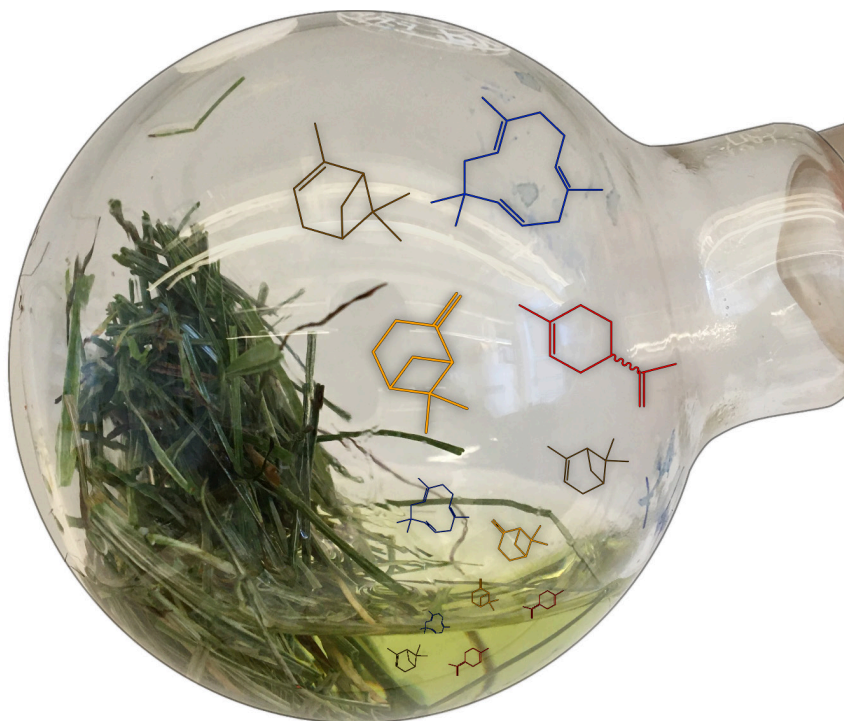


Doctoral Thesis in Chemistry

Sample preparation and analysis of metabolites from plants in the families of *Cyperaceae*, *Poaceae* and *Apiaceae*

LINUS SVENBERG



Sample preparation and analysis of metabolites from plants in the families of *Cyperaceae*, *Poaceae* and *Apiaceae*

LINUS SVENBERG

Academic Dissertation which, with due permission of the KTH Royal Institute of Technology, is submitted for public defence for the Degree of Doctor of Chemistry on Friday the 25th of March 2022, at 10:00. in E3, Osquar Backe 2, Stockholm.

Doctoral Thesis in Chemistry
KTH Royal Institute of Technology
Stockholm, Sweden 2022

© Linus Svenberg

ISBN: 978-91-8040-148-7

TRITA-CBH-FOU-2022:12

Printed by: Universitetsservice US-AB, Sweden 2022

Abstract

An important aspect of understanding how plants effect their environment is the study of volatile compounds and how these influence interactions between plants and other living organisms. The study of these secondary metabolites from plants can be used in practical applications, such as behaviour control of insects, and is an important part in the search for novel compounds that can be used to influence the behaviour of disease spreading vectors. The study of secondary metabolites can also be used as tools for introducing and teaching chemical ecology and analytical chemistry. The proper use of sample preparation methods and analysis has a key role in both these applications.

In this thesis, different sample collection methods are combined with gas chromatography-mass spectrometry to investigate the diversity of secondary metabolites emitted from plants. In **paper I**, two different sample collection methods, ultrasound assisted extraction and solid-phase microextraction, were used as a basis to collect six metabolites as a metric of comparison of the methods. The work compares the ultrasound assisted extraction and solid-phase microextraction of stored *Cyperus rotundus*, and also compares solid-phase microextraction collections from stored and live plant samples.

The static headspace solid-phase microextraction method used in **paper I** was then used to analyse the headspace samples of three other graminoid plants, namely *Cynodon dactylon*, *Cyperus exaltatus* and *Panicum repens*. In **paper III**, the identification of the headspace volatiles of the graminoid plants were determined, and the relative peak area percentages were analysed using one-way ANOVA. The result of this work allowed for a comparison between the three different plants to determine significant differences between the compounds detected based on relative peak area percentages.

Adding to the results of **paper I** and **III**, the results from **paper II** offers more insight into the chemicals present in the gas phase above the plants by employing dynamic headspace sampling from live plants. This adds another sampling method to the body of the work, which expands the knowledge of what volatile compounds are present around the plants. Further, in **paper II**, the use of two-port olfactometric and semi-field bioassays were used to compare which plants have the largest impact on the behaviour of the malaria vector *Anopheles gambiae*.

In **Paper IV**, the ultrasound assisted extraction method from **paper I** was used in order to evaluate the allelopathy of *Aegopodium podagraria*. This

work was used as an inquiry-based learning project for Swedish high school students, and the feedback of the students were collected for development of the project. The results of the work in **paper IV** demonstrated the implementation of the developed experimental methods as tools for teaching students of different academic levels about analytical chemistry and chemical ecology.

Keywords

Gas chromatography; Mass spectrometry; Ultrasound assisted extraction; Solid-phase microextraction; Volatile compounds; Allelopathy; Bioassay

Sammanfattning

En viktig aspekt för förståelsen av hur växter påverkar sin omgivning är studien av flyktiga ämnen och hur dessa påverkar interaktionen mellan växter och andra levande organismer. Studier av sekundära metaboliter från växter kan användas i praktiska applikationer, så som beetendekontroll av insekter, till exempel myggor, där denna kunskap är en viktig del i sökandet efter nya ämnen som kan användas för att påverka beteendet hos smittbärande insekter. Studier av sekundära metaboliter kan också användas som ett redskap för att introducera och undervisa i kemisk ekologi och analytisk kemi. God användning av provpreparering och provanalys har en viktig roll i båda applikationerna.

I denna avhandling så används olika provberedningsmetoder, i kombination med gaskromatografi-masspektometri, för att undersöka den mångfald av sekundära metaboliter som utsöndras från växter. I **paper I** används två olika provuppsamlingsmetoder, ultraljudsassisterad extraktion och fastfas-mikroextraktion, som grunden för uppsamling av sex metaboliter som mått för jämförelse mellan metoderna. Detta arbete jämför provuppsamling med ultraljudsassisterad extraktion och fastfas mikroextraktion på gräs som har förvarats en tid innan analys, men jämför även fastfas-mikroextraktion uppsamlingar från levande gräs med det som legat på förvaring.

