptanoate	106309		x				Yang et al., 2009
866	Ethyl benzoate	93890		x				Yang et al., 2009
867	Diethyl butanedioate	123251		x				Yang et al., 2009
868	Ethyl octanoate	106321		x				Yang et al., 2009
869	Ethyl benzeneacetate	101973		x				Yang et al., 2009
870	Ethyl 2-octenoate	2351908		x				Yang et al., 2009
871	2-Phenethyl acetate	103457		x				Yang et al., 2009
872	Diethyl pentanedioate	818382		x				Yang et al., 2009
873	4-Decenoic acid, ethyl ester	6142445		x				Yang et al., 2009
874	Ethyl decanoate	110383		x				Yang et al., 2009
875	Ethyl 2,4-decadienoate	37549749		x				Yang et al., 2009
876	Eucalyptol	470826		x				Yang et al., 2009
877	trans-Furan linalool oxide	34995772		x				Yang et al., 2009
878	cis-Furan linalool oxide	5989333		x				Yang et al., 2009
879	Linalool	78706		x				Yang et al., 2009
880	cis-Rose oxide	3033236		x				Yang et al., 2009
881	trans-Rose oxide	5258117		x				Yang et al., 2009
882	Nerol oxide	1786089		x				Yang et al., 2009
883	trans-Pyran linalool oxide	39028585		x				Yang et al., 2009
884	4-Terpineol	562743		x				Yang et al., 2009
885	cis-Pyran linalool oxide	14009713		x				Yang et al., 2009
886	alpha-Terpineol	98555		x				Yang et al., 2009
887	Nerol	106252		x				Yang et al., 2009
888	Citronellol	106229		x				Yang et al., 2009
889	Neral	106263		x				Yang et al., 2009
890	Geraniol	106241		x				Yang et al., 2009
891	Geranial	141275		x				Yang et al., 2009
892	Geranic acid	459803		x				Yang et al., 2009
893	Sitosterol	83465	x					Batovska et al., 2010
894	Sitosterol acetate	915059	x					Batovska et al., 2010
895	Campesterol	474624	x					Batovska et al., 2010
896	Stigmasterol	83487	x					Batovska et al., 2010
897	Cholesterol	57885	x					Batovska et al., 2010
898	Pinane	473552	x					Batovska et al., 2010
899	alpha-Tocopherol	1406184	x					Batovska et al., 2010
900	gamma-Tocopherol	7616220	x					Batovska et al., 2010
901	delta-Tocopherol	119131	x					Batovska et al., 2010
902	beta-Amyrine	559706	x					Batovska et al., 2010

Nr.	Name	CAS	l	b	f	s	other	reference
903	Lupeol	545471	x					Batovska et al., 2010
904	4,8,12,16-Tetramethylheptadecane-4-olide	200272613	x					Batovska et al., 2010
905	Tetradecanoic acid	544638	x					Batovska et al., 2010
906	Hexadecanoic acid	57103	x					Batovska et al., 2010
907	Octadecanoic acid	57114	x					Batovska et al., 2010
908	Decyl isobutyrate	5454228	x					Batovska et al., 2010
909	Methyl tetradecanoate	124107	x					Batovska et al., 2010
910	14,16-Hentriacontanedione	24724843	x					Batovska et al., 2010
911	2,3-Dihydro-3,5-dihydroxy-6-methylpyran-4-one	28564832	x					Batovska et al., 2010
912	2,3-Dihydrobenzofuran	496162	x					Batovska et al., 2010
913	Thymine	65714	x					Batovska et al., 2010
914	Ethyl acetate	141786		x				Fan et al., 2009
915	3-Methylbutanal	590863		x				Fan et al., 2009
916	ethyl butanoate	105544		x				Fan et al., 2009
917	Ethyl-2-methylbutanoate	nf		x				Fan et al., 2009
918	Hexanal	66251		x				Fan et al., 2009
919	2-Methylpropanol	78831		x				Fan et al., 2009
920	1-Butanol	71363		x				Fan et al., 2009
921	(E)-2-Hexenal	6728263		x				Fan et al., 2009
922	3-Methylbutanol	6423069		x				Fan et al., 2009
923	ethyl hexanoate	123660		x				Fan et al., 2009
924	1-Octen-3-one	4312996		x				Fan et al., 2009
925	1-Hexanol	111273		x				Fan et al., 2009
926	(E)-3-Hexen-1-ol	928972		x				Fan et al., 2009
927	(Z)-3-Hexen-1-ol	928961		x				Fan et al., 2009
928	ethyl octanoate	106321		x				Fan et al., 2009
929	Acetic acid	64197		x				Fan et al., 2009
930	1-Octen-3-ol	3391864		x				Fan et al., 2009
931	2-Ethyl-1-hexanol	104767		x				Fan et al., 2009
932	Decanal	112312		x				Fan et al., 2009
933	Benzaldehyde	100527		x				Fan et al., 2009
934	Linalool	78706		x				Fan et al., 2009
935	2-Methylpropanoic acid	79312		x				Fan et al., 2009
936	(E,Z)-2,6-Nonadienal	557482		x				Fan et al., 2009
937	4-Terpineol	562743		x				Fan et al., 2009
938	Butanoic acid	107926		x				Fan et al., 2009
939	Phenylacetaldehyde	122781		x				Fan et al., 2009
940	ethyl decanoate	110383		x				Fan et al., 2009
941	Acetophenone	98862		x				Fan et al., 2009
942	Ethyl benzoate	93890		x				Fan et al., 2009
943	1-Nonanol	143088		x				Fan et al., 2009

Nr.	Name	CAS	l	b	f	s	other	reference
944	3-Methylbutanoic acid	503742		x				Fan et al., 2009
945	alpha-Terpineol	98555		x				Fan et al., 2009
946	Naphthalene	91203		x				Fan et al., 2009
947	Ethyl-2-phenylacetate	101973		x				Fan et al., 2009
948	Methyl 2-hydroxybenzoate	119368		x				Fan et al., 2009
949	Anethole	104461		x				Fan et al., 2009
950	beta-Damascenone	36649635		x				Fan et al., 2009
951	Hexanoic acid	142621		x				Fan et al., 2009
952	(Z)-Geranylacetone	3879263		x				Fan et al., 2009
953	Benzyl alcohol	100516		x				Fan et al., 2009
954	2-Phenylethanol	60128		x				Fan et al., 2009
955	beta-Ionone	79776		x				Fan et al., 2009
956	Benzothiazolec	95169		x				Fan et al., 2009
957	gamma-Nonalactone	104610		x				Fan et al., 2009
958	Octanoic acid	124072		x				Fan et al., 2009
959	4-Methylphenol	106445		x				Fan et al., 2009
960	Nonanoic acid	112050		x				Fan et al., 2009
961	4-Ethylphenol	123079		x				Fan et al., 2009
962	Vanillin	121335		x				Fan et al., 2009
963	Acetovanillone	498022		x				Fan et al., 2009
964	14,16-Hentriacontanedione	24724843	x					Batovska et al., 2009b
965	2-Decenal	3913711	x					Batovska et al., 2009b
966	2-Pentenal	764396	x					Batovska et al., 2009b
967	2-Undecenal	2463776	x					Batovska et al., 2009b
968	4-(3-Cyclohexen-1-yl)-3-buten-2-one	nf	x					Batovska et al., 2009b
969	4-(3-Cyclohexen-1-yl)-3-buten-2-one (isomer)	nf	x					Batovska et al., 2009b
970	4,8,12,16-Tetramethylheptadecan-4-olide	200272613	x					Batovska et al., 2009b
971	4-Methyl-3-penten-2-one	141797	x					Batovska et al., 2009b
972	4-Methylacetophenone	122009	x					Batovska et al., 2009b
973	6,10,14-Trimethyl-pentadecan-2-one	502692	x					Batovska et al., 2009b
974	alpha-Tocopherol	1406184	x					Batovska et al., 2009b
975	beta-amyrine	559706	x					Batovska et al., 2009b
976	Decanal	112312	x					Batovska et al., 2009b
977	Decyl isobutyrate	5454228	x					Batovska et al., 2009b
978	delta-Tocopherol	119131	x					Batovska et al., 2009b
979	Docosane	629970	x					Batovska et al., 2009b
980	Eicosane	112958	x					Batovska et al., 2009b
981	Ethyl linoleate	544354	x					Batovska et al., 2009b
982	gamma-Terpinene	99854	x					Batovska et al., 2009b
983	Heneicosane	629947	x					Batovska et al., 2009b
984	Hentriacontane	630046	x					Batovska et al., 2009b

Nr.	Name	CAS	l	b	f	s	other	reference
985	Heptacosane	593497	x					Batovska et al., 2009b
986	Hexadecanoic acid	57103	x					Batovska et al., 2009b
987	Hexadecanol	36653824	x					Batovska et al., 2009b
988	Lupeol	545471	x					Batovska et al., 2009b
989	Methyl eicosanoate	1120281	x					Batovska et al., 2009b
990	Methyl hexadecanoate	112390	x					Batovska et al., 2009b
991	Methyl tetracosanoate	2442491	x					Batovska et al., 2009b
992	Methyl tetradecanoate	124107	x					Batovska et al., 2009b
993	Neophytadiene	504961	x					Batovska et al., 2009b
994	Neophytadiene (isomer)	504961	x					Batovska et al., 2009b
995	Nonacosane	630035	x					Batovska et al., 2009b
996	Nonadecan	629925	x					Batovska et al., 2009b
997	Nonadecanone	82986558	x					Batovska et al., 2009b
998	Nonanal	124196	x					Batovska et al., 2009b
999	Octacosane	630024	x					Batovska et al., 2009b
1000	Octadecan	593453	x					Batovska et al., 2009b
1001	Octadecanoic acid	57114	x					Batovska et al., 2009b
1002	Pentacosane	629992	x					Batovska et al., 2009b
1003	Pentacosanone	104921439	x					Batovska et al., 2009b
1004	Pentadecanone	76940915	x					Batovska et al., 2009b
1005	Sitosterol	83465	x					Batovska et al., 2009b
1006	Tetracosane	646311	x					Batovska et al., 2009b
1007	Tetradecanoic acid	544638	x					Batovska et al., 2009b
1008	Tetradecanol	112721	x					Batovska et al., 2009b
1009	Triacontane	638686	x					Batovska et al., 2009b
1010	Tricosane	638675	x					Batovska et al., 2009b
1011	Octadecane	593453		x				Todorova et al., 2010
1012	Octadecene	27070582		x				Todorova et al., 2010
1013	Nonadecane	629925		x				Todorova et al., 2010
1014	Icosane	112958		x				Todorova et al., 2010
1015	Icosene	112958		x				Todorova et al., 2010
1016	Henicosane	629947		x				Todorova et al., 2010
1017	Docosane	629970		x				Todorova et al., 2010
1018	Tricosane	638675		x				Todorova et al., 2010
1019	Pentacosane	629992		x				Todorova et al., 2010
1020	Hexacosene	64808919		x				Todorova et al., 2010
1021	Heptacosane	593497		x				Todorova et al., 2010
1022	Sitosterol	83465		x				Todorova et al., 2010
1023	alpha-Amorphene	483750		x				Todorova et al., 2010
1024	T-Muurolol	19912620		x				Todorova et al., 2010
1025	Cyperene	2387782		x				Todorova et al., 2010

Nr.	Name	CAS	l	b	f	s	other	reference
1026	Neophytadiene	504961		x				Todorova et al., 2010
1027	Phytol	7541493		x				Todorova et al., 2010
1028	beta-Amyrine	559706		x				Todorova et al., 2010
1029	Lupeol	545471		x				Todorova et al., 2010
1030	6,10,14-Trimethylpentadecan-2-one	502692		x				Todorova et al., 2010
1031	4,8,12,16-Tetramethylheptadecane-4-olide	200272613		x				Todorova et al., 2010
1032	Octadecanal	638664		x				Todorova et al., 2010
1033	Hexacosanal	26627850		x				Todorova et al., 2010
1034	Hexadecanoic acid	57103		x				Todorova et al., 2010
1035	1-Methylethyl dodecanoate	10233133		x				Todorova et al., 2010
1036	Isopropyl tetradecanoate	110270		x				Todorova et al., 2010
1037	Ethyl hexadecanoate	628977		x				Todorova et al., 2010
1038	Ethyl octadecanoate	111625		x				Todorova et al., 2010
1039	Ethyl octadecenoate	111625		x				Todorova et al., 2010
1040	Ethyl octadecadienoate	nf		x				Todorova et al., 2010
1041	Ethyl icosanoate	18281055		x				Todorova et al., 2010
1042	Methyl docosanoate	929771		x				Todorova et al., 2010
1043	Methyl tricosanoate	2433978		x				Todorova et al., 2010
1044	Methyl tetracosanoate	2442491		x				Todorova et al., 2010
1045	Ethyl tetracosanoate	24634955		x				Todorova et al., 2010
1046	Methyl 2,9-dimethylpentacosanoate	nf		x				Todorova et al., 2010
1047	2-Ethylhexyl benzoate	5444757		x				Todorova et al., 2010
1048	Guaiacol	90051		x				Hayasaka et al., 2010
1049	4-Methylguaiacol	93516		x				Hayasaka et al., 2010
1050	o-Cresol	95487		x				Hayasaka et al., 2010
1051	Phenol	108952		x				Hayasaka et al., 2010
1052	p-Cresol	106445		x				Hayasaka et al., 2010
1053	m-Cresol	108394		x				Hayasaka et al., 2010
1054	Syringol	91101		x				Hayasaka et al., 2010
1055	4-Methylsyringol	6638057		x				Hayasaka et al., 2010
1056	1,3,6-Octatriene, 3,7-dimethyl-	13877913					root	Du et al., 2009
1057	1,3-Cyclohexadiene, 1,3,5,5,6,6-hexamethyl-	959004898					root	Du et al., 2009
1058	1,3-Hexadiene, 3-ethyl-2,5-dimethyl-	62338072					root	Du et al., 2009
1059	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	nf					root	Du et al., 2009
1060	1-[2-Methyl-3-(methylthio) allyl] cyclohex-2-enol	nf					root	Du et al., 2009
1061	11-Hexadecenoic acid, methyl ester	55000425					root	Du et al., 2009
1062	14-Isopentyl-8,13-dimethylpodocarpane	nf					root	Du et al., 2009
1063	17-Pentatriacontene	6971400					root	Du et al., 2009
1064	1-Hentetracontanol	40710427					root	Du et al., 2009
1065	1-Pentanol, 4-methyl-	626891					root	Du et al., 2009
1066	1-Undecene	821954					root	Du et al., 2009

Nr.	Name	CAS	l	b	f	s	other	reference
1067	2,2-Dimethyl-3-methylenebicyclo[2.2.1]heptane	79925					root	Du et al., 2009
1068	2,3-Heptanedione	96048					root	Du et al., 2009
1069	2-Isopropyl-5-methyl-1-hexanol	2051334					root	Du et al., 2009
1070	2-Pinen-10-ol	515004					root	Du et al., 2009
1071	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	7541493					root	Du et al., 2009
1072	3,7-Dimethyl-6-nonen-1-ol	19550540					root	Du et al., 2009
1073	3-Hexen-1-ol	544127					root	Du et al., 2009
1074	3-Isopropyl-6,7-dimethyltricyclo[4.4.0.0(2,8)]decane-9,10-diol	nf					root	Du et al., 2009
1075	4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]unde	nf					root	Du et al., 2009
1076	4-Methyl-2-oxovaleric acid	816660					root	Du et al., 2009
1077	9,12-Octadecadienoic acid, methyl ester	2462853					root	Du et al., 2009
1078	9-Hexadecenoic acid, methyl ester, (Z)-	1120258					root	Du et al., 2009
1079	Acetaldehyde, (3,3-dimethylcyclohexylidene)-, (E)	26532252					root	Du et al., 2009
1080	Allopregnane	641850					root	Du et al., 2009
1081	alpha-Pinene	80568					root	Du et al., 2009
1082	Azulene	275514					root	Du et al., 2009
1083	Benzonorbornadiene	4453901					root	Du et al., 2009
1084	beta-Iso-methyl ionone	79890					root	Du et al., 2009
1085	Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-	5947364					root	Du et al., 2009
1086	Bicyclo[7.2.0]undecane,10,10-dimethyl-2,6-bis(methylene)-,[1S-(1R*,9S*)]	nf					root	Du et al., 2009
1087	Butyl aldoxime, 2-methyl-, syn-	53061044					root	Du et al., 2009
1088	Carbonic acid, allyl heptyl ester	nf					root	Du et al., 2009
1089	Caryophyllene	87445					root	Du et al., 2009
1090	Cholestane	481210					root	Du et al., 2009
1091	Cyclodocosane, ethyl-	934589421					root	Du et al., 2009
1092	Cyclohexene, 4-pentyl-1-(4-propylcyclohexyl)-	172098452					root	Du et al., 2009
1093	Cyclohexene,3-butyl-	3983071					root	Du et al., 2009
1094	Decanal	112312					root	Du et al., 2009
1095	Decane, 2,3,5,8-tetramethyl-	192823157					root	Du et al., 2009
1096	Dibutyl phthalate	84742					root	Du et al., 2009
1097	Diethyl Phthalate	84662					root	Du et al., 2009
1098	D-Limonene	5989275					root	Du et al., 2009
1099	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	959012078					root	Du et al., 2009
1100	Elaidic acid, methyl ester	1397628					root	Du et al., 2009
1101	Estradiol, 3-deoxy-	2529648					root	Du et al., 2009
1102	Ethanol, 1-(2-butoxyethoxy)-	54446785					root	Du et al., 2009
1103	Eucalyptol	470826					root	Du et al., 2009
1104	Geranyl isovalerate	109206					root	Du et al., 2009
1105	Heptadecane	629787					root	Du et al., 2009
1106	Heptadecane, 2,6,10,15-tetramethyl-	54833486					root	Du et al., 2009
1107	Hexadecanoic acid, 14-methyl-, methyl ester	2490495					root	Du et al., 2009