Den statistiska gasfas fastfas-mikroextraktionsmetod som användes i **paper I**, användes sedan också för att analysera den kemiska gasfasen för tre andra gräs-liknande växter, nämligen *Cynodon dactylon*, *Cyperus exaltatus* och *Panicum repens*. I **paper III** så utförs identifiering av flyktiga ämnen från de gräs-linkande växternas gasfas, och deras relativa toppareaprocent analyseras med *one-way* ANOVA. Resultaten från detta arbete möjliggör en jämförelse mellan de tre växter för att avgöra om man kan se signifikanta skillnader för de ämnen som detekteras baserat på den relativa toppareaprocenten.

Vidare, tillför arbetet från **paper II** mer insikt om de kemiska föreningar som kan finnas i gasfasen kring växterna utöver det som har visats i **paper I** och **paper III**, genom användning av dynamisk gasfassetuppsamling från levande växter. Detta bidrar med ytterligare en provuppsamlingsmetod, vilket vidare utökar informationen om vilka flyktiga ämnen som finns kring växterna. I **paper II** används två-ports olfaktometrisk-, samt *semi-field* bioanalyser för att jämföra vilken växt som har störst inverkan på malariavektorn *Anopheles gambiae* beteende.

I **paper IV** så används den ultraljudsassisterade extraktionsmetoden från **paper I** för att utvärdera den allelopatiska förmågan hos *Aegopodium podagraria*. Detta arbete användes som ett undersökande arbetsprojekt för svenska gymnasieelever, och återkoppling från eleverna användes för att utveckla projektet. Arbete från **paper IV** visar sig användbart som ett verktyg för utläring, på olika akademiska nivåer, i ämnena kemisk ekologi samt analytisk kemi.

Nyckelord

Gaskromatografi; Masspektrometri; Ultraljudsassisterad extraktion; fastfas-mikroextraktion; Flyktiga ämnen; Allelopati; Bioanalys

List of Publications

This thesis is based on the following papers, referred to as their corresponding Roman numeral in the text:

- I. **Analysis of six secondary metabolites from *Cyperus rotundus* - comparing different methods for determining volatile compounds in laboratory and field settings**
Svenberg, L., Bokore, G.E., Lindh, J., Emmer, Å., *Manuscript*
- II. **Grass-like plants release general volatile cues attractive for gravid *Anopheles gambiae* sensu stricto mosquitoes**
Bokore, G.E., Svenberg, L., Tamre, R., Onyango, P., Bukhari, T., Emmer, Å. and Fillinger, U.
Parasites & Vectors, 14(552), 2021, doi: **10.1186/s13071-021-04939-4**
- III. **Chemical Diversity between three graminoid plants found in Western Kenya analyzed by Headspace Solid-Phase Microextraction Gas Chromatography–Mass Spectrometry (HS-SPME-GC-MS)**
Svenberg, L. and Emmer, Å.
Plants, 10(11), 2423, 2021, doi: **10.3390/plants10112423**
- IV. **Investigation of the allelopathic effect of Ground Elder (*Aegopodium podagraria*) on Timothy (*Phleum pratense*) – Introducing high school students to analytical chemistry and chemical ecology**
Svenberg, L., Malm, L., Abdollahzadeh, N., Gohari, N., Almöv, M., Norin, A., Emmer, Å., *Manuscript*

The contributions of the author to these papers are:

- I. Major part of experiments and major part of writing.
- II. All GCMS analyses, evaluation of GCMS results, preparation of figures, tables and part of writing.
- III. All experiments and major part of writing.
- IV. Supervision of students, planning and performance of experiments, evaluation of data, major part of writing.

Work not included in this thesis:

Project Concrete and Clay: Degradation of organic materials in cementitious environments

Szabó, Z., Dvinskikh, S., Emmer, Å., Svenberg, L., Wold, S., Mårtensson, P.
Svensk Kärnbränslehantering, Stockholm, 2020, report nr. TR-20-02

Work not included in this thesis that has been presented at conferences:

Analysis of Proteins Related to Osteoporosis and CRPS Using CE, nLC, ESI-MS and MALDI-MS

Linus Svenberg, Johan Jacksén and Åsa Emmer

33rd International Symposium on Microscale Separations and Bioanalysis, MSB 2017, Nordwijkerhout, The Netherlands. Poster

Analysis of putative malaria vector oviposition attractants

Linus Svenberg, Jenny Lindh and Åsa Emmer

XIX Euroanalysis 2017, Stockholm, Sweden. Poster

Abbreviations

DHS – Dynamic Headspace
GC- Gas chromatography
MeCN – Acetonitrile
MeOH – Methanol
MS- Mass spectrometry
PDMS/DVB – Polydimethylsiloxane/Divinylbenzene
RI – Retention index
RT- Retention time
SPE – Solid-phase extraction
SPME – Solid-phase microextraction
UAE – Ultrasound assisted extraction
VOC – Volatile Organic Compound

An. – *Anopheles*

A. podagraria - *Aegopodium podagraria* (Ground Elder)

C. dactylon – *Cynodon dactylon* (Bermuda grass)

C. exaltatus – *Cyperus exaltatus* (Giant sedge)

C. rotundus – *Cyperus rotundus* (Nut grass)

C. setaceus – *Cenchrus setaceus* (Purple fountain grass)

P. pratense – *Phleum pratense* (Timothy)

P. repens – *Panicum repens* (Torpedo grass)

s.s – *Sensu Stricto*

Content

Abstract	iii
Sammanfattning	v
List of Publications	vii
Abbreviations	ix
1. Introduction	1
1.1 Malaria and malaria vectors	2
1.2 Plants	2
1.3 Semiochemicals	5
1.4 Sample collection and preparation	7
1.4.1 Ultrasound assisted extraction.....	7
1.4.2 Solid-phase extraction	7
1.4.3 Solid-phase microextraction	9
1.5 Gas Chromatography Mass Spectrometry	11
1.6 Sustainable development goals	12
2. Ultrasound extraction with solid-phase extraction sample concentration	13
2.1 Initial study of ultrasound assisted extraction and sample concentration	15
2.2 Ultrasound Assisted Extraction comparing roots and shoots of <i>Cyperus rotundus</i>	18
3. Olfactometric bioassays	21
3.1 Two-port olfactometric bioassays	21
3.2 Semi-field bioassays	22
4. Solid-phase microextraction analysis of graminoid plants	24
4.1 Chemical profile of <i>Cyperus rotundus</i> using SPME in a laboratory setting	24
4.2 Chemical profile of three graminoid plants using SPME in a laboratory setting	26

5. Headspace sampling in a field setting	32
5.1 Static headspace sampling of <i>Cyperus rotundus</i>	32
5.2 Dynamic headspace sampling of graminoid plants	34
6. Ultrasound used in inquiry-based learning.....	35
6.1 Analysis of extracts from <i>Aegopodium podagraria</i>	35
6.2 Allelopathic study of extracts	38
6.3 Allelopathic study of pure standards	39
6.4 Student evaluation	40
7. Conclusions and future prospects	42
8. Acknowledgements	43
9. References	44

1. Introduction

The relationship between chemical ecology and the behaviour of vectors, broadly defined as organisms that carries infectious diseases between other organisms[1], has been established and studied[2]. This relationship is an important part of developing strategies to combat the spread of diseases via these vectors[3]. Olfactory cues play a role in some of these strategies that are of interest. Thus, identifying olfactory cues from plants in the habitat of the vectors is a key aspect in the development of these strategies. The identification of olfactory cues demands a certain amount of prerequisite knowledge of both the vector and the plants, as well as methods for determining the secondary metabolites present and the plants' effect on the vectors. A secondary metabolite can be defined as "A metabolic intermediate or product, found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism, and biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism"[4]. The search for novel olfactory cues that are derived from plants therefore requires studies of the secondary metabolites in the plants, volatile compounds present in the headspace of the plants, and to put this information in context with behavioural trials of the vectors.

In this thesis, there are four aims that were used to guide the progress of the work. These aims are listed here:

- A1) To investigate methods of sample preparation for the extraction of secondary metabolites from plants
- A2) To compare the results from sample collections performed in a laboratory setting to those collected in a field setting
- A3) To investigate the volatile profile of graminoid plants associated with egg-laying sites of *An. gambiae*
- A4) To evaluate the use of the developed sample preparation methods as tools for teaching in inquiry-based learning

The following section provides an introduction to the concepts that are used in this thesis. This section will first discuss the malaria disease vector as well as the graminoid plants present in the environment of the vectors. This section also introduces the plants used for the allelopathic trials in the inquiry-based project and also introduces semiochemicals. The sample preparation methods and analysis method used in the thesis are briefly explained, introducing the sample preparation methods Ultrasound Assisted Extraction (UAE), Solid-Phase Extraction (SPE), Solid-phase Microextraction (SPME) and the sample analysis method Gas Chromatography-Mass Spectrometry (GC-MS).

1.1 Malaria and malaria vectors

The infection of the *plasmodium* parasite in humans, or as it is more commonly referred to, malaria, is a disease that continues to plague and inhibit the residents in many parts of the world. Although work is done to develop vaccines against the infectious disease, the development of methods for vector control is another viable option for decreasing the spread of the disease.

The definition of a vector, when discussing malaria, is an adult female mosquito of any species in which the *plasmodium* parasite undergoes its sexual cycle to reach its sporozoite stage[5]. At this point the parasite is ready to be transmitted by the blood-feeding of the mosquito on a vertebrate[5]. According to the World Health Organization (WHO), malaria is transmitted, in large part, by vectors of the *Anopheles* genus. The *Anopheles* genus is constituted of over 500 species of mosquitoes, where a large majority has been named[6]. Out of this large number of species, there have been over 50 species, and species complexes, that have been determined as dominant vector species (DVS)[7]. These DVS are described as the transmitters of the majority of the malaria infection in an area. In Africa, the presence of several *Anopheles* species has been reported at different geographical sites in the sub-Saharan areas[8]. Among these species are *An. gambiae s.s.*, *An. coluzzi* and *An. funestus s.s.*, which have been found to be some of the world's most efficient vectors of malaria[9]. Although several vectors are identified as DVS, the *An. gambiae* species is the one which has arguably been studied in most detail. The vectors' ability to transmit the disease is based on both intrinsic factors, such as genetics, and extrinsic factors, such as presence of rain[10]. In the development of novel control methods targeted towards the disease vector, one angle is the use of semiochemicals, which are chemicals that elicit a behavioural response from other organisms[11, 12], present at the natural egg-laying sites of the vector. Such naturally occurring volatile organic compounds (VOCs) could be used to influence the choice of egg-laying sites and are discussed more in section 1.3 in this thesis. One source of VOCs that could influence the ovipositional choice are the plants that grow in the egg-laying sites of the vectors.

1.2 Plants

In this work, the study of different plants has been performed with similar sample preparation methods. It has been shown that vector habitats containing vegetation also contain a larger number of the *Anopheles* larvae, compared to habitats without vegetation[13, 14]. The type of vegetation that is found in the habitats of the vector are that of graminoid plants defining the wetlands of Africa, and its association has been studied in Western Kenya[15]. Graminoid plants such as the grass-like sedges *Cyperus rotundus (L.)*[16] (*Cyperaceae Juss.*)[17] and *Cyperus exaltatus*

(Retz.)[18] (*Cyperaceae* Juss.) as well as the true grasses *Cynodon dactylon* (Pers.)[19] (*Poaceae* Barnhart)[20], and *Panicum repens* (L.)[21] (*Poaceae* Barnhart) are examples of vegetation that occurs in the habitats of the vectors[15]. In figure 1, examples of these plants are shown.

Apart from the abovementioned plants, the ornamental dry-land plant *Cenchrus setaceus* (Morrone)[22] (*Poaceae* Barnhart) and the flowering plant *Aegopodium podagraria* (L.)([23] (*Apiaceae* Lindl.)) [24] have been studied. *C. setaceus* has been used as a comparative plant to the ones found in the habitats of the vectors, as it is used as an ornamental plant in parts of Kenya. It is therefore suggested that it should have no ovipositional effect on the vectors and was used for comparison in the investigation of the behavioural effect of the other plants mentioned above. *A. podagraria*, referred to as Goutweed or Ground Elder, has been studied with the same sample preparation techniques as used for the study of *C. rotundus*. Instead of investigating its effect on malaria vectors, the study of *A. podagraria* has been focused on its allelopathic effect on other plants, in this case *Phleum pratense* (L.)([25] (*Poaceae* Barnhart)). This is because of the observed invasive nature of *A. podagraria* [26].