Nr.	Name	CAS	l	b	f	s	other	reference
1108	Hexadecanoic acid, 15-methyl-, methyl ester	6929040					root	Du et al., 2009
1109	Hexadecanoic acid, methyl ester	112390					root	Du et al., 2009
1110	Hexanal	66251					root	Du et al., 2009
1111	Hexatriacontane	630068					root	Du et al., 2009
1112	Hexyl chloroformate	6092542					root	Du et al., 2009
1113	L-Camphor	464482					root	Du et al., 2009
1114	L-Fenchone	7787204					root	Du et al., 2009
1115	Linoleic acid, methyl ester	112630					root	Du et al., 2009
1116	m-Cymene	535773					root	Du et al., 2009
1117	Methyl 8-(2-furyl)octanoate	38199507					root	Du et al., 2009
1118	Nonanal	124196					root	Du et al., 2009
1119	Nonanoic acid, 9-oxo-, methyl ester	1931631					root	Du et al., 2009
1120	Octadecanoic acid, methyl ester	112618					root	Du et al., 2009
1121	Oxalic acid, dodecyl 3,5-difluorophenyl ester	nf					root	Du et al., 2009
1122	Pregn-20-yn-17-ol	127995395					root	Du et al., 2009
1123	Sclareoloxide	nf					root	Du et al., 2009
1124	Stigmastane	601581					root	Du et al., 2009
1125	Sulfurous acid, cyclohexylmethyl hexadecyl ester	959302411					root	Du et al., 2009
1126	Tetradecane, 2,6,10-trimethyl-	14905567					root	Du et al., 2009
1127	Tetratriacontane	14167590					root	Du et al., 2009
1128	Triacontane	638686					root	Du et al., 2009
1129	Tritetracontane	7098217					root	Du et al., 2009
1130	Ylangene	14912448					root	Du et al., 2009
1131	alpha-Cedrene	469614					root	Du et al., 2009
1132	Hexanal	66251		x				Kalua and Boss, 2010
1133	E-2-Hexenal	6728263		x				Kalua and Boss, 2010
1134	Hexanol	111273		x				Kalua and Boss, 2010
1135	Z-3-Hexenyl butanoate	16491364		x				Kalua and Boss, 2010
1136	Hexyl acetate	142927		x				Kalua and Boss, 2010
1137	Geraniol	106241		x				Kalua and Boss, 2010
1138	beta-Ionone	79776		x				Kalua and Boss, 2010
1139	alpha-Caryophyllene	6753986		x				Kalua and Boss, 2010
1140	alpha-Muurolene	31983229		x				Kalua and Boss, 2010
1141	Calamene	1406504		x				Kalua and Boss, 2010
1142	Octanal	124130		x				Kalua and Boss, 2010
1143	2,4-Hexadienal	80466348		x				Kalua and Boss, 2010
1144	Z-3-Hexen-1-ol	928961		x				Kalua and Boss, 2010
1145	2,2,6-Trimethyl-cyclohexanone	2408379		x				Kalua and Boss, 2010
1146	Eucalyptol	470826		x				Kalua and Boss, 2010
1147	alpha-Copaene	3856255		x				Kalua and Boss, 2010
1148	alpha-Cubebene	17699148		x				Kalua and Boss, 2010

Nr.	Name	CAS	l	b	f	s	other	reference
1149	alpha-Gurjunene	489407		x				Kalua and Boss, 2010
1150	gamma-Muurolene	30021740		x				Kalua and Boss, 2010
1151	alpha-Muurolene	31983229		x				Kalua and Boss, 2010
1152	Galamenene	nf		x				Kalua and Boss, 2010
1153	Nonanal	124196		x				Kalua and Boss, 2010
1154	2-Heptanol	543497		x				Kalua and Boss, 2010
1155	Methyl hexanoate	106707		x				Kalua and Boss, 2010
1156	Benzyl alcohol	100516		x				Kalua and Boss, 2010
1157	Benzaldehyde	100527		x				Kalua and Boss, 2010
1158	2-Phenyl ethanol	60128		x				Kalua and Boss, 2010
1159	Geraniol	106241		x				Gunata et al., 1985
1160	Linalool	78706		x				Gunata et al., 1985
1161	Nerol	106252		x				Gunata et al., 1985
1162	alpha-Terpineol	98555		x				Gunata et al., 1985
1163	Citronellol	106229		x				Gunata et al., 1985
1164	2-Phenylethynol	nf		x				Gunata et al., 1985
1165	Benzyl alcohol	100516		x				Gunata et al., 1985
1166	Geraniol	106241	x					Gunata et al., 1985
1167	Linalool	78706	x					Gunata et al., 1985
1168	Nerol	106252	x					Gunata et al., 1985
1169	alpha-Terpineol	98555	x					Gunata et al., 1985
1170	Citronellol	106229	x					Gunata et al., 1985
1171	2-Phenylethynol	nf	x					Gunata et al., 1985
1172	Benzyl alcohol	100516	x					Gunata et al., 1985
1173	p-Cymene	99876		x				Parker et al., 2007
1174	Limonene	138863		x				Parker et al., 2007
1175	beta-Phellandrene	555102		x				Parker et al., 2007
1176	1,8-cineole	470826		x				Parker et al., 2007
1177	cis-Rose oxide	3033236		x				Parker et al., 2007
1178	Camphor	76222		x				Parker et al., 2007
1179	cis-linalool oxide (pyran)	14009713		x				Parker et al., 2007
1180	trans-Linalool oxide (pyran)	39028585		x				Parker et al., 2007
1181	Carvone	99490		x				Parker et al., 2007
1182	Geraniol	106241		x				Parker et al., 2007
1183	Cyclosativene	22469529		x				Parker et al., 2007
1184	alpha-Ylangene	14912448		x				Parker et al., 2007
1185	beta-Bourbonene	5208593		x				Parker et al., 2007
1186	beta-Ylangene	20479065		x				Parker et al., 2007
1187	beta-Copaene	18252443		x				Parker et al., 2007
1188	Guaia-6,9-diene	37839648		x				Parker et al., 2007
1189	Selina-3,7-diene	10064343		x				Parker et al., 2007

Nr.	Name	CAS	l	b	f	s	other	reference
1190	gamma-Muurolene	30021740		x				Parker et al., 2007
1191	Bicyclosesquiphellandrene	54324037		x				Parker et al., 2007
1192	alpha-Amorphene	483750		x				Parker et al., 2007
1193	epizonarene	41702630		x				Parker et al., 2007
1194	Zonarene	nf		x				Parker et al., 2007
1195	gamma-Cadinene	39029419		x				Parker et al., 2007
1196	Isocalamenene	68566701		x				Parker et al., 2007
1197	alpha-Cadinene	24406051		x				Parker et al., 2007
1198	alpha-Calacorene	21391991		x				Parker et al., 2007
1199	beta-Calacorene	50277344		x				Parker et al., 2007
1200	Cadelene	483783		x				Parker et al., 2007
1201	1,2,3-Butanetriol	4435501	x					Batovska et al., 2008, suppl. info
1202	1,3-Butanediol	107880	x					Batovska et al., 2008, suppl. info
1203	1,3-Propanediol	504632	x					Batovska et al., 2008, suppl. info
1204	11-Butyl docosane	13475768	x					Batovska et al., 2008, suppl. info
1205	1-Cyclohexene-1-carboxylic acid	636828	x					Batovska et al., 2008, suppl. info
1206	1-Hydroxy-4-ethyloxy-phenyl-beta-D-glucopyranoside	nf	x					Batovska et al., 2008, suppl. info
1207	2,3-Dihydro-3,5-dihydroxy-6-methylpyrane-4-one	28564832	x					Batovska et al., 2008, suppl. info
1208	2,3-Dihydrobenzofurane	496162	x					Batovska et al., 2008, suppl. info
1209	2,3-Dihydroxybutanoic acid	3413976	x					Batovska et al., 2008, suppl. info
1210	2,4-Butanediol	107880	x					Batovska et al., 2008, suppl. info
1211	2,5-Furandione	108316	x					Batovska et al., 2008, suppl. info
1212	24-Methylene-9,19-cyclolanostan-3-ol	nf	x					Batovska et al., 2008, suppl. info
1213	2-Butenal	4170303	x					Batovska et al., 2008, suppl. info
1214	2-Ethylhexanoic acid	149575	x					Batovska et al., 2008, suppl. info
1215	2-Methoxypropanoic acid	4324372	x					Batovska et al., 2008, suppl. info
1216	2-Methyleicosane	1560845	x					Batovska et al., 2008, suppl. info
1217	2-Methylheptadecane	1560890	x					Batovska et al., 2008, suppl. info
1218	2-Methylnaphtalene	91576	x					Batovska et al., 2008, suppl. info
1219	2-Methylpentadecane	1560936	x					Batovska et al., 2008, suppl. info
1220	3,4,5-Trimethoxyacetophenone	1136863	x					Batovska et al., 2008, suppl. info
1221	3-Hydroxy-2,4-dimethylpentanoic acid	nf	x					Batovska et al., 2008, suppl. info
1222	3-Hydroxybutanoic acid	300856	x					Batovska et al., 2008, suppl. info
1223	3-Hydroxycaproic acid	10191249	x					Batovska et al., 2008, suppl. info
1224	3-Methylnaphtalene	nf	x					Batovska et al., 2008, suppl. info
1225	3-Methyltetradecane	18435228	x					Batovska et al., 2008, suppl. info
1226	4,8,12,16-Tetramethylheptadecan-4-olide	200272613	x					Batovska et al., 2008, suppl. info
1227	4-Methylcyclohexan-1-ol	7731295	x					Batovska et al., 2008, suppl. info
1228	5,6,7,7a-Tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	15356748	x					Batovska et al., 2008, suppl. info
1229	5-Hydroxy-7-methoxy-2-phenyl-4H-1-benzopyran-4-one	520285	x					Batovska et al., 2008, suppl. info
1230	5-Hydroxymethyl-2-furanecarboxaldehyde	67470	x					Batovska et al., 2008, suppl. info

Nr.	Name	CAS	l	b	f	s	other	reference
1231	6-Methyl-2H-1-benzopyran-2-one	92488	x					Batovska et al., 2008, suppl. info
1232	8-Methyloctahydronaphtalen-2-one	nf	x					Batovska et al., 2008, suppl. info
1233	9,19-Cyclolanostan-24-en-3-ol	nf	x					Batovska et al., 2008, suppl. info
1234	9,19-Cyclolanostan-7-en-3-ol	nf	x					Batovska et al., 2008, suppl. info
1235	Aldohexose	50997	x					Batovska et al., 2008, suppl. info
1236	Arabinoic acid	13752835	x					Batovska et al., 2008, suppl. info
1237	Butyl butanoate	109217	x					Batovska et al., 2008, suppl. info
1238	Caffeic acid	331395	x					Batovska et al., 2008, suppl. info
1239	Campesterol	474624	x					Batovska et al., 2008, suppl. info
1240	Chloroacetophenone	532274	x					Batovska et al., 2008, suppl. info
1241	Cholesterol	57885	x					Batovska et al., 2008, suppl. info
1242	Dihydroactidiolide	nf	x					Batovska et al., 2008, suppl. info
1243	Dimethylnaphtalene	65338081	x					Batovska et al., 2008, suppl. info
1244	Docosane	629970	x					Batovska et al., 2008, suppl. info
1245	Dodecane	112403	x					Batovska et al., 2008, suppl. info
1246	Eicosane	112958	x					Batovska et al., 2008, suppl. info
1247	Eicosene	27400788	x					Batovska et al., 2008, suppl. info
1248	Erythronic acid	nf	x					Batovska et al., 2008, suppl. info
1249	Ethyl palmitate	628977	x					Batovska et al., 2008, suppl. info
1250	Ethyl stearate	111615	x					Batovska et al., 2008, suppl. info
1251	Ethylenediol	nf	x					Batovska et al., 2008, suppl. info
1252	Fructose	7660255	x					Batovska et al., 2008, suppl. info
1253	Gallic acid	149917	x					Batovska et al., 2008, suppl. info
1254	Glyceric acid	473814	x					Batovska et al., 2008, suppl. info
1255	Glycerol	56815	x					Batovska et al., 2008, suppl. info
1256	Glyceryl 3-caprylate	nf	x					Batovska et al., 2008, suppl. info
1257	Glycine-*N*-phenylethyl ester	nf	x					Batovska et al., 2008, suppl. info
1258	Glycolic acid	79141	x					Batovska et al., 2008, suppl. info
1259	Henicosane	629947	x					Batovska et al., 2008, suppl. info
1260	Heptacosane	593497	x					Batovska et al., 2008, suppl. info
1261	Heptadecane	629787	x					Batovska et al., 2008, suppl. info
1262	Hexacosane	630013	x					Batovska et al., 2008, suppl. info
1263	Hexadecane	544763	x					Batovska et al., 2008, suppl. info
1264	Hexadecene	68855594	x					Batovska et al., 2008, suppl. info
1265	Hexahydrofarnesylacetone	502692	x					Batovska et al., 2008, suppl. info
1266	Isopropyl myristate	110270	x					Batovska et al., 2008, suppl. info
1267	Isopropyl palmitate	142916	x					Batovska et al., 2008, suppl. info
1268	Lactic acid	50215	x					Batovska et al., 2008, suppl. info
1269	Lauric acid	143077	x					Batovska et al., 2008, suppl. info
1270	Linoleic acid	60333	x					Batovska et al., 2008, suppl. info
1271	Linolenic acid	463401	x					Batovska et al., 2008, suppl. info