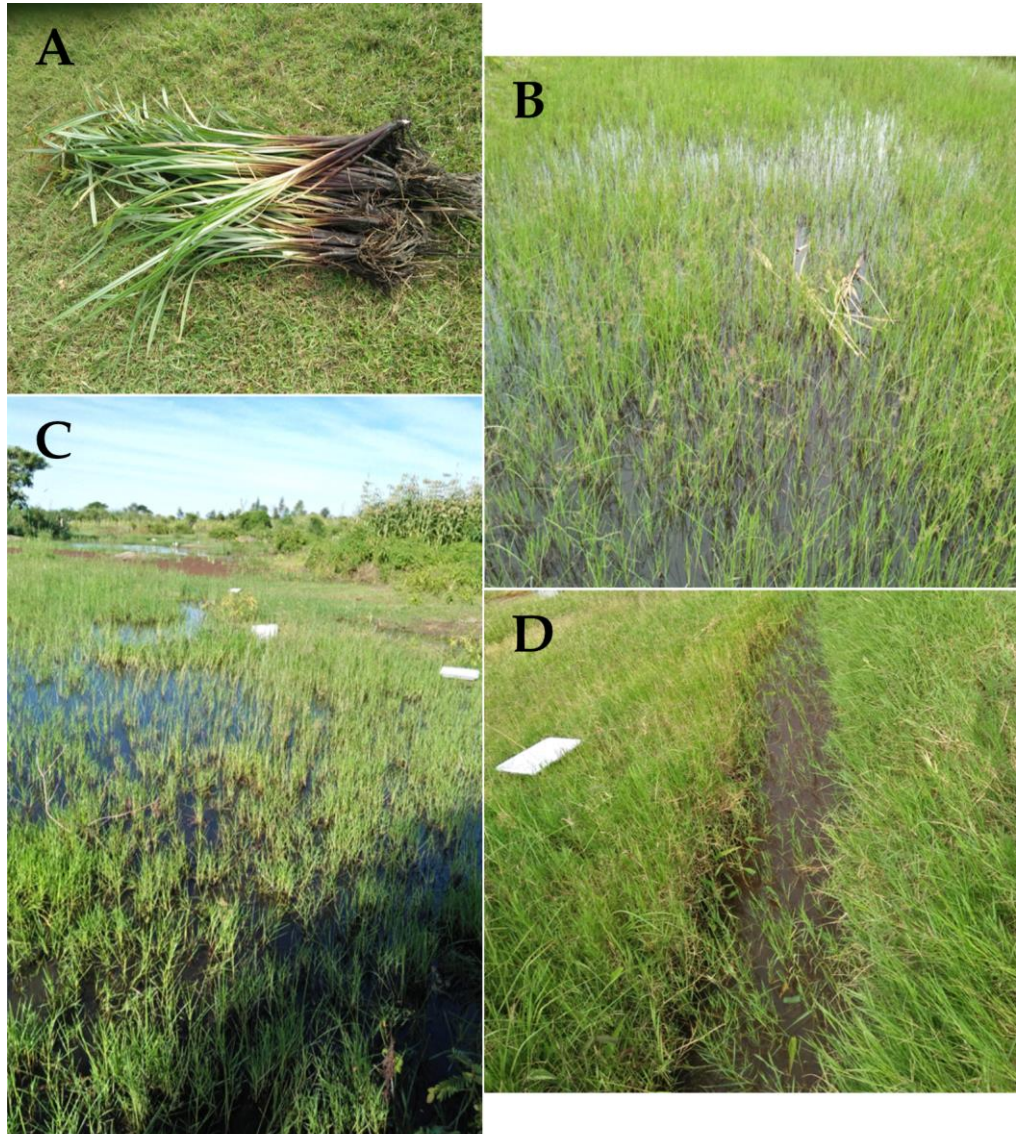


Figure 1: Graminoid plants that have been identified growing in proximity of egg-laying sites of the malaria vector. **A**- *Cyperus exaltatus*, **B** – *Cyperus rotundus*, **C** – *Panicum repens* and **D** – *Cynodon dactylon*. Pictures provided courtesy of Getachew Etichia Bokore, figure B and D appears in: Bokore, G. E., et al. (2020). "A cross-sectional observational study investigating the association between sedges (swamp grasses, Cyperaceae) and the prevalence of immature malaria vectors in aquatic habitats along the shore of Lake Victoria, western Kenya." F1000Res **9**: 1032.

1.3 Semiochemicals

Plants produce a variety of compounds which can be described as primary or secondary metabolites. The secondary metabolites, as described previously, can be involved in several types of interactions with the plant's environment. The secondary metabolites have been shown to be part of both plant-plant interactions and plant-organism interactions. These compounds are referred to as semiochemicals, which are chemicals that are involved in the communication between organisms[27]. In the case of plant-organism interactions, examples of this includes feeding, breeding and oviposition[12]. Semiochemicals can also be classified as allo- and kairomones. These semiochemicals are emitted from a plant which results in a behavioural or physiological response in a plant that receives the semiochemicals. What differentiates them is that the response to an allomone results in a change that is beneficial for the plant emitting the semiochemicals, while a kairomone causes a beneficial change for the receiving plant[27]. These compounds can be named according to their functional groups, following a IUPAC definition, for classification. An alternative way to classify the compounds that are involved in the fragrance profile of plants is by using the following seven groups[28, 29]: Aliphatic, Benzenoids and phenylpropanoids, C5-branched compounds, Nitrogen containing compounds, Sulphur containing compounds, Terpenoids, and miscellaneous Cyclic compounds, examples of which are shown in figure 2.

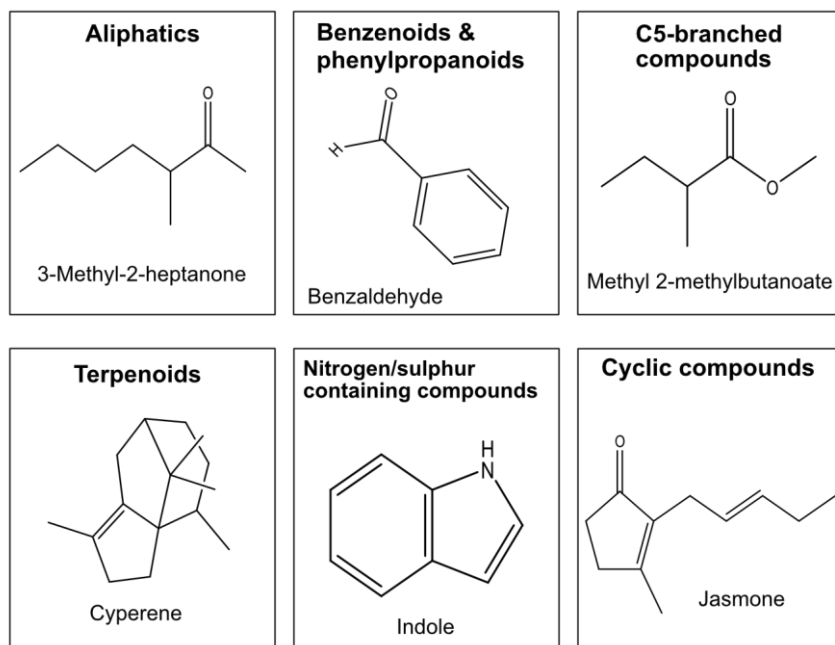


Figure 2: Examples of compounds from the different classes of volatile organic compounds

In the work by Wooding et al, a summary of the semiochemicals and their mediated behavioural on mosquitoes have been presented[30]. In that work, there are compounds from different classes that have shown to be semiochemicals for one or several of the vectors. As discussed in section 1.1, the use of volatiles found at the egg-laying sites of the *An. gambiae* mosquito can be the basis for developing strategies that could be utilized to control the spread of disease. Therefore, the semiochemicals that are listed as oviposition semiochemicals becomes more pertinent. The sesquiterpene alcohols, a subclass of terpenoids, can be exemplified by cedrol, which has been shown to be an ovipositional attractant of *An. gambiae*[31]. Furthermore, compounds such as limonene, β -pinene and β -caryophyllene have been shown to elicit antennal responses from gravid female *An. arabiensis*[32], which suggests their ovipositional influence on the vector. Although these terpenoids have not been tested for their behavioural effect on *An. gambiae*, their influence on a member of the same species complex[33] makes them a point of interest for further investigation.

1.4 Sample collection and preparation

1.4.1 Ultrasound assisted extraction

In order to analyse the secondary metabolites found in the plants, there are different methods available for the extraction of these chemical compounds. Examples of methods utilised are maceration, Soxhlet extraction, hydrodistillation, pressurised hot water extraction (PHWE)[34], microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE). Extraction methods, such as hydrodistillation and Soxhlet extraction, albeit still used for extraction of essential oil, have their limitations. These extraction techniques usually require a larger amount of sample biomass, i.e. the plants that are under investigation, as well as large quantities solvents and energy than methods such as UAE does[35, 36]. In UAE, different types of solvents can be used, depending on target analytes, much like with Soxhlet extractions. A limitation of UAE is that the solvents have to meet requirements regarding physical properties, such as their viscosity and vapour pressure, which are parameters that effect the effectiveness of UAE method[36].

The application of UAE as an effective extraction method has been used for different plants as sample material[37-39], and the application of the UAE has been discussed and reviewed[35, 40-42]. When subjugating the sample to UAE, the generation of microbubbles that are caused by the application of ultrasound in the extraction solvent is used by causing the implosion of these microbubbles[36]. These implosions are a result of repeated rarefaction and compression of the solvent due to the oscillation of the ultrasound. The use of ultrasound adds an acoustic pressure to the solvent. This, in turn, allows the small bubbles to expand in the rarefactions of the ultrasound, as the pressure decreases in the solvent. As the pressure increases, due to compression caused by the oscillations of ultrasound, the bubbles are forced to decrease in size. This mechanism continues to occur until, in a rarefaction stage, the bubble reaches a critical size due to repeated increase and decrease in size. The following implosion of the bubble, due to its inability to maintain its structure, is the driving force with which the extraction occurs. Via the asymmetrical implosions, microjets of the extractant are targeted to the surface of the plant material[36]. This causes pitting and erosion[35], which breaks down the cell walls and allows for the secondary metabolites to be extracted. Additionally, ultrasound causes a shaking at a macroscopic level, which further aids the extraction[35].

1.4.2 Solid-phase extraction

Solid-phase extraction (SPE) is a sample preparation technique that is employed in order to purify and concentrate a sample. It is a technique that has been used since the 1960s, and has shown its usefulness by partly replacing methods such as liquid-liquid extraction. The use of SPE makes

a wider range of applications possible, as well as being a greener alternative to liquid-liquid extraction due to the decreased volumes of solvents needed[43]. SPE methods commonly consists of five steps[44], which are illustrated in figure 3. The first step is the pre-conditioning step, which is shown as step A in the figure. This step is performed with an appropriately selected solvent, based on the stationary phase of the bed, and its purpose is to wet the stationary phase as well as fill the void volume of the cartridge. A common practice is to use the same solvent for both the pre-conditioning and the elution of the sample at the end of the protocol. The advantage of pre-conditioning the dry bed with the elution solvent is that any impurities from the bed will be washed out in this step, rather than co-eluting with the target analytes in the final elution step.

After pre-conditioning, a first washing step is performed. In this step, it is important to ensure that the surface of the solid phase is properly wetted, which is achieved by using a solvent that is appropriate for the solid phase used[45]. In the case of extractions in this work, using acetonitrile (MeCN) or methanol (MeOH), the wetting/initial washing stage can be performed using these as solvent, which is visualized in the figure as step B. However, a step between A and B can be necessary if the extraction solvent is not suitable for wetting of the stationary phase. The next step is to load the sample and allow it to pass through the sorbent at a low enough rate to allow the sample to interact with the stationary phase. Shown in figure 3-C.2, it can be seen that as the sample passes through, both the target analytes and non-target-analytes, are bonding to the sorbent. This is assuming that there are non-target analytes in the sample that have the ability to interact with the stationary phase. These non-target analytes can be matrix compounds and/or compounds that are weakly bound to the sorbent, that are also extracted from the plant samples. In order to remove these auxiliary compounds, a second washing step is performed, shown as step D in figure 3, where a solvent that is a slightly stronger eluent than the extraction solvent is used. The strength of this solvent is dependent on the SPE protocol, but in the case of this work, a stronger solvent would be a more non-polar solvent. This is done in order to remove the weaker bound non-target analytes. This solvent can, however, not be as strong an eluent as the final elution solvent, as this would lead to loss of the target analytes bound to the stationary phase. The final step is the elution of the target analytes using the elution solvent, shown as step E, which can be chosen depending on the analysis that will be performed after the SPE treatment. However, the selected solvent must be able to elute and dissolve the target analytes first and foremost.