Nr.	Name	CAS	l	b	f	s	other	reference
1272	Lupeol	545471	x					Batovska et al., 2008, suppl. info
1273	Malic acid	6915157	x					Batovska et al., 2008, suppl. info
1274	Methyl ester of 11,14,17-eicosatrienoic acid	55682887	x					Batovska et al., 2008, suppl. info
1275	Methyl linolenate	301008	x					Batovska et al., 2008, suppl. info
1276	Monoethyl phosphate	1623149	x					Batovska et al., 2008, suppl. info
1277	Myoinositol	87898	x					Batovska et al., 2008, suppl. info
1278	Myristic acid	544638	x					Batovska et al., 2008, suppl. info
1279	Naphtalene diol	nf	x					Batovska et al., 2008, suppl. info
1280	Naphtalene diol (isomer)	nf	x					Batovska et al., 2008, suppl. info
1281	Nonacosane	630035	x					Batovska et al., 2008, suppl. info
1282	Nonadecane	629925	x					Batovska et al., 2008, suppl. info
1283	Norpristane	3892000	x					Batovska et al., 2008, suppl. info
1284	Octacosane	630024	x					Batovska et al., 2008, suppl. info
1285	Octadecane	593453	x					Batovska et al., 2008, suppl. info
1286	Octadecane	593453	x					Batovska et al., 2008, suppl. info
1287	Palmitic acid	57103	x					Batovska et al., 2008, suppl. info
1288	p-Coumaric acid	7400080	x					Batovska et al., 2008, suppl. info
1289	Pentacosane	629992	x					Batovska et al., 2008, suppl. info
1290	Pentadecane	629629	x					Batovska et al., 2008, suppl. info
1291	Pentadecylcyclohexane	6006957	x					Batovska et al., 2008, suppl. info
1292	Phosphoric acid	78400	x					Batovska et al., 2008, suppl. info
1293	p-Hydroxybenzaldehyde	123080	x					Batovska et al., 2008, suppl. info
1294	Phytane	638368	x					Batovska et al., 2008, suppl. info
1295	Phytol	7541493	x					Batovska et al., 2008, suppl. info
1296	Pinene	80568	x					Batovska et al., 2008, suppl. info
1297	Pristane	1921706	x					Batovska et al., 2008, suppl. info
1298	Sacharose	nf	x					Batovska et al., 2008, suppl. info
1299	Sitosterol	83465	x					Batovska et al., 2008, suppl. info
1300	Sitosterol acetate	915059	x					Batovska et al., 2008, suppl. info
1301	Sorbopyranose	7270771	x					Batovska et al., 2008, suppl. info
1302	Squalene	94016350	x					Batovska et al., 2008, suppl. info
1303	Stearic acid	57114	x					Batovska et al., 2008, suppl. info
1304	Stigma-4-en-3-one	nf	x					Batovska et al., 2008, suppl. info
1305	Stigmasta-3,5-dien-7-one	2034722	x					Batovska et al., 2008, suppl. info
1306	Stigmasterol	83487	x					Batovska et al., 2008, suppl. info
1307	Succinic acid	110156	x					Batovska et al., 2008, suppl. info
1308	Taraxerol	127220	x					Batovska et al., 2008, suppl. info
1309	Tartaric acid	87694	x					Batovska et al., 2008, suppl. info
1310	Tetracosane	646311	x					Batovska et al., 2008, suppl. info
1311	Tetradecane	629594	x					Batovska et al., 2008, suppl. info
1312	Tetradecyl palmitate	adecyl ester	x					Batovska et al., 2008, suppl. info

Nr.	Name	CAS	l	b	f	s	other	reference
1313	Thymine	65714	x					Batovska et al., 2008, suppl. info
1314	Tributylphosphate	80094399	x					Batovska et al., 2008, suppl. info
1315	Tricosane	638675	x					Batovska et al., 2008, suppl. info
1316	Tridecane	629505	x					Batovska et al., 2008, suppl. info
1317	Undecyl laurate	3658444	x					Batovska et al., 2008, suppl. info
1318	Vanillin	121335	x					Batovska et al., 2008, suppl. info
1319	alpha-Amyrine	638959	x					Batovska et al., 2008, suppl. info
1320	alpha-Tocopherol	1406184	x					Batovska et al., 2008, suppl. info
1321	beta-Amyrine	559706	x					Batovska et al., 2008, suppl. info
1322	gamma-Tocopherol	7616220	x					Batovska et al., 2008, suppl. info
1323	delta-Tocopherol	119131	x					Batovska et al., 2008, suppl. info
1324	Z-3-Hexenal	6789806					whole plant	Loughrin et al., 1996
1325	E-2-Hexenal	6728263					whole plant	Loughrin et al., 1996
1326	Z-3-Hexenol	928961					whole plant	Loughrin et al., 1996
1327	E-Hexenol	nf					whole plant	Loughrin et al., 1996
1328	Z-3-Hexenyl acetate	3681718					whole plant	Loughrin et al., 1996
1329	Benzyl alcohol	100516					whole plant	Loughrin et al., 1996
1330	E-beta-Ocimene	3779611					whole plant	Loughrin et al., 1996
1331	Linalool	78706					whole plant	Loughrin et al., 1996
1332	E-4,8-Dimethyl-1,3,7-nonatriene	19945610					whole plant	Loughrin et al., 1996
1333	Indole	120729					whole plant	Loughrin et al., 1996
1334	E,E-alpha-Farnesene	502614					whole plant	Loughrin et al., 1996
1335	Nerolidol	7212444					whole plant	Loughrin et al., 1996
1336	Z-3-Hexenyl benzoate	25152856					whole plant	Loughrin et al., 1996
1337	alpha-terpinolene	586629	x					Hernádez-Orte et al., 2008
1338	Linalool	78706	x					Hernádez-Orte et al., 2008
1339	alpha-Terpineol	98555	x					Hernádez-Orte et al., 2008
1340	beta-Citronellol	106229	x					Hernádez-Orte et al., 2008
1341	Nerol	106252	x					Hernádez-Orte et al., 2008
1342	Geraniol	106241	x					Hernádez-Orte et al., 2008
1343	Z-Linalool oxide	5989333	x					Hernádez-Orte et al., 2008
1344	E-Linalool oxide	34995772	x					Hernádez-Orte et al., 2008
1345	Nerol oxide	1786089	x					Hernádez-Orte et al., 2008
1346	Linalool acetate	115957	x					Hernádez-Orte et al., 2008
1347	2,6-Dimethyl-1,7-octadien-3,6-diol	51276336	x					Hernádez-Orte et al., 2008
1348	delta-Terpineol	7299425	x					Hernádez-Orte et al., 2008
1349	Neric acid	37349294	x					Hernádez-Orte et al., 2008
1350	beta-Damascenone	36649635	x					Hernádez-Orte et al., 2008
1351	beta-Ionone	79776	x					Hernádez-Orte et al., 2008
1352	Vitispirane A	99944793	x					Hernádez-Orte et al., 2008
1353	Vitispirane B	99881853	x					Hernádez-Orte et al., 2008

Nr.	Name	CAS	l	b	f	s	other	reference
1354	Riesling acetal	129601941	x					Hernádez-Orte et al., 2008
1355	TDN	30364386	x					Hernádez-Orte et al., 2008
1356	TPB	644976705	x					Hernádez-Orte et al., 2008
1357	3-Oxo-beta-ionone	29790292	x					Hernádez-Orte et al., 2008
1358	Actinidols	nf	x					Hernádez-Orte et al., 2008
1359	3-Oxo-alpha-ionone	79734433	x					Hernádez-Orte et al., 2008
1360	Z-Whiskylactone	nf	x					Hernádez-Orte et al., 2008
1361	delta-Octalactone	698760	x					Hernádez-Orte et al., 2008
1362	gamma-Nonalactone	104610	x					Hernádez-Orte et al., 2008
1363	delta-Nonalactone	3301948	x					Hernádez-Orte et al., 2008
1364	gamma-Decalactone	706149	x					Hernádez-Orte et al., 2008
1365	Vanillin	121335	x					Hernádez-Orte et al., 2008
1366	Methyl vanillate	3943746	x					Hernádez-Orte et al., 2008
1367	Ethyl vanillate	617050	x					Hernádez-Orte et al., 2008
1368	Acetovanillone	498022	x					Hernádez-Orte et al., 2008
1369	Zingerone	122485	x					Hernádez-Orte et al., 2008
1370	Homovanillyl alcohol	2380781	x					Hernádez-Orte et al., 2008
1371	Syringaldehyde	134963	x					Hernádez-Orte et al., 2008
1372	Acetosyringone	2478388	x					Hernádez-Orte et al., 2008
1373	Guaiacol	90051	x					Hernádez-Orte et al., 2008
1374	Eugenol	97530	x					Hernádez-Orte et al., 2008
1375	o-Cresol	95487	x					Hernádez-Orte et al., 2008
1376	m-Cresol	108394	x					Hernádez-Orte et al., 2008
1377	4-Ethylphenol	123079	x					Hernádez-Orte et al., 2008
1378	4-Vinylguaiacol	7786610	x					Hernádez-Orte et al., 2008
1379	2,6-Dimethoxy phenol	91101	x					Hernádez-Orte et al., 2008
1380	E-Isoeugenol	5932683	x					Hernádez-Orte et al., 2008
1381	4-Vinylphenol	2628173	x					Hernádez-Orte et al., 2008
1382	Dihydromethyl eugenol	nf	x					Hernádez-Orte et al., 2008
1383	Benzaldehyde	100527	x					Hernádez-Orte et al., 2008
1384	Phenylacetaldehyde	122781	x					Hernádez-Orte et al., 2008
1385	Benzyl alcohol	100516	x					Hernádez-Orte et al., 2008
1386	beta-Phenylethanol	60128	x					Hernádez-Orte et al., 2008
1387	Z-3-Hexen-1-ol	928961	x					Hernádez-Orte et al., 2008
1388	E-2-Hexen-1-ol	928950	x					Hernádez-Orte et al., 2008
1389	2-Methylbutyric acid	116530	x					Hernádez-Orte et al., 2008
1390	Ethyl cinnamate	103366	x					Hernádez-Orte et al., 2008
1391	Benzyl alcohol	100516					cutting secretion	Jirovetz et al., 1994
1392	Butanol	71363					cutting secretion	Jirovetz et al., 1994
1393	Butylacetate	nf					cutting secretion	Jirovetz et al., 1994
1394	Carvacrol	499752					cutting secretion	Jirovetz et al., 1994

Nr.	Name	CAS	l	b	f	s	other	reference
1395	beta-Caryophyllene	87445					cutting secretion	Jirovetz et al., 1994
1396	beta-Caryophyllene oxide	1139306					cutting secretion	Jirovetz et al., 1994
1397	1,8-Cineol	470826					cutting secretion	Jirovetz et al., 1994
1398	Cuminaldehyde	122032					cutting secretion	Jirovetz et al., 1994
1399	1,2-Dimethylbenzol	95476					cutting secretion	Jirovetz et al., 1994
1400	Acetic acid	64197					cutting secretion	Jirovetz et al., 1994
1401	Ethylacetate	141786					cutting secretion	Jirovetz et al., 1994
1402	beta-Farnesene	18794848					cutting secretion	Jirovetz et al., 1994
1403	beta-Farnesol	58181763					cutting secretion	Jirovetz et al., 1994
1404	Hexanol	111273					cutting secretion	Jirovetz et al., 1994
1405	1-Hexen-3-ol	4798441					cutting secretion	Jirovetz et al., 1994
1406	Hexylacetate	142927					cutting secretion	Jirovetz et al., 1994
1407	Campher	nf					cutting secretion	Jirovetz et al., 1994
1408	Myristic acid	544638					cutting secretion	Jirovetz et al., 1994
1409	Octanol	11875					cutting secretion	Jirovetz et al., 1994
1410	Palmitic acid	57103					cutting secretion	Jirovetz et al., 1994
1411	Palmitic acid methyl ester	112390					cutting secretion	Jirovetz et al., 1994
1412	alpha-Pinene	80568					cutting secretion	Jirovetz et al., 1994
1413	Propanol	71238					cutting secretion	Jirovetz et al., 1994
1414	Sabinene	3387415					cutting secretion	Jirovetz et al., 1994
1415	Stearic acid	57114					cutting secretion	Jirovetz et al., 1994
1416	alpha-Teroineol	98555					cutting secretion	Jirovetz et al., 1994
1417	Terpinolene	586629					cutting secretion	Jirovetz et al., 1994
1418	Terpinyl acetate	80262					cutting secretion	Jirovetz et al., 1994
1419	Tetradecane	629594					cutting secretion	Jirovetz et al., 1994
1420	Thymol	89838					cutting secretion	Jirovetz et al., 1994
1421	Tridecane	629505					cutting secretion	Jirovetz et al., 1994
1422	1,2,3-Trimethylbenzol	526738					cutting secretion	Jirovetz et al., 1994
1423	t-2-Hexenal	6728263		x				Ibarz et al., 2006
1424	1-Hexen-3-ol	4798441		x				Ibarz et al., 2006
1425	*c*-3-Hexenol	928961		x				Ibarz et al., 2006
1426	t-2-Hexenol	928950		x				Ibarz et al., 2006
1427	*c*-Whiskylactone	nf		x				Ibarz et al., 2006
1428	delta-Octalactone	698760		x				Ibarz et al., 2006
1429	gamma-Nonalactone	104610		x				Ibarz et al., 2006
1430	gamma-Decalactone	706149		x				Ibarz et al., 2006
1431	delta-Nonalactone	3301948		x				Ibarz et al., 2006
1432	Wine lactone	182699770		x				Ibarz et al., 2006
1433	delta-Decalactone	705862		x				Ibarz et al., 2006
1434	Ethyl isobutyrate	97621		x				Ibarz et al., 2006
1435	Isobutyl acetate	110190		x				Ibarz et al., 2006

Nr.	Name	CAS	l	b	f	s	other	reference
1436	Ethyl decanoate	110383		x				Ibarz et al., 2006
1437	Hexanoic acid	142621		x				Ibarz et al., 2006
1438	2-Ethylhexanoic acid	149575		x				Ibarz et al., 2006
1439	Octanoic acid	124072		x				Ibarz et al., 2006
1440	Nonanoic acid, 9-oxo-, ethyl ester	3433167		x				Ibarz et al., 2006
1441	3-Methyl-2-hexanone	2550212		x				Ibarz et al., 2006
1442	1,5-Heptadiene-3,4-diol	51945983		x				Ibarz et al., 2006
1443	(E,Z)-2,6-Nonadienal	557482		x				Ibarz et al., 2006
1444	8-Hydroxy-2-octanone	25368541		x				Ibarz et al., 2006
1445	Benzaldehyde	100527		x				Ibarz et al., 2006
1446	Phenylacetaldehyde	122781		x				Ibarz et al., 2006
1447	1-Phenyl-propanone	nf		x				Ibarz et al., 2006
1448	Phenylethyl acetate	103457		x				Ibarz et al., 2006
1449	Benzyl alcohol	100516		x				Ibarz et al., 2006
1450	2-Phenylethanol	60128		x				Ibarz et al., 2006
1451	2-Phenoxyethanol	122996		x				Ibarz et al., 2006
1452	4-Methoxybenzenemethanol	105135		x				Ibarz et al., 2006
1453	Benzoic acid	65850		x				Ibarz et al., 2006
1454	Phenylacetic acid	103822		x				Ibarz et al., 2006
1455	3,4-Dimethoxybenzenemethanol	93038		x				Ibarz et al., 2006
1456	3-Hydroxybenzaldehyde	100834		x				Ibarz et al., 2006
1457	Ethyl paraben	120478		x				Ibarz et al., 2006
1458	Guaiacol	90051		x				Ibarz et al., 2006
1459	o-Cresol	95487		x				Ibarz et al., 2006
1460	4-Ethylguaiacol	2785899		x				Ibarz et al., 2006
1461	p-Cresol	106445		x				Ibarz et al., 2006
1462	m-Cresol	108394		x				Ibarz et al., 2006
1463	4-Propylguaiacol	2785877		x				Ibarz et al., 2006
1464	Eugenol	97530		x				Ibarz et al., 2006
1465	4-Ethylphenol	123079		x				Ibarz et al., 2006
1466	2-(1,1-Dimethylethyl)-phenol	nf		x				Ibarz et al., 2006
1467	4-Vinylguaiacol	7786610		x				Ibarz et al., 2006
1468	Isoeugenol(a)	nf		x				Ibarz et al., 2006
1469	2,6-Dimethoxyphenol	91101		x				Ibarz et al., 2006
1470	Isoeugenol(b)	nf		x				Ibarz et al., 2006
1471	4-Vinylphenol	2628173		x				Ibarz et al., 2006
1472	4-Allyl-2,6-dimethoxyphenol	6627889		x				Ibarz et al., 2006
1473	Dihydromethyleugenol	nf		x				Ibarz et al., 2006
1474	Vanillin	121335		x				Ibarz et al., 2006
1475	Methyl vanillate	3943746		x				Ibarz et al., 2006
1476	Ethyl vanillate	617050		x				Ibarz et al., 2006