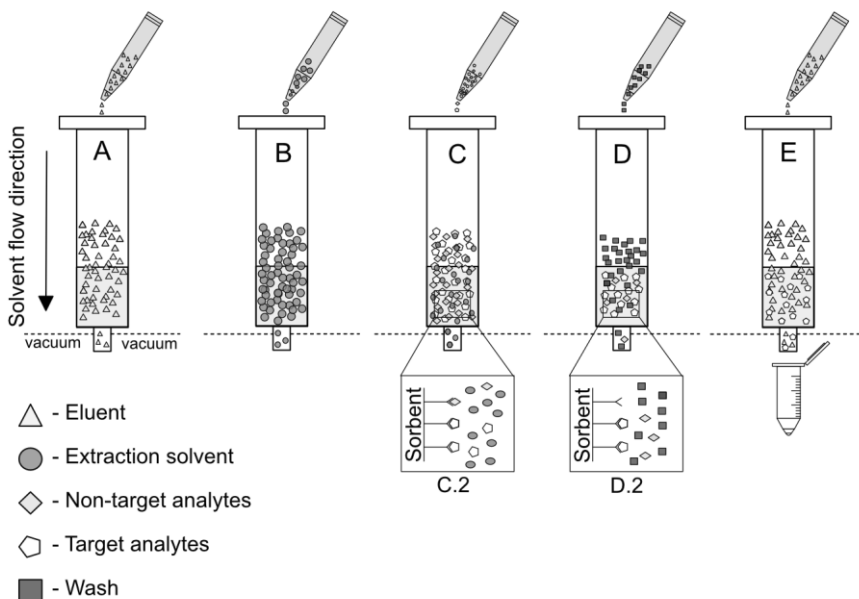


Figure 3: Schematic representation of the workflow of solid-phase extraction. **A** - Pre-conditioning step, **B** - Initial washings/Wetting step, **C** - Sample loading step, **C.2** - Visualization of analytes and sample matrix adsorbing to the stationary phase, **D** - Second washing step, **D.2** - Visualization of matrix analytes being eluted in the second wash step, **E** - Elution step.

1.4.3 Solid-phase microextraction

Solid-phase microextraction (SPME) was first introduced in the work by Arthur and Pawliszyn, presenting it as an alternative method to that of thermal desorption of compounds from solid phase extraction supports[46]. The use of SPME fibres allows for a rapid and solvent-free sample collection[47], and has been used for fragrance analysis of plants[48, 49]. Examples of applications of SPME is direct extraction, headspace extraction and extractions using membrane protection[50]. The methods applied in this work has been focused on using headspace sample collection, which is relevant for collecting compounds that are relatively volatile[50]. In figure 4, the headspace sample collection workflow for this work is visualized. It is initiated by allowing the analytes to start moving into the headspace, prior to introduction of the fibre. After the fibre has been injected and exposed to the headspace, it remains in the headspace for a pre-determined period of time to allow for absorption of sample. The principle of absorption of analytes to the fibre relies on mass transfer

between the headspace and the solid phase of the fibre. Volatile analytes are extracted faster than semi-volatiles due to the faster mass transport rates through the headspace as a result of concentration differences[50]. After the extraction time has elapsed, the fibre is inserted into the injector port of the analysis instrument, and the absorption of the analytes is reversed via thermal desorption.

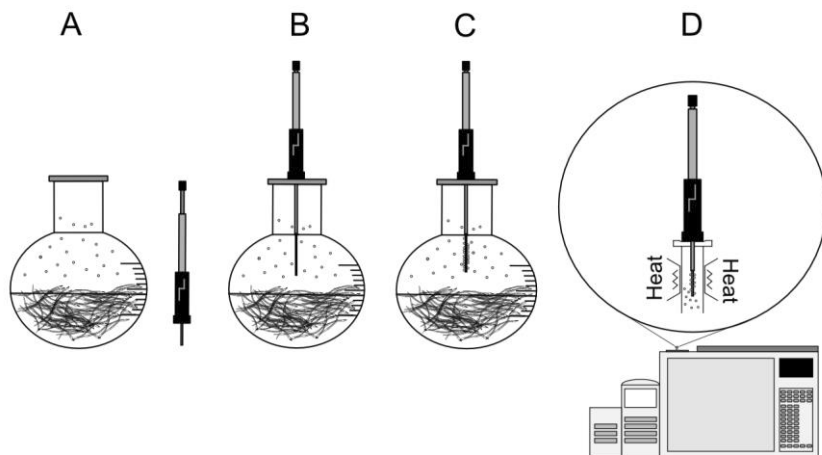


Figure 4: Schematic figure of workflow for sample collection using Headspace-SPME. **A** - the headspace is allowed to equilibrate, **B** - the introduction of the fibre to the headspace of the plant material, **C** - After a pre-determined time has passed, the analytes that have absorbed to the stationary phase are withdrawn with the fibre, **D** - The analytes are introduced to the analytical instrument via thermal desorption of the fibre.

There are several facets of SPME analysis that requires optimisation. Parameters such as sample volume, extraction time, and choice of fibre coating must be considered in order to obtain the optimal sample collection method[51]. The choice of fibre is based on volatility, polarity and molecular weight, for which there is a selection of commercially available fibres to choose from. There is also development of new concepts for fibre-coating[52-54]. Sample volume plays an important role in the determination of the distribution constant between the fibre and sample[55]. Therefore, changing the sample volume can change the amount of analyte that is extracted, which will affect the precision of the analysis[55]. Extraction times is selected depending on whether the extraction should be performed in the time prior to equilibrium or once equilibrium in the headspace has reached. The advantage of allowing the system to reach equilibrium is that differences in absorption of analytes is minimized in relation to precision in extraction time. However, allowing

the system to reach equilibrium might be too large of a time-sink to be applicable[56]. Other examples of parameters that should be considered for the method development is temperature and agitation[50].

There are a large number of applications of the analysis of VOCs from different plant parts, such as leaves, flowers, fruits and, in fewer cases, roots presented in the literature[57]. There are several advantages with headspace collections compared to that of other extraction methods, such as the avoidance of problems associated with interference from matrix effects[50]. Moreover, it is solvent-free and minimizes loss of VOCs[58]. Nonetheless, there are challenges in the application of SPME, for example, the loadability of the coating and to maintain a consistent sample volume for *in vivo* sampling[57]. Therefore, there is no need to select one method over the other, but rather evaluate what information that can be gained by using different methods of sample preparation together or in parallel. This approach could ensure the largest amount of information regarding e.g. the presence of metabolites in plants is obtained.

1.5 Gas Chromatography Mass Spectrometry

One of the earliest works based on the concept of separation of analytes in gas-phase was shown by James and Martin, who are generally attributed with the invention of gas chromatography[59, 60] (GC). The main constituents of the modern instrumentation are the inlet, the column and the detector. In addition to this, there is a carrier gas and pneumatic controls as well as an oven for temperature control of the column. Finally, there is a software unit that is used for the collection of data and for controlling the analytical methods executed by the instrument. In this work, the instrumentation will be viewed from its application within metabolite profiling of compounds from plants.

The use of GC, in the field of metabolomic profiling, is the analysis method that is perhaps most commonly employed. Due to the ability to separate complex mixtures of volatiles with the use of capillary columns, and the versatility, the technique has been used for analysis of many compounds, like terpenoids[61]. The use of mass spectrometry (MS), in combination with GC, has enabled the identification of compounds, and MS has become a common detector in combination with GC for the analysis of volatile organic compounds[62]. In addition to its sensitivity, MS also provides structural information, and via the use of spectral libraries, the identity of the compounds can be suggested[61, 63]. MS instruments generally consists of four parts; ion source, mass analyser, detector and a vacuum system[64]. In the case of metabolic profiling, a commonly used combination of ion source and mass analyser is that of an Electron Ionization (EI) source[65], coupled to a single Quadrupole mass analyzer[62, 65].

The features of GC-MS that are important when developing a method for analysis are many, but certain parameters requires more attention than others. These parameters include choice of stationary phase of the column, temperature program in the oven, choice of injector and scan mode in the MS, to name a few. The choice of stationary phase in the column is dictated by the properties of the target analytes, as the separation occurs due to the partition between the stationary phase of the column and mobile phase of the inert carrier gas. Due to the properties of many of the secondary metabolites, the more common stationary phases are non-polar phases, but separation on polar phases can be done, both as a compliment to analysis with a non-polar column, or as its own analysis method. The temperature program of the oven also influences the separation of the analytes, as volatility plays a part in the separation[66], and is an important aspect to keep in mind for further handling of data from the analysis.

Once a method is established, the structural information, as well as the retention time (RT) of the analytes can be determined. Although there is a substantial amount of information available, obtained by the analysis method, it is often not enough for the identity of an analyte peak to be determined. Therefore, additional information must be provided for verifying the identity of an analyte peak. One of the methods employed for further determining the identity of an analyte is using retention index (RI), which relates the RT of an analyte to a reference series of compounds. Most commonly, this series of compounds are n-alkanes of increasing sizes, which allows for the calculation of the RI. Kovats RI is the calculations performed for isothermal temperature profiles and Van Den Dool/Kratz RI is the calculations performed for temperature gradients[67]. This allows for comparison with standards that have been analysed with similar instrumental methods and can further verify the identity of the analyte. Even though RI allows for comparison, the analysis of pure standards of the suspected analyte using the same analysis method as for the sample, is the most desirable. To confirm the metabolites retention time on the method used for the metabolite profiling, comparison with external standard is perhaps the most reliable way of verifying the identity of analyte peak.

1.6 Sustainable development goals

In 2015, the United Nations adopted the new 2030 agenda for sustainable development[68]. In this new agenda, 17 Sustainable Development Goals (SDGs) were defined to help promote sustainable development, and with this came 169 targets that were agreed to be enacted. The work presented in this thesis can be related to certain targets and goals from the 2030 Agenda, when put into the context of implementing the findings in the development of malaria vector control.

The use of solvent-free headspace sampling for the identification of VOCs in the headspace of the graminoid plants in **paper I** and **III** relates to goal 12, named “responsible consumption and production”. This is due to the minimized amount of solvent used, while still gaining valuable information with regards to the diversity of secondary metabolites available from the plants. This concept also applies to the use of ultrasound in the extraction of metabolites in **paper I** and **IV**, where UAE has been shown to be a greener alternative to other methods of extraction[69, 70].

The shaping of the educational project presented in **paper IV** relates to goal 4, which is named “quality education”[68, 71]. One of the targets of quality education is target 4.7, which is defined as “By 2030, ensure that all learners acquire the knowledge and skills needed to promote sustainable development, including, among others, through education for sustainable development and sustainable lifestyles, human rights, gender equality, promotion of a culture of peace and non-violence, global citizenship and appreciation of cultural diversity and of culture’s contribution to sustainable development”[68, 72]. By showing the responsible application of more environmentally friendly analytical techniques in a project aimed for students, some knowledge of sustainable development can be made available to the students.

Finally, the results presented in **paper II** shows more evidence for the use of semiochemicals from plants to control the behaviour of the disease vector, and through it, limiting the transmission of the disease. The long-term goal of this work relates it to goals 1, “no poverty”[73-75] and to goal 3, “good health and well-being”[76-78].

2. Ultrasound extraction with solid-phase extraction sample concentration

To identify and select the secondary metabolites that could be tested as ovipositional attractants for *An. gambiae*, the analysis of the chemicals available in the graminoid plants must be investigated. In **paper I**, the use of UAE was investigated as a method to extract secondary metabolites in different plant tissues for further separation and identification. Shoots are referring to the parts of the plant that are protruding above the soil, while the roots refer to the parts of the plant that are embedded in the soil. The findings in **paper I** was used as the foundation for the UAE and SPE protocol used in **paper IV**. In order to offer an overview of what methods are used for what samples throughout the entirety of this work, figure 5 shows a flowchart describing the different methods used, which paper they are used in and what section of this thesis discusses them.