Nr.	Name	CAS	l	b	f	s	other	reference
1477	Acetovanillone	498022		x				Ibarz et al., 2006
1478	Zingerone	122485		x				Ibarz et al., 2006
1479	Homovanillyl alcohol	2380781		x				Ibarz et al., 2006
1480	Syringaldehyde	134963		x				Ibarz et al., 2006
1481	Vitispirane A	99944793		x				Ibarz et al., 2006
1482	Vitispirane B	99881853		x				Ibarz et al., 2006
1483	Riesling acetal; 2,2,6,8-Tetramethyl-7,11-dioxatricyclo[6.2.1.0(1,6)]undec-4-e	129601941		x				Ibarz et al., 2006
1484	TDN	30364386		x				Ibarz et al., 2006
1485	beta-Damascenone	36649635		x				Ibarz et al., 2006
1486	TPB	644976705		x				Ibarz et al., 2006
1487	alpha-Ionone	127413		x				Ibarz et al., 2006
1488	3-Oxo-beta-ionone	29790292		x				Ibarz et al., 2006
1489	beta-Ionone	79776		x				Ibarz et al., 2006
1490	Actinidols	nf		x				Ibarz et al., 2006
1491	OH-TDN	nf		x				Ibarz et al., 2006
1492	Dihydroactinidiolide	17092921		x				Ibarz et al., 2006
1493	3-Hydroxy-beta-damascone	35734613		x				Ibarz et al., 2006
1494	*c*-3-Oxo-alpha-ionol	896107690		x				Ibarz et al., 2006
1495	t-3-Oxo-alpha-ionol	8961073		x				Ibarz et al., 2006
1496	3-Hydroxy-7,8-dihydro-beta-ionol	113110024		x				Ibarz et al., 2006
1497	3-Oxo-7,8-dihydro-alpha-ionol	nf		x				Ibarz et al., 2006
1498	3-Hydroxy-7,8-dehydro-beta-ionol	58023726		x				Ibarz et al., 2006
1499	2,3-Dehydro-4-oxo-beta-ionol	nf		x				Ibarz et al., 2006
1500	Blumenol A	23526456		x				Ibarz et al., 2006
1501	gamma-Terpinene	99854		x				Ibarz et al., 2006
1502	alpha-Terpinolene	586629		x				Ibarz et al., 2006
1503	Rose oxide	1640943		x				Ibarz et al., 2006
1504	*c*-Linalool oxide	5989333		x				Ibarz et al., 2006
1505	Nerol oxide	1786089		x				Ibarz et al., 2006
1506	*t*-Linalool oxide	68780916		x				Ibarz et al., 2006
1507	Linalool	78706		x				Ibarz et al., 2006
1508	1-Terpinenol	586823		x				Ibarz et al., 2006
1509	Terpinen-4-ol	562743		x				Ibarz et al., 2006
1510	2,6-Dimethyl-1,7-octadiene-3,6-diol	51276336		x				Ibarz et al., 2006
1511	Myrcenol	543395		x				Ibarz et al., 2006
1512	delta-Terpineol	7299425		x				Ibarz et al., 2006
1513	Ocimenol	5986389		x				Ibarz et al., 2006
1514	alpha-Terpineol	98555		x				Ibarz et al., 2006
1515	Terpinyl acetate	80262		x				Ibarz et al., 2006
1516	beta-Citronellol	106229		x				Ibarz et al., 2006
1517	Nerol	106252		x				Ibarz et al., 2006

Nr.	Name	CAS	l	b	f	s	other	reference
1518	Geraniol	106241		x				Ibarz et al., 2006
1519	p-Cymen-8-ol	1197019		x				Ibarz et al., 2006
1520	Geranyl acetone	3796701		x				Ibarz et al., 2006
1521	3,7-Dimethyl-1,5-octadien-3,7-diol	13741214		x				Ibarz et al., 2006
1522	6,7-Dihydroxylinalool	nf		x				Ibarz et al., 2006
1523	Terpin	80535		x				Ibarz et al., 2006
1524	8-Hydroxylinalool	64142785		x				Ibarz et al., 2006
1525	Neric acid	37349294		x				Ibarz et al., 2006
1526	Farnesol(2*E*,6*E*)	106285		x				Ibarz et al., 2006
1527	*c*-2,7-Dimethyl-4-octene-2,7-diol	160790498		x				Ibarz et al., 2006
1528	3-Methyl-2-butenol	556821		x				Ibarz et al., 2006
1529	2,4,4-Trimethylcyclopentanol	56470838		x				Ibarz et al., 2006
1530	2-Methyl-1-penten-3-ol	2088075		x				Ibarz et al., 2006
1531	5-Methylfurfural	620020		x				Ibarz et al., 2006
1532	Pantolactone	599042		x				Ibarz et al., 2006
1533	5-Hydroxy-methylfurfural	67470		x				Ibarz et al., 2006
1534	2,5,8-Trimethyl-1,2-dihydronaphthalene	30316235		x				Ibarz et al., 2006
1535	4-(4-Hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]-hept-1-yl)-3-buten-2-one	397869884		x				Ibarz et al., 2006
1536	5-Methyl-2-furfural	620020	x					Wildenradt et al., 1975
1537	2-Methyl-2-propanol	75650	x					Wildenradt et al., 1976
1538	Iso-butyl alcohol	78831	x					Wildenradt et al., 1977
1539	Benzene	71432	x					Wildenradt et al., 1978
1540	3-Pentanone	96220	x					Wildenradt et al., 1979
1541	trans-2-Hexenal	6728263	x					Wildenradt et al., 1980
1542	Furfural	98011	x					Wildenradt et al., 1981
1543	cis-3-Hexen-1-ol	928961	x					Wildenradt et al., 1982
1544	n-Hexanol	111273	x					Wildenradt et al., 1983
1545	trans-2-Hexen-1-ol	928950	x					Wildenradt et al., 1984
1546	-Hexa-2,4-dienal (cis or trans)	nf	x					Wildenradt et al., 1985
1547	2-Furyl methyl ketone	1192627	x					Wildenradt et al., 1986
1548	-2-Heptenal (cis or trans)	nf	x					Wildenradt et al., 1987
1549	Benzaldehyde	100527	x					Wildenradt et al., 1988
1550	Myrcene	123353	x					Wildenradt et al., 1989
1551	Hexyl acetate	142927	x					Wildenradt et al., 1990
1552	Nonanal	124196	x					Wildenradt et al., 1991
1553	Linalool	78706	x					Wildenradt et al., 1992
1554	tert-Butyl benzene	98066	x					Wildenradt et al., 1993
1555	Safranal	116267	x					Wildenradt et al., 1994
1556	alpha-Terpineol	98555	x					Wildenradt et al., 1995
1557	Isopulegone	29606799	x					Wildenradt et al., 1996
1558	Citral (cis or trans)	5392405	x					Wildenradt et al., 1997

Nr.	Name	CAS	l	b	f	s	other	reference
1559	Geraniol (or nerol)	nf	x					Wildenradt et al., 1998
1560	alpha-Ionone	127413	x					Wildenradt et al., 1999
1561	beta-Ionone	79776	x					Wildenradt et al., 2000
1562	-3,5-Dimethyl-1,2,4-trithiolane	23654924	x					Wildenradt et al., 2001
1563	1-Hexanal	66251	x					Wildenradt et al., 2002
1564	Phenethyl butyrate	103526	x					Wildenradt et al., 2003
1565	Tetrathiocane	nf	x					Wildenradt et al., 2004
1566	Trimethyl-dihydronaphthalene	nf	x					Wildenradt et al., 2005
1567	Pentanal	110623		x				Kalua and Boss, 2009
1568	n-Heptan-2-ol	543497		x				Kalua and Boss, 2009
1569	Methyl butanoate	623427		x				Kalua and Boss, 2009
1570	Hexyl acetate	142927		x				Kalua and Boss, 2009
1571	Hexanal	66251		x				Kalua and Boss, 2009
1572	Hexan-1-ol	111273		x				Kalua and Boss, 2009
1573	heptanal	111717		x				Kalua and Boss, 2009
1574	gamma-Muurolene	30021740		x				Kalua and Boss, 2009
1575	Eucalyptol (1,8-cineole)	470826		x				Kalua and Boss, 2009
1576	Ethyl acetate	141786		x				Kalua and Boss, 2009
1577	Ethanol	64175		x				Kalua and Boss, 2009
1578	Ethanal	75070		x				Kalua and Boss, 2009
1579	beta-Ionone	79776		x				Kalua and Boss, 2009
1580	beta-Cymene	535773		x				Kalua and Boss, 2009
1581	beta-Cyclocitral	432257		x				Kalua and Boss, 2009
1582	beta-Caryophyllene	87445		x				Kalua and Boss, 2009
1583	Benzyl alcohol	100516		x				Kalua and Boss, 2009
1584	alpha-muurolene	31983229		x				Kalua and Boss, 2009
1585	alpha-Caryophyllene	6753986		x				Kalua and Boss, 2009
1586	7-Oxabicyclo[4.1.0]heptane	286204		x				Kalua and Boss, 2009
1587	3-Methylbutanal	590863		x				Kalua and Boss, 2009
1588	2-Phenylethanol	60128		x				Kalua and Boss, 2009
1589	2-Phenylethanal	122781		x				Kalua and Boss, 2009
1590	2,2,6-Trimethylcyclohexanone	2408379		x				Kalua and Boss, 2009
1591	(Z)-3-Hexenyl butanoate	16491364		x				Kalua and Boss, 2009
1592	(Z)-3-Hexenyl acetate	3681718		x				Kalua and Boss, 2009
1593	(Z)-3-Hexen-1-ol	928961		x				Kalua and Boss, 2009
1594	(E)-2-Hexenal	6728263		x				Kalua and Boss, 2009
1595	(-)-alpha-Cubebene	17699148		x				Kalua and Boss, 2009
1596	(-)-alpha-Copaene	3856255		x				Kalua and Boss, 2009
1597	Ethanol	54175	x					Hebash et al., 1991
1598	2-Methylbutanal	96173	x					Hebash et al., 1991
1599	3-Methylbutanal	590863	x					Hebash et al., 1991

Nr.	Name	CAS	l	b	f	s	other	reference
1600	1-Propanal	123386	x					Hebash et al., 1991
1601	2-Methylpropanal	78842	x					Hebash et al., 1991
1602	2-Methylpentanal	123159	x					Hebash et al., 1991
1603	1-Pentanal	110623	x					Hebash et al., 1991
1604	1-Butanol	71363	x					Hebash et al., 1991
1605	1-Pentanol	71410	x					Hebash et al., 1991
1606	2-Pentenal	764396	x					Hebash et al., 1991
1607	2-Hexanone	591786	x					Hebash et al., 1991
1608	2-Pentenol	20273249	x					Hebash et al., 1991
1609	E-2-Hexenal	6728263	x					Hebash et al., 1991
1610	2-Heptanone	110430	x					Hebash et al., 1991
1611	1-Hexenol	nf	x					Hebash et al., 1991
1612	2-Hexenol	nf	x					Hebash et al., 1991
1613	Heptanal	111717	x					Hebash et al., 1991
1614	Z-3-Hexenal	6789806	x					Hebash et al., 1991
1615	Octanal	124130	x					Hebash et al., 1991
1616	2-Heptenol	22104774	x					Hebash et al., 1991
1617	1-Octanol	111875	x					Hebash et al., 1991
1618	1-Nonanal	124196	x					Hebash et al., 1991
1619	E-2-Nonenal	18829566	x					Hebash et al., 1991

Study of the volatile metabolome in plant-insect interactions

Georg J.F. Weingart, Nora C. Lawo, Astrid Forneck, Rudolf Krska, Rainer Schuhmacher

Abstract
The study of the molecular basis of plant-insect interactions is a rewarding research area which is fundamental in character and can lead to an improved understanding of these interaction processes. While earlier studies frequently focused on the investigation of a limited number of substances, the so called “omics-techniques” led to a shift to system-wide approaches which aim to describe whole biological systems. The first part of this chapter presents a general overview of the methodology used for the study of volatile plant metabolomes. The protocol covers the profiling of volatiles induced in plant tissue by insect feeding and is exemplified with grapevine roots attacked by the piercing/sucking phylloxera (grape louse). Volatiles were extracted from root tissue with solid phase microextraction (SPME), separated with gas chromatography (GC) and detected with mass spectrometry (MS). Data were processed with AMDIS software, which enables to deconvolute mass spectra of coeluting metabolites. In addition, the software matches the recorded mass spectra with library entries and compares the retention index (RI) with reference values. This led to a list of annotated compounds whereof the identity of the majority could be confirmed with authentic standards. Finally, univariate and multivariate statistics were applied to provide differentially expressed metabolites comparing a non infested control group of plants with plants attacked by phylloxera. Most of the described procedures are general in character and can be applied to other plant-insect interaction studies after slight modifications. All methods and instruments employed are well established and easy to handle in order to enable also non-specialists to utilise the developed protocol.

1
Introduction

1.1
Plant-insect interactions

The interaction between plants and insects is a highly complex and fascinating research area. These interactions can be mutually beneficial (symbiotic) but also pathogenic, i.e. leading to plant diseases or affecting the insect negatively [1, 2]. An example for an interaction beneficial for both partners is the attraction of insects by pollinating flowers: the insect takes care for the pollination of the plant and thereby receives nutrition. Plant-insect interactions can also be beneficial for just one of the interaction partners. Some plants for example permanently emit volatiles (repellents) to be less attractive to herbivores, others actively defend against herbivory by producing metabolites, which are toxic to the attacking insect. As the permanent synthesis and emission of repellents, for example comes at considerable metabolic energy costs, many plants have developed defence strategies, which are only activated when the plant is attacked - the so called induced defence [3]. Induced defence can either work in a direct mode against the attacker [4, 5] or indirectly, by the attraction of natural enemies of the attacker [6, 7]. The herbivore does not only cause a metabolic change in the directly affected part of the plant but could also lead to a response in other plant parts - a phenomenon called systemic defence [8, 9]. The changes in the metabolite pattern due to herbivore attack are reported to last from days to years [3, 10].

There are various further ways to classify plant-insect interactions. It can be differentiated between chewing and piercing/sucking insects for example, or interaction below ground (roots) or above ground (shoot, e.g. leaves). Recent studies have shown that the metabolic activity of different plant organs can be affected simultaneously, as e.g. Bezemer et al. [11] have demonstrated that the attack by root feeding insects can lead to increased non-volatile terpenoid concentration in leaves.

1.2
Significance of volatile plant metabolites

Volatile organic compounds (VOCs) originating from plants are frequently involved in plant-insect interactions since plants, being sessile organisms, make use of volatiles for signalling either within the plant or by sending signals over relatively long distances [12, 13]. Besides the use of brightly coloured flowers, plants can also emit volatiles to attract potential pollinators [1] or as a defence mechanism against herbivory [14]. The attack of a feeding insect usually induces a signalling cascade, whereby the attacked leaf and in some plants also leaves nearby the attacked leaf start to produce VOCs for defence [13]. Typical plant derived VOCs consist of low molecular weight, mostly lipophilic substances such as terpenoids, phenylpropanoids, benzenoids and other aliphates with a high vapour pressure of ≥ 0.01 kPa at 20°C [15, 16]. Several reviews recently published discuss VOCs concerned in plant-insect interactions, e.g. [15, 17-21]).

Plant VOCs generally consist of complex mixtures and play a key role in plant-insect interactions and therefore the comprehensive study of these metabolites is of major importance for a better understanding of the underlying molecular mechanisms. The analytical methodology for the study of volatile plant metabolomes will be described in more detail in the following sections.

1.3
Study of the plant volatile metabolome in plant-insect interactions

The study of the volatile metabolome, i.e. the entirety of the volatile metabolites produced by an insect-affected plant requires a comprehensive analytical approach. After thoroughly planning and realising the biological experiment, plant samples must be taken and, if plant tissue is physically harvested, efficiently quenched to freeze the plants' metabolic state. These steps are followed by homogenisation and further sample preparation, measurement of the metabolites and processing of raw data. In a targeted metabolomics approach, which will be described here, parts of the metabolome are detected, identified or annotated and (relatively) quantified. Relative or absolute concentrations of the detected metabolites are then compared by various statistical methods with the aim to identify metabolites being differentially expressed in the tested biological samples. Finally, the analytical and statistical results require biological interpretation, which again leads to the setup of new biological experiments. The analytical workflow of such a metabolomics approach covers all of the above mentioned steps and is schematically illustrated in Figure 1.

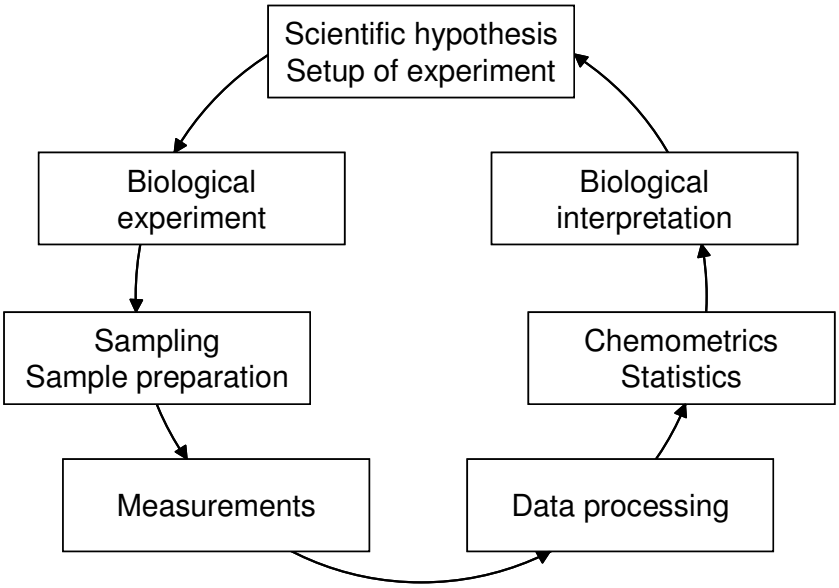

Figure 1 Typical steps in a general metabolomics workflow. Starting with a biological question the experiment is designed and carried out. Samples are harvested, treated and measured. The data is processed and analysed by means of various data processing software and statistic/chemometric tools. The results are interpreted in relation to the initial biological questions. Eventually new scientific questions can be defined which then can be addressed by additional experiments.

It is worth to mention that comprehensive metabolomics studies require numerous analytical techniques to cover other parts of the plant metabolome like non-volatile polar (mostly primary) or non-volatile apolar secondary metabolites. Additionally, metabolomics studies are frequently complemented by the simultaneous measurement of further biological parameters. For example, in addition to metabolite identification and quantification, water potential, gas exchange or photosynthetic activity are recorded to describe the physiological state of the tested plants. Moreover, plant metabolomics is frequently carried out in combination with other "-omics techniques" such as functional genomics, transcriptomics or proteomics.
In the presented protocol a major focus is laid on the VOCs produced within the plant tissue after attack by an insect. In the following, the setup of biological experiments, sampling, sample preparation and measurement of volatiles by GC-MS will be discussed.

1.3.1 Setup of biological experiments

Since most metabolomics methods aim at the measurement of as many low molecular weight metabolites as possible with the method in use, the simultaneous absolute quantification of all detected metabolites is not feasible. Instead, a typical metabolomics experiment affords the differential comparison of a defined number of replicate samples (i.e. relative quantification of metabolites), which have been grown in parallel and represent different states, e.g. "control vs. treatment" or "wildtype vs. mutant" [22]. The ultimate goal of this type of metabolomics study generally is to link the differentially expressed metabolites (i.e. the differences detected in the metabolome) to the experimental factor which was varied to generate the different sample states.
Therefore, all factors potentially influencing the metabolome (i.e. genotype, developmental and physiological states as well as environmental conditions) have to be controlled carefully during a biological experiment. Compared to biological experiments in the field, greenhouse experiments can be better standardised than field trials, the latter however offers the advantage of having more natural experimental conditions. If the metabolomics study aims at the investigation of the interaction between different organisms, the experiment has to consider the tested organisms alone as well as combinations thereof. In plant-insect interaction studies for example, in which the response of a plant to an insect is to be tested, the metabolome of a set of insect-affected plants (treatment) can be compared to the metabolome of plants grown in absence of the insect

(control). Furthermore, in experiments addressing plant response directly at the interaction site, it can be difficult to differentiate between plant and insect derived metabolites. In this case, the sampled and analysed plant tissue might contain metabolites from both interaction partners. This type of study ideally includes both types of control groups, plants and insects versus a group of treated plants.
During the setup of the biological experiment it also has to be considered that some volatiles can act as signalling molecules which might affect the metabolome of plants being exposed to these metabolites. Thus, if necessary, e.g. treated and non-treated plants have to be grown separately without direct contact.
For both, control and treated groups, the number of replicates should be large enough to allow the estimation of technical and biological variability. Moreover, (biological) quality control samples should be included in every measurement sequence to monitor and verify proper performance of the analytical process.

1.3.2 Sampling, quenching and sample preparation

Sampling and quenching of plant material. Sampling and quenching and are crucial steps in plant metabolomics. Numerous factors, such as metabolic turnover rates, diurnal or nocturnal rhythm of plants, tissue specificity of metabolic activity, or induction of metabolic change by invasive sampling techniques have to be considered [23].
Various techniques have been described for sampling of plant volatiles, which can be classified in two main categories: non-invasive and invasive methods. The former technique usually makes use of dynamic headspace enrichment of volatiles and allows the (online/onsite) investigation of the living plant without largely influencing the biological system, while the latter affords sampling of the whole plant or selected detached plant organs/tissue in combination with subsequent extraction of volatiles. For a detailed description and discussion of the techniques available for sampling and measurement of volatiles, the reader is referred to the excellent review of Tholl et al. [16]. Here, some general aspects of sampling in plant metabolomics shall briefly be discussed.
The formation of green leave volatiles for example, is induced by mechanical damage of plant tissue and can be extremely fast (within seconds). This means that sampling of leaf- or root tissue for example, requires efficient quenching (i.e. freezing of the metabolic state at the time point of sampling). Immediate cooling of the plant tissue with liquid nitrogen (lq. N_2) after sampling is the most simple and common method today [23], which can also be applied in the field. Many secondary metabolites undergo a diurnal or nocturnal rhythm as described for example by Urbanczyk-Wochniak et al. [24]. Therefore, the time of day for sampling should be kept constant and has to be chosen carefully according to the metabolic cycle and the metabolites of interest.
Another important point to consider is the choice of the plant tissue to be sampled. Choi et al. [25] for example reported different metabolomes in terms of metabolite concentrations as well as the type of metabolites in younger versus older leaves or within the same leaf when leaf veins were compared to other leaf tissue. While it should be kept in mind that the metabolomes of different plant tissues can vary significantly, analysis of whole plants or pooling of tissue from multiple plants can also be useful. In case of small sample weight, root tips for example, pooling might help to collect enough material for further analysis. If large variability of metabolite concentrations can be expected e.g. for field samples, pooling might also facilitate to detect differences between the metabolomes of the different sample groups.

Sample preparation. Here, the term sample preparation is used to describe all sample manipulation steps between quenching and instrumental analysis. In general it can be recommended to reduce sample preparation to a minimum since every step carries the risk of loosing metabolites or introducing artefacts. Usually, quenching is followed by measures to stabilise the plant sample. This can be done by permanent cooling with lq. N_2, storage in the freezer at -80 °C or freeze drying. The latter technique is well suited for stabilising plant material, as many enzymes need water to be active. However, freeze drying has been reported to lead to a loss of volatiles [26] and is therefore not appropriate for the determination of VOCs.

Usually, sample stabilisation is followed by homogenisation of the frozen or dried plant samples under cooled conditions, either by manual crushing in a mortar or by the aid of a ball mill.
While headspace analysis can directly be carried out with defined amounts of the homogenised plant powder, methods employing the analysis of liquid sample extracts require an additional solvent extraction step e.g. with acetone, dichloromethane or butanol, e.g. [27, 28].
If the non-volatile metabolome shall also be analysed, a procedure involving a mixture of methanol/chloroform/water (e.g. 2.5/1/1 v/v/v) [29] has been used frequently in plant metabolomics. This allows the separation of DNA and proteins from polar (mostly primary) and non-polar metabolites, among which also most of the volatile metabolites can be found.

1.3.3 Headspace extraction and measurement by GC-MS

In metabolomics, extraction and measurement of volatile metabolites is most frequently carried out by headspace techniques in combination with gas chromatography-mass spectrometry (GC-MS).
GC-MS provides highly efficient chromatographic separation. In capillary GC using standard wall coated open tubular columns, peak capacities typically range between 120 and 250 for a standard GC temperature gradient programme of 5 - 60 minutes runtime [30]. Full scan mass spectrometric detection adds extra and complementary selectivity to the chromatographic separation and furthermore leads to standardised mass spectra, independent of the type of GC-MS instrument manufacturer. Large spectra libraries e.g. NIST (http://www.nist.gov) or Wiley MS libraries (http://www.wiley.com) containing some 100,000s of mass spectra are available which can be used for the automated comparison with measured spectra.
Headspace extraction techniques can be roughly divided in static and dynamic approaches. **Dynamic headspace** techniques make use of a continuous gas stream flowing through a container in which the sample is located. The gas stream transports the headspace constituents to some kind of trap (e.g. adsorbent or cryogenic) where the volatiles are enriched during the extraction process. Dynamic headspace extraction can be carried out in a closed (i.e. closed loop stripping) or open (i.e. purge and trap) system. Trapped VOCs, then have to be eluted from the trap by organic solvents or the loaded trap is directly connected to the carrier gas stream of a GC instrument and the VOCs are desorbed onto the GC column by rapidly heating the trap to high temperatures (thermodesorption). Due to the exhaustive extraction and enrichment of volatiles, dynamic headspace techniques are extremely sensitive but are also prone to artefacts as e.g. impurities which are present in the gas stream are also enriched.
In comparison, **static headspace** extraction is a discontinuous process which makes use of passive sampling. The plant, parts of it or detached plant tissue is sealed in a container (e.g. headspace vial, plastic bag, bell jar). After a time period for equilibration the headspace is sampled by either withdrawal of a defined volume aliquot from the headspace (e.g. by sample loop or gas tight syringe) or the introduction of an ad(/ab)sorptive material attached to the sampling device. For this purpose solid phase microextraction (SPME) [31] or stir bar sorptive extraction (SBSE) [32] are used most frequently. Compared to dynamic headspace sampling, static techniques are generally less sensitive but also less prone to artefacts due to less enrichment of contaminants.
Both, dynamic as well as static headspace extraction techniques can be used to extract VOCs from (parts of) living plants or from detached, powdered plant samples. For metabolomics studies of living, intact plants the dynamic approaches offer the advantage of better control of relative humidity as well as temperature of the experimental setup.
A detailed discussion of these two concepts can be found e.g. in [16]. In this protocol, the cheap and easy to use SPME technique has been used and will therefore be described in more detail in the next section.

Solid phase microextraction (SPME). Developed by Arthur and Pawliszyn in the late 1980s [31], SPME is a very widespread technique for the extraction of trace amounts of all kinds of low molecular weight compounds. Headspace (HS)-SPME consists of two steps: First, a fibre with an adsorptive/absorptive coating is inserted into the sample container and exposed to the headspace above a solid or liquid sample. After partitioning of the volatile metabolites between the sample, headspace and fibre coating for a defined period of time, the fibre is withdrawn from the sample container. In a second step the fibre is introduced into the injector of a gas

chromatograph for desorption of the extracted volatiles and subsequent analysis. This technique is well suited to be used for both, manual or fully automated profiling of plant VOCs and can also be used for on-site sampling in the field. As there is an enrichment of substances on the fibre, very low concentrations (<ppb) can be detected, e.g. [16]. Various fibres combining different kinds of coating materials with different polarities are available [33] (Table 1). Depending on the coating material, the volatiles are either adsorbed in pores at the surface of the coating material (solid coating, e.g. carboxene) or absorbed into the coating material (liquid coating, e.g. polydimethyl siloxane). As the SPME efficiency of a certain volatile metabolite is mainly a function of the extraction temperature, extraction time and polarity of the fibre coating, these experimental parameters have to be evaluated carefully, selected and kept constant during method application in order to obtain acceptable extraction efficiency and precision of the SPME step. Moreover, it shall be noted that any selection of these experimental parameters always leads to a discrimination of some volatiles, whose polarity or volatility do not match the extraction conditions. Generally, with shorter extraction times, low boiling, highly volatile low molecular weight compounds are sampled preferentially. Longer extraction times lead to the preferential extraction of higher molecular weight molecules with lower boiling points [34].
HS-SPME is excellently suited for qualitative and semi-quantitative analysis of VOCs. Absolute quantification of volatiles is not straightforward and generally needs a thorough control of sampling parameters and a detailed evaluation of matrix effects [16, 35, 36]. The accurate quantification of a few target compounds can be achieved by the use of internal standards, but the simultaneous quantification of a great number of compounds is more difficult. One option enabling quantification is multiple headspace extraction (MHE). With this technique, all metabolites for which a standard is available can be quantified by external calibration [37].
As only volatile compounds are enriched during HS-SPME, this technique yields relatively "clean" extracts and contamination of the GC inlet or MS detector is usually not an issue.

Table 1 Commercial available SPME fibre coatings and their recommended application (according to the supplier).

Fibre	Application
Polydimethylsiloxane (PDMS)	Nonpolar coating for nonpolar analytes
Polydimethylsiloxne/Divinylbenzene (PDMS/DVB)	Polar analytes, especially amines
Polyacrylate (PA)	Highly polar coating for general use, ideal for phenols
Carboxen/Polydimethylsiloxane (CAR/PDMS)	For gaseous/volatile analytes, high retention for trace analysis
Carbowax/Divinylbenzene (CW/DVB)	For polar analytes, especially for alcohols, low temperature limit
Divinylbenzene/Carboxen/PDMS (DVB/CAR/PDMS)	For broad range of analyte polarities, good for C3-C20 range
Carbowax/Templated Resin (CW/TPR	Developed for HPLC applications, e.g. surfactants

Stir bar sorptive extraction (**SBSE)** [32] is based on the same extraction principle as SPME and can be used as an alternative. Through the higher amount of coating material on the stir bar compared to an SPME fibre coating, SBSE is more sensitive. A major drawback of this technique is that it can not be fully automated and for the desorption step into the GC inlet additional equipment is necessary. Because of the larger amount of substances ab(/ad)sorbed to the stir bar coating the absorbed volatiles can be eluted subsequently with liquid solvents and then multiple liquid injections of the same sample extract are possible.

1.3.4 Data handling

Data handling can be divided in data processing and data analysis steps [38] and shall be briefly addressed below.
Data processing. A typical metabolomics experiment comprises the analysis of at least two groups of biological samples (with several replicates per sample group) plus standards, blanks

and quality control samples. Each of the measurements results in large raw data files, which have to be processed in various ways. This makes manual data evaluation too labour intensive. Instead, efficient software tools are needed to process and analyse metabolomics data appropriately.
For example, the large number of volatiles extracted by HS-SPME of a plant sample usually exceeds the chromatographic peak capacity and therefore leads to the co-elution of volatiles into the mass spectrometer. Consequently, efficient software for peak picking and deconvolution of mass spectra is needed which helps to eliminate background signals as well as MS signals from interfering compounds. Additionally, annotation/identification of metabolites is also necessary. In GC-MS of volatiles, this is generally realised by the comparison of experimental mass spectra and retention indices (RI) with reference spectra and RI values from the literature [39, 40]. Various platform independent software tools are available, e.g. AMDIS (Automated Mass Spectral Deconvolution and Identification System [41]), Tagfinder [42] or MetaboliteDetector [43], which can be used for these data processing steps.
Data analysis (statistics/chemometrics). Data analysis is usually carried out with the aim to identify differences between the investigated groups of biological samples and to identify the metabolites that significantly contribute to these observed differences. For this purpose, various statistical methods are available. Depending on the setup of the biological experiment and the number and design of analytical measurement sequences as well as the used analytical techniques, simple univariate or more complex multivariate statistics are required.
In the presented protocol univariate t-tests and principal component analysis (PCA) have been carried out using the freely available R-software (www.r-project.org) [44].

1.3.5 Biological interpretation

In the last step of the metabolomics workflow the obtained results have to be put into biological context. Reliable metabolite annotation/identification, accurate relative quantification of the detected metabolites as well as proper statistics forms the basis of any meaningful biological interpretation. Usually, this last but essential step requires a joint effort of researchers from various disciplines such as plant biology, entomology, analytical chemistry and statistics.

2 Methods and protocols

For the scientific hypothesis and the design of the biological experiment, no general protocol is available. Therefore, we want to briefly exemplify these steps of the workflow with a setup for a thought study of roots of grapevine plants attacked by the piercing/sucking insect phylloxera. The majority of the presented procedures within this protocol are general in character and can be applied to other plant-insect interaction studies after appropriate (slight) modifications.
For grapevine roots attacked by phylloxera, for example the following hypothesis can be defined: The attack of phylloxera to roots of the grapevine cultivar Teleki 5C leads to the induction of volatile metabolites in the roots (local response) and leaves (systemic response) of the infested plants. It is further hypothesised that this metabolic change in the volatile metabolome can be measured by HS-SPME GC-MS. Therefore, the setup of the following thoughtbiological experiment could be assumed: Root tips of 24 plants, 12 of which inoculated with phylloxera, 12 serving as an untreated control group are sampled in regular intervals. At the end of the experiment, also the leaves shall be sampled.
Comments or hints to crucial steps in the protocol are referred to at the appropriate text passage. If company names are mentioned, they shall serve as an example for a suitable supplier. Other companies might be appropriate as well. Location of the respective company is given only when first mentioned.

Biological material

Permanent breed of insects

A single founder lineage of phylloxera (*Daktulosphaira vitifoliae* Fitch) was collected in Großhoeflein (Austria) in 2007 and has since been maintained in the greenhouse on the leaf-forming rootstock Teleki 5C with no additions of field-collected insects. Four plants were kept in

one plastic container (height: 125 cm, diameter: 50 cm), containing five holes (three for ventilation, 10 cm diameter) and two allowing operation (20 cm diameter). All holes were covered with a fine-mesh netting (149 µm diameter), except the ones for handling which were closed via plastic foil. Plants were watered and fertilized depending on the weather conditions. To prevent insects from escaping the containers were maintained in a water containing tray.

Cultivation of grapevine plants and inoculation with phylloxera
The following protocol describes the routine procedure to produce root and leaf tissue samples for subsequent HS-SPME GC-MS analysis. It is based on a procedure published in [45].

Materials
- Seradix B2 (3g/kg indole-3-butyric acid, Kwizda, Austria) to support one-node cuttings in rooting
- "Jiffy-7" pots (40 mm diameter)
- Water + fertilizer (e.g. FERTY 3 Mega, N+P+K+Mg (18+12+18+2) + micronutrient, diluted with water to a concentration of 0.5 g/L, Planta, Regenstauf, Germany)
- Small spray bottle allowing gentle watering
- Rooting Teleki 5C (*V. berlandieri* Planch. x *V. riparia* Michx.) one-node cuttings 6 per plant tray, 24 in total
- Substrate (6 parts commercial potting soil, 1 part sand, 1 part perlite) for planting
- Tray-A: plant tray (including 6 pots, 26x31 cm, 4 pieces in total) with big holes on the bottom to allow roots to grow through
- Net (1x1 cm per hole) to prevent soil from falling out when turning the plant tray-A during harvest for 90°
- Wire to fix the net
- Perlite to fill box-A and box-B
- Box-A: plastic box (25x30x7 cm, 4 L, 4 pieces in total) filled ¾ with perlite and spacers to provide a space between the perlite and the plant tray-A
- Tray-B: big tray (68x63x12.5 cm, 2 pieces in total, providing space for 2 plant trays-A) filled 2 cm with water to prevent insect from escaping
- Box-B: large box (48x31x42 cm, 65 L, 2 pieces in total) to cover everything, the top of the box contains a vent hole (30x15 cm) covered with a fine-mesh netting plus 4 additional holes for ventilation (10 cm diameter)
- Ethanol (70%) to clean the working material
- Dish to provide a working platform
- Gloves
- Scalpel to open the leaf galls
- 4 heavily infested phylloxera leaves from a permanent single founder phylloxera lineage (as described above)

Procedures
Propagate one-node dormant cuttings from the rootstock variety Teleki 5C vegetatively in the greenhouse. Therefore, dip cuttings in indole-3-butyric acid for rooting and place them into "Jiffy-7" pots. Grow them for 4 - 5 weeks until rooting (depending on temperature) before preparing them for the experiment.
Take care that no insect can escape from the experimental setup (quarantine pest). Therefore, cover all venting holes with a fine net and settle the entire setup in water filled containments. During the experiment, water and fertilize the plants depending on the weather conditions to provide optimal conditions. Figure 2A illustrates how such a setup could look like. To have an appropriate number of biological replicates perform the experiment either several times consecutively or in parallel.

Setup of the plants:
1. Plant 6 rooting plants into plant tray-A including 6 pots in substrate, prepare 4 such trays with a total of 24 plants.
2. Cover and fix rootstocks with net (1x1 cm), fix net with wire.
3. Put tray with plants in box-A filled with perlite.

4. Store every 2 setups in a bigger tray-B. Fill tray-B 2 cm with water.
5. Cover each of the 2 boxes of type A containing the plants with a bigger box-B. The result are two independent but identical setups for the further experiment.
6. Let the roots grow for 1 – 2 weeks (until a sufficient root biomass is grown through the net).
7. Separate the 2 setups to avoid any sort of communication between the plants with each other via air.

Inoculation with insects:

1. Remove box-B and take out box-A with plants.
2. Turn the plant tray-A carefully for 90° (Figure 2B).
3. Prepare the inoculum (phylloxera eggs) by cutting off the tops of all leaf galls of a medium sized leaf with a scalpel.
4. Place the prepared leaves upside down on the perlite layer of the boxes-A (2 leaves per 6 plants).
5. Rebuild setup.
6. After a defined time interval (e.g. 3 days) of inoculation, remove leaves.

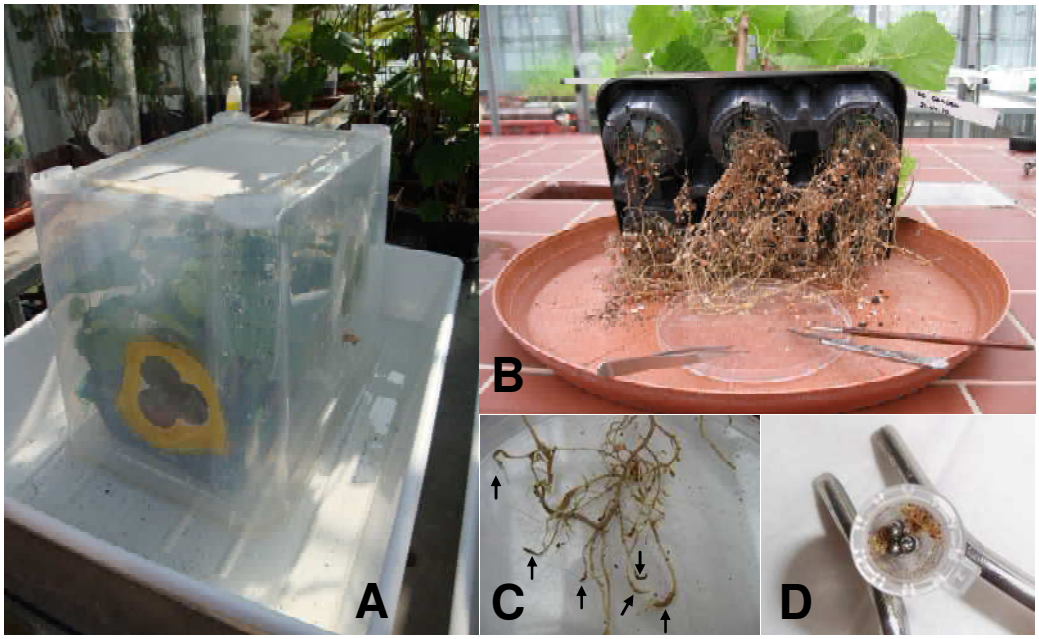

Figure 2 Setup of the biological experiment (A). For sampling of root tissue, the tray with the grapevine plants is removed from the perlite underground and turned for 90° (B). After cleaning the roots (C, arrows show some root galls) they can be sampled, pooled and filled into an Eppendorf tube. Finally, steel balls are added prior to the milling step (D).

Sampling and quenching of plant tissue (roots and leaves)

The typical mass of a root tip is in the range of a few mg. Thus around 50 root tips have to be collected for GC-MS measurements. In case of leaves, which weigh around 3-4 g, one single leaf offers enough material for >10 GC-MS measurements. This suggests different sampling strategies for roots and leaves. Additionally, due to the significant change of the physiological state of a plant, which is caused by the detachment of a leaf, the plant should be excluded from further sampling. In case of the root sampled plants the changes in the metabolome induced by the cutting can be regarded much smaller. Due to the simultaneous cutting of roots of the control group, it can be assumed that the influence does not appear in differentially expressed metabolites.

It shall be noted that for the handling of liquid nitrogen (lq. N_2) always safety gloves and a face shield should be used to protect against cold. Additionally, wear appropriate clothing in order to protect legs and feet.

Sampling and quenching of root tips
Materials

- Polystyrene box with lid (20x15x10 cm) with lq.N_2
- Scalpel
- Fine tweezers
- Fine brush
- Ethanol (70%) and tissue to clean the working material
- Magnifying glass (12x)
- Dish to provide a working platform
- Gloves
- Lamp to provide additional light
- Plastic tubes (e.g. Eppendorf tubes 1.5 mL)
- Rack for holding the Eppendorf tubes in lq. N_2

Procedures

1. Take samples in regular intervals (e.g. daily) at the same time of day (e.g. 10 - 12 am): young root galls (fed on by second nymphal instar phylloxera), mature root galls (feed on by one adult phylloxera producing maximal five eggs) or uninfested root tips.
2. For sampling, remove box-B and take out box-A with plants.
3. Turn the plant tray-A carefully for 90° (Figure 2B).
4. Free all roots foreseen for sampling from any soil with a fine brush.

Infested root tips:

5. Remove insect with a fine brush
6. Hold root tip with tweezers, cut root tips above the root gall with a scalpel (Figure 3), insert root into lq. N_2 immediately.
7. Pool root tissue from several plants until appr. 200 mg per Eppendorf tube. Separate different developmental stages (young root galls, mature root galls). If not enough material collected at one sampling date, pool with samples of the next sampling date.

Uninfested root tips:

8. Hold with tweezers, cut with scalpel at 1.5 cm length (Figure 3), insert in lq. N_2 immediately.
9. Pool root tissue from several plants until appr. 200 mg per Eppendorf tube. Cut as many roots as from the infested plants. If not enough material collected at one sampling date, pool with samples of the next sampling date.

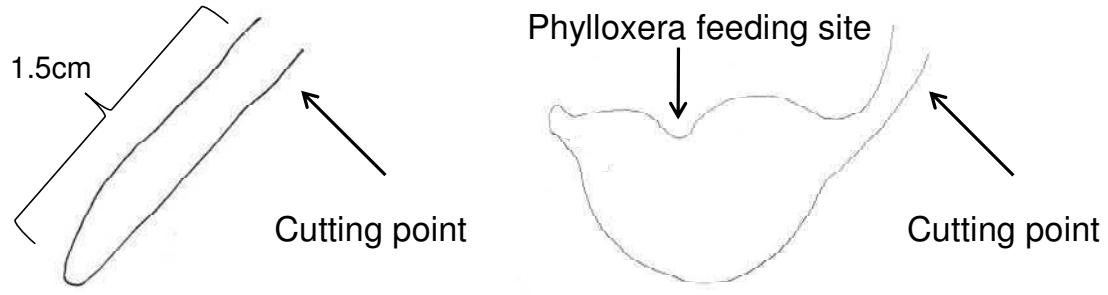

Figure 3 Illustration of uninfested (left) and infested (right) root tips. Arrows indicate where roots should be cut for sampling.

Sampling and quenching of grapevine leaves
Many plant metabolites undergo a diurnal rhythm. Therefore, it is essential for comprehensive studies to sample always at the same time of day and within a short time interval.

Materials

- Polystyrene box with lid (40x35x25 cm) with lq. N_2
- Scalpel (or scissors)
- Plastic tubes with screw cap (e.g. Sarstedt 50 mL or aluminium foil folded to an envelope, which can hold one leaf)
- Racks for holding 50 mL Sarstedt tubes in lq. N_2

Procedures

1. Sample always at the same time of day, (e.g. 10 - 12 am).
2. Cut leaf with 1 cm stalk remaining and insert the cutting site in lq. N_2 immediately (within seconds).
3. Put sample into 50 mL Sarstedt tube, AVOID FURTHER DAMAGE of the sampled leaf!
4. Cool Sarstedt tube by inserting it into lq. N_2.

Milling and weighing of plant tissue (roots and leaves)

During the homogenisation and weighing steps care has to be taken that the plant material does not thaw. Therefore, all materials are pre-cooled with lq. N_2. It is also very important to minimise contamination. It is recommended to clean all material (except disposables) carefully (e.g. with methanol/water (50/50 v/v)) before use. Bake out HS vials, caps and septa at 120 °C for at least 1 hour.

The HS vials should also be pre-cooled before weighing in the plant tissue but this time ice is to be preferred. The weighing process should be carried out very fast to avoid thawing and the condensation of air humidity on the plant material or the HS vials. It is worth to practice this beforehand and to use a spatula that takes approximately the amount that should be weighed in.

Milling and weighing of root samples

When using the ball mill for homogenisation of root tips a sample mass of appr. 200 mg per Eppendorf tube is necessary to receive 100 - 150 mg of root tissue powder that can be weighed into HS vials for subsequent GC-MS measurements. If necessary, smaller sample amounts down to single root tips can be ground with a glass rod directly in the HS vial.

Materials

- Polystyrene boxes with lid (10x10x10 cm and 2 pieces 30x25x20 cm)
- Lq. N_2
- Frozen root samples in Eppendorf tubes
- Ball mill (e.g. MM 301, Retsch, Haan, Germany)
- Adapter for Eppendorf tubes fitting into ball mill (e.g. Retsch)
- Stainless steel balls (3 mm diameter, e.g. Retsch), 5 per Eppendorf tube
- Spatula
- HS vial 20 mL
- Magnetic screw cap with hole and 1.3 mm silicone/PTFE septa for HS vial
- Box with crushed ice for pre-cooling of HS vials before weighing step
- Analytical balance (e.g. RC 2010 P, Sartorius, Göttingen, Germany)
- Beaker holding HS vial in balance
- Tissue, methanol/water (50/50 v/v), for cleaning of the equipment
- Boxes for storage of HS vials and remaining root powder at -80 °C

Procedures

1. Fill one small and one large polystyrene box to a height of 5 – 10 cm with lq. N_2.*
2. Place -80 °C storage box for HS vials and Eppendorf tubes in one large polystyrene box and fill to a height of ca. 2 cm with lq. N_2.*
3. Cool Eppendorf tube in lq. N_2 (small box).
4. Cool stainless steel balls and adapter in lq. N_2 (large box).
5. Add 5 stainless steel balls to every Eppendorf tube (Figure 2D).
6. Close Eppendorf tubes tightly and put into adapter.
7. Cool adapter with tubes in lq. N_2.

8. Fix adapter into ball mill. Both arms of the mill should carry the same weight to avoid damage.
9. Run ball mill at 30Hz for 3 minutes.
10. Remove adapter with Eppendorf tubes from ball mill and cool in lq. N_2.
11. Place HS vial into a beaker located on the balance to avoid canting of the vial.
12. Weigh 50±5 mg sample powder into pre-cooled (ice) HS vial.
13. Note the exact weight.
14. Tightly seal HS vial with septum containing screw cap.
15. Place HS vial into -80 °C storage box, so that the bottom of HS vial is cooled in lq. N_2.
16. Remove balls from Eppendorf tube, close tightly and cool in lq. N_2.
17. Store HS vial and remaining root powder at -80 °C till analysis.

* Always close the lid of the boxes and refill lq. N_2 from time to time.

Milling and weighing of leaf samples
Materials

- Polystyrene boxes with lid (10x10x10 cm and 2 pieces 30x25x20 cm)
- Lq. N_2
- Frozen leaf samples in 50 mL Sarstedt tubes
- Glass rod
- Ball mill (e.g. MM 301, Retsch)
- Stainless steel beaker 10 mL (e.g. Retsch)
- Stainless steel ball 9mm diameter (1 per beaker, e.g. Retsch)
- Spatula
- HS vial 20 mL
- Magnetic screw cap with hole and 1.3 mm silicone/PTFE septa for HS vial
- Box with crushed ice for pre-cooling of HS vials before weighing step
- Analytical balance (e.g. RC 2010 P, Sartorius)
- Beaker holding HS vial in balance
- Tissue, methanol/water (50/50 v/v), for cleaning of the equipment
- Plastic tube (e.g. Sarstedt 10 mL) for storage of the remaining leaf powder
- Boxes for storage of HS vials and remaining leaf powder at -80 °C

Procedures

1. Fill both a small and large polystyrene box to a height of 5-10 cm with lq. N_2.*
2. Place -80 °C storage box for HS vials and Eppendorf tubes in one large polystyrene box and fill to a height of ca. 2 cm with lq. N_2.*
3. Cool tubes with leaf samples in lq. N_2.
4. Pre-homogenise leaves with pre-cooled glass rod in Sarstedt tube (Figure 4A).
5. Cool stainless steel beakers and balls in lq. N_2 (large box).**
6. Transfer leaf sample to stainless steel beaker (Figure 4C).
7. Close beaker and cool in lq. N_2.
8. Fix beakers into ball mill. Both arms of the mill should carry the same weight to avoid damage.
9. Run ball mill at 30Hz for 3 minutes.
10. Remove beaker from ball mill and cool in lq. N_2.
11. Place HS vial into a beaker located on the balance to avoid canting of the vial.
12. Weigh 100±5 mg sample powder into pre-cooled (ice) HS vial (Figure 4F).
13. Note the exact weight.
14. Tightly seal HS vial with septum containing screw cap.
15. Place HS vial into -80 °C storage box, so that the bottom of HS vial is cooled in lq. N_2.
16. Remove ball from beaker and transfer remaining powder to pre-cooled (lq. N_2) Sarstedt tube (10 mL).
17. Cool 10 mL Sarstedt tube in lq. N_2.
18. Store HS-vial and 10 mL Sarstedt tube with remaining leaf powder at -80 °C.

* Always close the lid of the boxes and refill lq. N_2 from time to time.
** Take care that beakers are completely dry before cooling in lq. N_2.

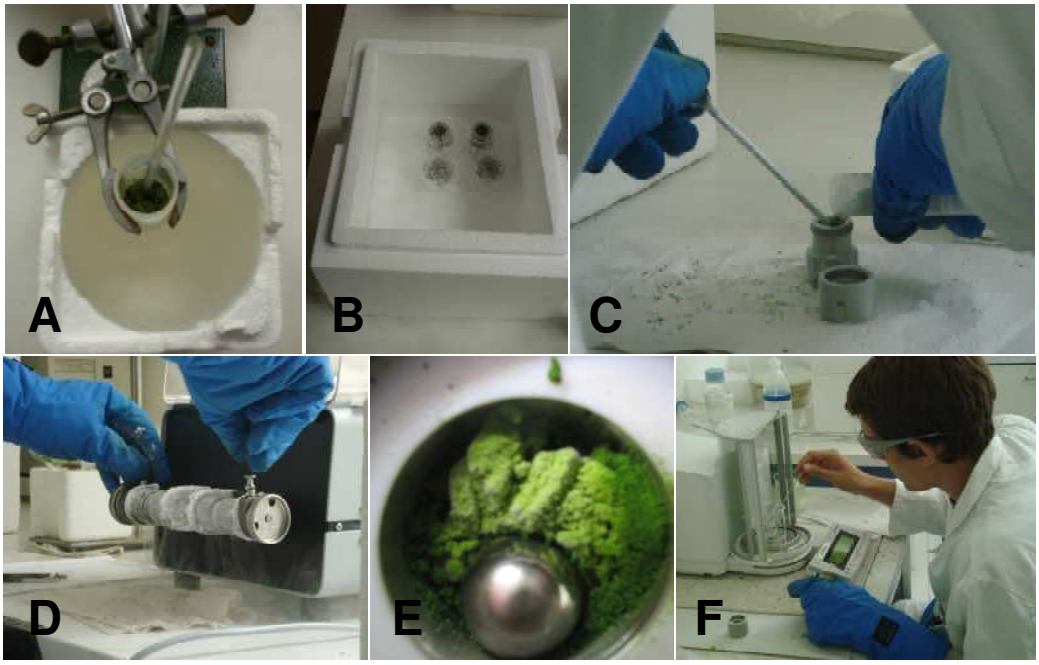

Figure 4 Leaf sample homogenisation and weighing. The leaf is crushed with a glass rod in a cooled (lq. N_2) 50-mL Sarstedt tube (A). Thereafter the leaf tissue is transferred (C) into pre-cooled stainless steel beakers (B). Milling with a ball mill (D) results in fine powder (E) which can be easily weighed into HS-vials (F).

Measurement – automated HS-SPME extraction and GC-MS analysis
Materials

- GC-MS instrument (e.g. Agilent 6890N GC coupled to 5975B MSD, Agilent, Waldbronn, Germany)
- Autosampler with cooled tray, HS-vial heating station, needle heater, SPME holder (e.g. MPS2XL from Gerstel, Mühlheim a.d. Ruhr, Germany)
- Apolar column, 95% dimethyl-, 5% diphenyl polysiloxane (e.g. DB-5MS, 30 m length, 0.25 mm inner diameter, 0,25 µm film thickness, Agilent)
- Polar column, polyethylene glycole, e.g. Optima-WAX 30 m length, 0.25 mm inner diameter, 0,25 µm film thickness, Machery-Nagel, Düren, Germany)
- Alkanes for RI calibration covering the range of C7 – C30 (e.g. C5-C10: in-house mix, C8-C20, 40 mg each in hexane, Fluka, from Sigma-Aldrich, Vienna, Austria), C21-C40 (40 mg each in toluene, Fluka)
- Quality control samples (e.g. pooled root or leaf samples from both treatments)
- SPME-liner 0.75 mm inner diameter
- SPME-fibre 2 cm 50/30 µm Carboxen/Divinylbenzene/Polydimethylsiloxan (CAR/DVB/PDMS, Supelco, from Gerstel) for autosampler, conditioned to suppliers recommendation
- Mobile phase GC: He 5.0

SPME-method

- Equilibration time: 30 min
- Extraction time: 60 min
- Equilibration and extraction temperature: 90 °C
- Needle heater: 270 °C, N_2 flushed, 10 min beak out prior to extraction
- Desorption: 2 min at 250 °C in GC-inlet, splitless mode

SPME is an easy to use, rather cheap technique for metabolite extraction. Further advantages are the enrichment of metabolites on the fibre and the possibility to automate the extraction and enrichment process. No liquid solvents are necessary which preserves costs and environment.

HS-SPME causes only little contamination of the GC inlet liner and the MS source. The disadvantages are the need of permanent monitoring of fibre performance and the somewhat lower precision of GC-MS peak areas compared to liquid injection techniques. Absolute and simultaneous quantification of several ten to hundreds metabolites with SPME is difficult. One possibility is the use of multiple headspace extraction (MHE) [37]. Also the selection of the SMPE conditions (fibre type, equilibration time and temperature, extraction time and temperature) require optimisation depending on sample characteristics as sample amount, water content and metabolites of interest.

GC-Method

- Flow: 1 mL/min He 5.0
- Inlet: 250 °C, splitless for 2 min (during desorption of VOCs from fibre coating)
- Oven programme: starting temperature: 35 °C hold 2 min
- Temperature ramp: 5 °C/min to 260 °C, hold 5 min
- Transfer line: 270 °C

MS-settings

- Electron ionisation mode (EI), ionisation energy: 70 eV
- Source temperature: 230 °C
- Quadrupole temperature: 150 °C
- Scan range: 35 – 500 m/z
- Scan speed: 3 scans/s
- Weekly tuning with PFTBA

At the beginning of every sequence check the condition of the column (blank of column) and the fibre (blank of fibre). Then continue with e.g. 5 quality control samples to check performance of instrument. Proceed with alkane standards for RI calibration.
Include a quality control sample at regular intervals (e.g. every 5-10 samples) to check for method performance. For a typical measurement sequence, see Table 2.
Measurement of the quality control sample is important if no standards for quantification are measured within the sequence. This helps to monitor data variability associated with the weighing step, the SPME process or the separation/detection with GC-MS Further on, these QC samples can serve as basis for calculation of the technical variability of peak areas and RIs. Of course, also standard solutions containing single pure substances can be used for QC purposes. In this case the handling of volatile standards has to be evaluated and optimised since volatiles tend to evaporate during handling. This might result in low precision between HS-SPME GC-MS measurements. Additionally, the SPME conditions have to be optimised for the standard mix. The simplest and easiest way is to use pooled and homogenised plant material, which is available in appropriate amounts as in our example the leaf tissue.

If possible, measure all samples in one sequence or at least within a short time interval.
Due to the fact that a lot of plastics are used at various occasions of this protocol, it can be anticipated to find volatiles originating from these materials also when measuring the plant samples. Additionally, contaminants or artefacts from the lab environment, equipment (e.g. septa, SPME fibre coating or the GC instrument) might be found. The measurement of blank samples provides information which chromatographic peaks must be excluded from the results. The surrounding lab air for example can be measured by placing an open HS vial nearby the balance during the whole weighing procedure and subsequent treating it as the other samples.

Table 2 Illustration of the first 18 measurements of a typical GC sequence table for measuring leaf samples.

sequence 100409			
method	**file**	**sample info**	**sample weight/volume**
columnblank_HP5.M	100409_01	blank of column	-
fibreblank_HP5.M	100409_02	blank of fibre	-
30'60'90°_HP5.M	100409_03	empty vial	-
30'60'90°_HP5.M	100409_04	quality control	10µL
30'60'90°_HP5.M	100409_05	quality control	10µL
30'60'90°_HP5.M	100409_06	quality control	10µL
30'60'90°_HP5.M	100409_07	quality control	10µL
30'60'90°_HP5.M	100409_08	quality control	10µL
C5_10_HP5.M	100409_09	Alkane C5-10	1µL
C8_20_HP5.M	100409_10	Alkane C8-20	10µL
C21_40_HP5.M	100409_11	Alkane C21-40	50µL
30'60'90°_HP5.M	100409_12	quality control	10µL
30'60'90°_HP5.M	100409_13	leaf sample	101mg
30'60'90°_HP5.M	100409_14	leaf sample	105mg
30'60'90°_HP5.M	100409_15	leaf sample	104mg
30'60'90°_HP5.M	100409_16	leaf sample	108mg
30'60'90°_HP5.M	100409_17	leaf sample	102mg
30'60'90°_HP5.M	100409_18	quality control	10µL

Data processing with AMDIS

For the data processing procedures described in the following, AMDIS (Automated Mass Spectral Deconvolution and Identification System, version 2.65, www.amdis.net, Stein 1999 [41]), a freely available software for GC-MS peak picking, mass spectral deconvolution, RI calculation and annotation of compounds by comparing measured mass spectra and RI with library entries was used.

An in-house reference library has to be established in advance. In the targeted approach described below, data evaluation by AMDIS requires a reference library with a list of VOCs, which can be expected to occur in the plant samples. This library is limited to approx. 8000 entries each consisting of a unique chemical identification number (Chemical ID), corresponding reference mass spectra and reference RI value. It serves as a positive list of potential VOCs. Only metabolites contained in the library can be found by the below described "AMDIS batch job" analysis. VOCs of the library can be taken from own measurements of typical plant samples and subsequent annotation/identification of metabolites and/or from the literature. A more detailed description of how this library is established can be found in e.g. [46, 47]. When using more than one stationary GC-phase it is necessary to create separate libraries with RIs for each column type.

In the following a detailed description of typical settings and the operating steps required by the AMDIS programme will be given. This might be helpful during analysis since this type of documentation has not been found in the literature by the authors so far. Words in italic preceded by an arrow (→) refer to either opening the respective menu or using the respective command in the software.
Underlined words refer to the headlines of register tabs in software dialog windows.

Generation of RI calibration file
First, generate a RI calibration file from the alkane standards included in every GC sequence (see Table 2). The RI calibration file is needed to automatically calculate and assign RI values of the deconvoluted metabolite peaks later on in the data evaluation process.

1) Open AMDIS
2) Check presence of alkane standards
    a. Load GC-MS raw data: → *File* → *open* select GC-MS raw data file of alkane standard.
    b. → *Analyze* → *Settings* → *Default*, Identif. tab: Minimum match factor: 90; Type of analysis: Simple, Libr. tab: Target Compounds Library: .MSL file containing mass spectra of the alkanes → *Save*. The software should now deliver a list with all alkane standards, their retention time and their match factor. Check, whether the identifications are correct, and in the correct order.

3) Create alkane standard calibration file for calculation of retention time indices (RI)
    → *Analyze* → *Settings* → *Default*, except: Identif. tab: Minimum match factor: 90; Type of analysis: RI Calibration/Performance; Libr. tab: Target Compounds Library: .MSL file containing mass spectra of the alkanes; RI Calibration Data: .CAL file where AMDIS writes the information about retention time and corresponding retention index → *Save*.

Batch job analysis for the simultaneous processing of multiple sample chromatograms
Second, evaluate all samples against the self-compiled library.
We want to point out here that it is very important to prove the identity of the detected metabolites. Otherwise, it might be that in the case of false positive identifications also the interpretation of the underlying biological processes is wrong. Unfortunately, there are no standardised rules how to identify a metabolite correctly. Different approaches are discussed e.g. in [46-49].
This protocol uses rather strict criteria to avoid false positives. According to [46, 47] we use a minimum match factor of 90 and a maximum relative RI deviation of ±2% if the reference value is from literature. In case of authentic standards measured within a short time interval to the sequence containing the samples, a lower minimum mass spectral match factor of e.g 60 and a narrower RI window of e.g ±5 RI units has been used in the authors laboratory.
Although AMDIS has not been developed especially for quantification, we have checked reliability of the software's peak integration by comparison with manually integrated peaks using Agilent Chemstation software and found no significant difference.

4. AMDIS batch job analysis
    a. Load GC-MS raw data: → *File* → *open* select GC-MS raw data file of sample.
    a. → *Analyze* → *Settings* → *Default*, except: Identif. tab: Minimum match factor: 90; Type of analysis: Use Retention Index Data; RI window: 1 + 2 x 0.01 RI (to allow a RI deviation of ±2% of the measured RI value from the reference value in the library); Match factor penalties: Level: Infinite; Maximum penalty: 100; No RI in library: 100; Libr. tab: Compounds Library: .MSL file containing mass spectra and RI of the metabolites of interest; RI Calibration Data: the .CLA file created in step 3 → *Save*.
    b. → *File* → *Batch Job* → *Create and Run Job…*, Data files: → *Add* all GC-MS files to be processed; Analysis type: Use RI calibration data (CAL); Generate report: mark; Include only first 1 hits; → *Save as…* → *Run*.

    The batch job function automatically generates a text file with the results in the same directory where the batch job is stored.

Statistics/Chemometrics
The last step of this protocol is carried out with the aim to find the metabolites that are differentially expressed between the control group and the group of phylloxera-infested plants. To this end, statistical data analysis is necessary. It is convenient to bring the text file generated by AMDIS batch job in a form where the samples are in rows and the corresponding metabolite

peak areas in columns. This can be done either manually, for example with Microsoft Excel or an R script. The resulting matrix can then easily be processed by several statistical or chemometric software programmes.
In the follwing, statistical data analysis shall be exemplified with a t-test and principal component analysis (PCA) using the software R (version 2.13.0). For a detailed description how to perform a t-test or a PCA with R, the reader can refer to websites dealing with R (e.g. www.r-project.org*),* to literature about statistics and chemometrics in metabolomics, e.g. [40, 50, 51] and to the bioinformatics chapter in this book.

Univariate statistics
To check which metabolites in the matrix show significantly different peak areas a simple t-test can be applied. First divide metabolite peak areas (integrated signal) by the exact sample weight to standardise peak areas. Keep only substances in the matrix, which have been found in most of the samples within the same sample group (e.g. ≥50% of the samples of the same group (control or treatment)).

Use for example the following R command to calculate an unpaired Welch's t-test:

```
> t.test(group1, group2, alternative=c("two.sided"), var.equal=FALSE,
+ conf.level=0.95)
```

where group1 and group2 refer to the normalised peak areas of a metabolite of the control and treated group, respectively. The variances of the peak areas of the two groups are assumed to be unequal and the confidence level is set to 95%.
In case of many metabolites to be tested, an R script for automated application of t-tests to all metabolites in the matrix might be useful.

Multivariate statistics
Principal component analysis (PCA) is a non-supervised statistical method which can be used to classify different sample groups and to provide information about the variance in the data. Ideally, the scores plot shows the two differentially treated samples in form of two separate clusters. Then the loadings plot provides information, which metabolites mainly contribute to this separation. In contrast to t-test no direct information about the statistical significance is provided by PCA.
For PCA also the same matrix with the rows representing the objects (i.e. samples) and the columns corresponding to variables (i.e. metabolites) is needed. Furthermore, the data has to be auto scaled and mean centred. Thus, each cell in the matrix contains the corresponding peak areas of the respective metabolites after auto scaling and mean centring. Keep only metabolites in the matrix, which have been found in most of the samples within the same sample group (e.g. ≥50% of the samples of the same group (control or treatment)).

The R commands are:

```
> scale(matrix)
```

and for PCA:

```
> svd(scaled_matrix)
```

3 Applications of the Technology

The presented protocol was applied by the authors to study the metabolic response of grapevine roots to phylloxera attack [47]. 38 metabolites have been annotated whereas 32 have been confirmed with authentic standards. Applying univariate statistics (t-test) to the results obtained for the two sample groups (phylloxera infested root tips and non-infected root tips) resulted in 14 metabolites showing significantly different concentration levels. The differential expression of some terpenoids like eugenol, beta-myrcene, methyl salicylate, geraniol and beta-

caryophyllene is a hint of activation of the mevalonate (MEV) or the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [52]. Future investigations for example could aim to find JA which can be postulated to be present at an elevated level in infected tissue. Furthermore, the GLVs hexanal and (E)-2-hexenal were found at elevated levels in infested tissue, which indicated the involvement of the lipoxygenase (LOX) pathway [53].

HS-SPME based profiling of plant VOCs was successfully carried out in several studies. A recent metabolomics study for example showed how the terpenoid pattern emitted by *Mentha aquatica* L. changes either under attack of *Chrysolina herbacea* or mechanical damage [54]. The authors collected VOCs over 6 hours from the living plants with SPME followed by GC-MS analysis. They found eight terpenoids with significantly different levels in undamaged, mechanical damaged and herbivore damaged plants. Fernandes et al. [55] investigated the VOCs emitted by kale (*B. oleracea L. var. acephala*) plants before, during and after herbivore (*Pieris brassicae*) attack as well as the VOCs emitted from the insects alone by HS-SPME-GC-MS. A maximum in total terpene level was found after 1 h of herbivore feeding while the mechanical wounding showed no change of the total terpene level. More examples of studies investigating plant-insect interaction in terms of induced VOCs are discussed in e.g. [5, 7, 56-58].

In addition to the technology presented in this protocol, several other approaches have been used to investigate the metabolic response of plants to insects as for example dynamic HS trapping and subsequent two-dimensional GCxGC-ToF-MS [59] or investigation of primary metabolites using LC-MS e.g. [60, 61] or NMR technique e.g. [62, 63].

In recent years a shift from target analysis of a few metabolites towards more comprehensive metabolomics approaches can be observed.

4 Perspectives

Although VOCs play a major role in plant-insect interactions, studies of volatile plant metabolomes on a system level are still sparse. Most plant metabolomics studies, which are carried out on a systems biology level focus on the determination of polar metabolites (GC/MS after derivatisation) as well as non-volatile secondary metabolites (reversed phase LC/MS(/MS)) [64]. The approach presented in this chapter is well suited to be integrated with existing metabolomics platforms and the future consideration of VOCs will significantly extend the coverage of the investigated metabolomes.

Plant-insect interactions are very complex and comprise at least two organisms. The unambiguous assignment of the detected metabolites to one of the interaction partners is still a major challenge in plant-insect metabolomics research. The recently proposed technology of *in vivo* stable isotopic labelling (e.g. ^{13}C- or ^{15}N-labelling) offers a powerful tool to circumvent this limitation by introducing a mass spectral feature that is only observable for metabolites originating from the labelled organism [65, 66]. *In vivo* stable isotopic labelling will also help to develop quantitative HS-SPME GC-MS methods. Fully ^{13}C-labelled plant material can be mixed with each of the plant samples from a biological experiment and used for internal standardisation. The precision of HS-SPME GC-MS based metabolomics methods (i.e. technical variability) will also be significantly improved by the concept of internal standardisation of biological samples.

The study of plant-insect interactions on a systems level requires the integration of the presented technology with both complementary analytical techniques and other "-omics technologies". In close co-operation with researchers from biological and computer disciplines, this type of metabolomics research will lead to an improved understanding of the complex molecular interactions between insects and plants.

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14 Conclusion and Outlook

The presented thesis provides a GC-MS based metabolomics workflow for the investigation of volatiles in plant samples. The aims of this work can be summarised as follows:

1. Comprehensive **literature survey.**
2. **Development** of an **HS-SPME-GC-MS method** for measurement of **volatiles produced** by **grapevine leaves.** This part of the thesis also comprised **optimisation** of method parameters and **evaluation** of the method.
3. Automated **metabolite annotation.**
4. **Integration** of the analytical **method in a workflow** covering all steps from **sampling** to detection of **biomarkers.**
5. **Application** of the developed workflow **to selected biological samples** of **grapevine** with the aim to:
    a. **identify volatile metabolites** of Pinot Noir leaves
    b. **test** if leaf samples originating from different **sampling dates can be differentiated**
    c. **detect volatile biomarkers** indicating **drought** and **UV-B stress** in grapevine
    d. **identify root metabolites** the concentration of which is directly **influenced due to insect attack** (phylloxera, grapevine louse)

Ad 1 The literature study resulted in a database with 1619 entries covering 39 studies referring to the investigation of grapevine by GC. It is available as supplementary data to paper #3.

Ad. 2, 3, 4 The workflow starts with sampling and quenching of biological processes by cooling the samples in lq. nitrogen as fast as possible. For homogenisation a ball mill was used. Samples were milled 3 minutes at 30 Hz. I avoided thawing by cooling all equipment in lq. N_2. After milling, samples were weighed into 20-mL HS-vials, which were tightly sealed with magnetic screw caps containing septa. The samples were stored at -80 °C until further analysis. A HS-SPME-GC-MS method was developed and applied to the samples. The SPME parameters were optimised to extract as many metabolites as possible resulting in an equilibration time of 30 minutes, an extraction time of 60 minutes, both at a temperature of 90 °C. Recorded mass spectra were compared with combined Wiley-NIST 2008 reference mass spectra library and metabolites with

a match factor ≥90 were termed annotated. To ensure the trueness of annotations the maximum deviation of measured RIs (measured on two GC columns, a apolar DB5-MS and a polar Optima-WAX) from reference values was ±2%. Several annotated metabolites have been identified with authentic standards.

Ad 5 The investigation of *Vitis vinifera* cv. Pinot Noir (clone 18 Gm) leaves (paper #3) sampled in open field resulted in the identification of 47 and the annotation of 16 metabolites. Thereof 19 metabolites have been described in *Vitis* spp. for the first time. Technical variability of the method was <40% for the majority of metabolites, biological variability ranges from 7 – 119% between plants. Applying multivariate statistic (PCA) to the detected metabolite showed two clearly separated clusters corresponding to the two sampling dates.

My favourite and very exciting application of the developed workflow was the investigation of volatiles in grapevine roots under attack of phylloxera (paper #1). The publication is the first report on the metabolic response of volatiles in grapevine roots due to phylloxera attack. I was able to identify 32 and annotate 6 root metabolites. The majority of them (32) was described in this paper for the first time to occur in grapevine roots, 4 of them have not been found in *Vitis* spp. until then. 14 metabolites showed significantly differences in their concentrations compared between attacked and non-attacked roots. Several if them are involved of defence related pathways. This is a marvellous example for a metabolomics study ending up with biological interpretation of the analytical results.

Ad 5c Potential stress markers have been found. As the data analysis is still under progress these two experiments have not been discussed in this thesis. The results are going to be published soon.

An additional very interesting application of the workflow out of the grapevine context was the investigation of dust samples. Thereby the matrix was non-living material but consisted largely of organic material. Settled floor dust (SFD) contains a lot of metabolites originating from microorganisms. Therefore, SFD can serve to detect contamination of buildings affected by fungi. The workflow developed in this work also functioned for other than plant samples and my colleague identified 27 and annotated 44 VOCs in dust samples (paper #2).

Several improvements and alternative or complementary procedures are possible for future applications. Shortening of the SPME method and decreasing of equilibration and extraction temperature might result in a two fold improvement. On the one hand, it will offer the possibility to measure more samples per day and on the other hand, lower temperature during equilibration and extraction will reduce the probability of oxidation product formation. The addition of antioxidants or an inert gas (e.g. nitrogen) to the HS-vial containing the sample to be measured might also be tested. Eventually, another fibre

coating might provide a better reproducibility of peak areas possibly leading to the detection of additional metabolites showing significantly different concentrations in differentially treated sample groups.

Improving the accuracy of metabolite quantification, at best establishing absolute quantification would be satisfying. The addition of internal standards or labelled substances might serve as a suitable approach for this purpose and is under investigation already at the institute by another colleague.

A rewarding long term goal in the investigation of the volatile plant metabolome consists in the cultivation of fully labelled living plants to see exactly which metabolites origin from the plant and which do not. Additionally, it will be of great interest to sample the living plants with dynamic HS techniques. E.g. volatiles emitted by leaves can be collected by enveloping a leaf with a bag where continuously air is pulled through and VOCs are trapped in adsorbent tubes.

Besides comprehensive quality control measures and appropriate choice of the analytical instruments and applied techniques, the key for high quality metabolomics studies is to keep an eye on the biological context. Hence, the collaboration with scientists from other disciplines, as biology, informatics, and statistics is necessary and was one of the parts of the PhD thesis where I learned most. I hope that the work done during this thesis has a positive impact to future investigations of the grapevine metabolome.

Appendix

Entscheidend ist, was hinten rauskommt.

Helmut Kohl, 1984

15 List of abbreviations

AMDIS	Automated Mass spectral Deconvolution and Identification System
ArMet	Architecture for metabolomics
CAR	Carboxene
DNA	Desoxyribonucleic acid
DVB	Divinylbenzene
EI	Electron ionisation
EIC	Extracted ion current
GC	Gas chromatography or gas chromatograph
GLV	Green leaf volatiles
HCA	Hierarchical cluster analysis
HS	Headspace
IT	Ion trap
lq.	liquid
LTPRI	Linear temperature programmed retention index
m/z	Mass to charge ratio
MIAMET	Minimum Information About a METabolomics experiment
MS	Mass spectrometry
MSD	Mass selective detector
MSI	Metabolomics Standards Initiative
N_2	Nitrogen
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
PA	Polyacrylate
PBM	Probability based matching
PC	Principal component
PCA	Principal component analysis
PDMS	Polydimethyl-siloxane
PEG	Polyethylene glycol
PLS	Partial least-squares
QqQ	Triple quadrupole
RI	Retention index
RNA	Ribonucleic acid
RSD	Relative standard deviation
SFD	Settled floor dust
SPME	Solid phase microextraction
TIC	Total ion current
TOF	Time of flight
VOC	Volatile organic compound

16 Curriculum vitae

Personal information

First name / Surname	**Georg WEINGART**
Address	Rührsdorf 14 3602 Rossatz AUSTRIA
Mobile	++43 69910568652
E-mail	sg.weingart@yahoo.com
Nationality	Austrian
Date of birth	14.12.1977
Gender	Male

Education and training

Dates	October 2007 – September 2011
Title of qualification to be awarded	Dr.nat.tech. (PhD)
Principal subjects/occupational skills covered	Thesis title: "Development and Application of Analytical Methods for the Metabolic Profiling of Volatiles Produced by Grapevine", Identification of leave metabolites and search for stress markers with GC-MS, part of the project: "Physiological Fingerprinting in Viticulture", http://www.physiological-fingerprint.com/pf-02-project-goals-en.html Compilation of a database for *Vitis* metabolites, about 1600 entries.
Name and type of organisation providing education and training	University of Natural Resources and Life Sciences, Vienna, Department for Agrobiotechnology, IFA Tulln, Center for Analytical Chemistry, http://www.ifa-tulln.ac.at/index.php?id=6&L=1
Level in national or international classification	ISCED 6
Dates	October 1995 – May 2007
Title of qualification awarded	Mag.rer.nat. (MSc)
Principal subjects/occupational skills covered	Study of chemistry, focus on food, analytic, organic chemistry Master thesis: "Analytical and Organoleptic Determination of 2,4,6-Trichloroanisole and Geosmin in Wine", quantitative and qualitative determination of off-flavours in white and red wine with GC-MS, determination of olfactory threshold levels
Name and type of organisation providing education and training	University of Vienna, Department of Chemistry Master thesis at the Teaching and Research Center for Viticulture and Horticulture Klosterneuburg
Level in national or international classification	ISCED 5

Work experience

Dates	July – August 2004, July – September 2003
Occupation or position held	Internship
Main activities and responsibilities	Synthesis of antibiotic agent, cleanup, prepHPLC
Name and address of employer	Sandoz, Vienna, Austria
Type of business or sector	Pharmacy
Dates	July – September 2002
Occupation or position held	Internship
Main activities and responsibilities	Synthesis of pharmaceutical agent, cleanup, prepHPLC
Name and address of employer	Novartis, Vienna, Austria
Type of business or sector	Pharmacy
Dates	November 2001 – June 2002
Occupation or position held	Part time job
Main activities and responsibilities	Recyclable and hazardous waste collecting point, sorting the waste for later treatment, advising people on how to avoid, reduce waste
Name and address of employer	Municipality of Vienna, Austria
Dates	August – September 2001
Occupation or position held	Internship
Main activities and responsibilities	Synthesis of perfume ingredient, cleanup, GC, preparation of olfactory tests
Name and address of employer	KCCS Krems Chemie Chemical Services, Krems, Austria
Type of business or sector	Chemical industry
Dates	September 1998
Occupation or position held	Internship
Main activities and responsibilities	Laboratory for water analysis, checking quality of water needed in different processes of a refinery
Name and address of employer	OMV refinery Schwechat, Austria
Type of business or sector	Chemical industry

Special experience

Dates	May 2010 – September 2010
Occupation or position held	Paternity leave
Main activities and responsibilities	Changing nappies, cooking, doing the laundry, cleaning, entertaining, first aid
Name and address of employer	Maximilian Weingart
Type of business or sector	Household, parents

Personal skills and competences

Mother tongue **German**

Other language

Self-assessment	Understanding				Speaking				Writing	
European level (*)	Listening		Reading		Spoken interaction		Spoken production			
English	B2	Independent user	C1	Proficient user	B2	Independent user	C1	Proficient user	C1	Proficient user

(*) Common European Framework of Reference for Languages

Social skills and competences

Team work experience at University with project partners, at volunteer fire brigade and mountain rescue service, both, team leader and team member
Courses concerning team work, leadership, and communication at the University and at fire fighters school
Supervision of students during a practical course at the University and during their internship at our lab, taking care of a baccalaureate student
Increasing interest in public relation work for fire brigade and civil defence

Organisational skills and competences

Keeper of the minutes of monthly meeting of MS group during PhD
Assisting in the organisation of annual fire brigade festival

Technical skills and competences

Responsible for regularly maintenance of GC-MS and LC-MS (Orbitrap) during PhD
Trainer for technical equipment at the volunteer fire brigade

Computer skills and competences

Competent with most Microsoft Office programmes (Word, Excel, PowerPoint),
Agilent Chemstation, Thermo Xcalibur, AMDIS, Spectconnect
Basic knowledge in R, XCMS, SIEVE, MassFrontier, Tagfinder, MetaboliteDetector

Other skills and competences

1994 onwards
Member of volunteer fire brigade Rossatz, several courses and trainings
Year 2000: commander of the fire brigade
2001-2010: responsible for training of the fire brigade members (about 40 persons)
Regularly attending competitions, in a team and individual
Assisting in organisation of annual fire brigade festival

2008 - 2011
Member of mountain rescue service

2009 - 2011
Civil defence deputy, community of Rossatz

Driving licence

Austrian drivers licence A, B, C, E, F, G

Additional information

PUBLICATIONS

Lawo* NC, Weingart* GJF, Schuhmacher R, Forneck A **2011** The volatile metabolome of grapevine roots: First insights into the metabolic response upon phylloxera attack. Plant Physiology and Biochemistry 49, 9, 1059-1063 * shared first authorship

Vishwanath V, Sulyok M, Weingart G, Kluger B, Täubel M, Mayer S, Schuhmacher R, Krska R **2011** Evaluation of settled floor dust for the presence of microbial metabolites and volatile anthropogenic chemicals in indoor environments by LC-MS/MS and GC-MS methods. Talanta 85, 4, 2027-2038

Weingart, G., H. Schwartz, R. Eder, G. Sontag **2010** Determination of geosmin and 2,4,6-trichloroanisole in white and red Austrian wines by headspace SPME-GC/MS and comparison with sensory analysis. European Food Research and Technology 231, 5, 771 - 779, DOI: 10.1007/s00217-010-1321-8

Brandes W; Nauer S; Weingart G **2010** Characterization of tertiary education and wine flavors topic: SCHIMMEL – KORK. Mitteilungen Klosterneuburg 60 (1) 160-164

Eder, R., E.M. Hütterer, G. Weingart, W. Brandes **2008** Reduction of 2,4,6-trichloroanisole and geosmin contents in wine by means of special filter layers. Mitteilungen Klosterneuburg 58, 12

PERSONAL INTERESTS

Cooking, hiking, wine, reading (crimes), running, my family