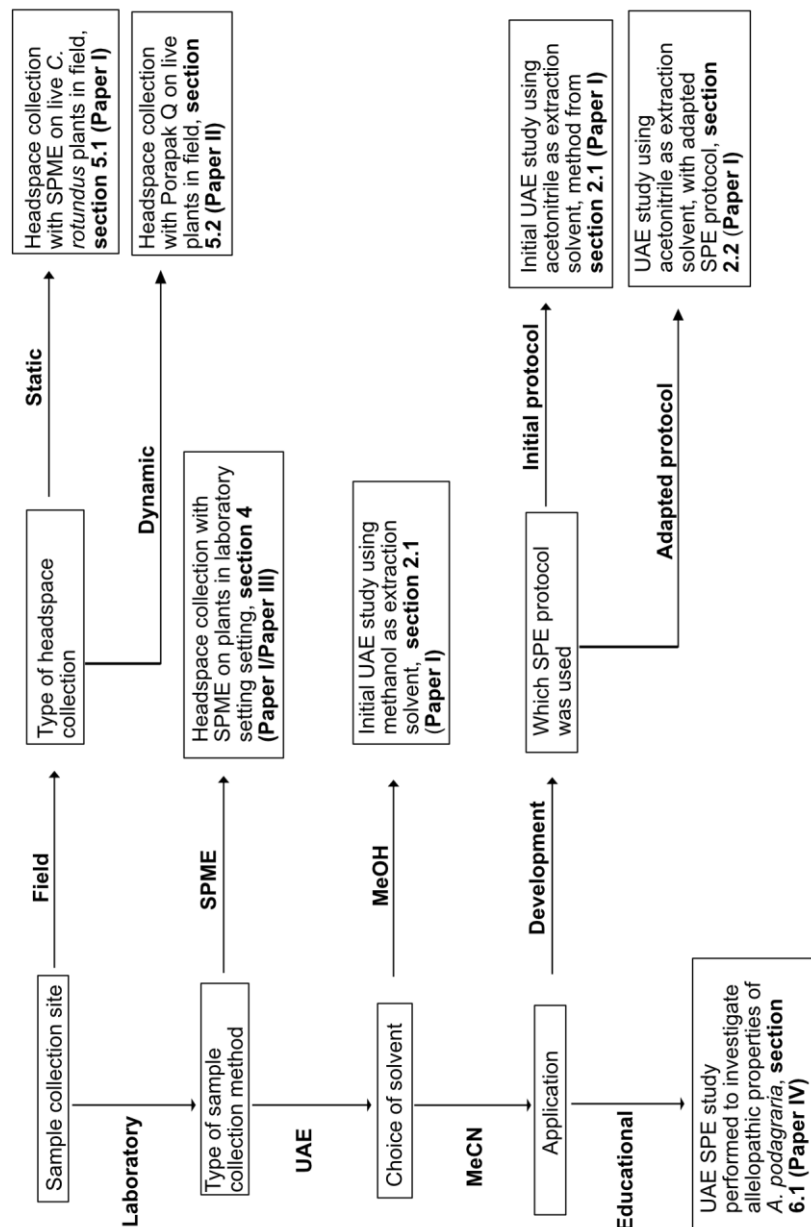


Figure 5 A flowchart showing what sample preparation methods are related to what samples, which section of this thesis and which paper contains more details regarding the method and the results.

2.1 Initial study of ultrasound assisted extraction and sample concentration

As described in the aims of the thesis, A1 is targeted towards the extraction of secondary metabolites from plants. The initial study, using UAE as extraction method, was performed to obtain information regarding individual secondary metabolites and blends that could be used as ovipositional substrates. This initial study did not include optimisation of the SPE protocol and was tested to investigate if UAE-SPE was a suitable technique for the plant sample. Here, the goal was to extract the chemicals present in the plant biomass by using a more energy and solvent efficient method, as mentioned in section 1.4.1. The initial plant sample studied was *C. rotundus*, where the roots and the shoots of the plant had not been separated. The first iteration of extraction parameters was performed with both MeOH and MeCN, using roughly 5 grams of plant mass, and covering the volume of the mass with the solvent, which required around 20 mL. This was done as no relationship between mass and volume had been established yet from the bioassays. Fully submerging the volume of the plant mass would ensure that as much of the plant as possible was exposed to the mechanisms of the UAE, as discussed in section 1.4.1. Two replicates of extractions with MeOH and two replicates of extractions with MeCN were performed. From the solvent volume of the samples, 1 μL was aspirated and diluted in 9 μL hexane, while the remaining volume of the extracts were concentrated with a non-optimized SPE protocol. From the 10 μL of prepared solution, 1 μL was injected and analysed for the two extractions with MeOH and the two extractions with MeCN.

In figure 6, the comparison between UAE with and without SPE is shown, as well as the comparison between the solvents used. In figure 6, the chromatogram timeframe that is shown is between 10-16 minutes of the analysis, due to the large cluster of peaks eluting here, but the total analysis time was 21.3 minutes. In figure 6A, the comparison of MeOH and MeCN is shown, where similar peaks were extracted in the two solvents. The differences between the two extraction solvents seems to be MeCN extracting a higher number of unique peaks. Examples of this are the three peaks eluting just after 10 minutes in figure 6A. Figure 6B shows two replicates of MeOH extractions, illustrating that in this initial test study, the results of the method appear to be repeatable. However, only two extractions were made for the initial study, so repeatability cannot be confirmed. Figure 6C-D shows the comparison between UAE extracts and their SPE-concentrated counterpart. It can be determined that the implementation of the SPE procedure affects different analyte peaks in different ways, causing some to increase and others to decrease in signal intensity. This can be attributed to the non-optimized parameters of the SPE-protocol, such as the solvent chosen for the different steps. Despite this, the advantage of removal of particles, the possibility of solvent

exchange, and the reduction of sample volumes advocates for the continued implementation of the SPE protocol. However, in order to improve the SPE protocol, the composition of the wash and elution solvent must be chosen so to displace the non-targeted analytes, while retaining the target analytes[79]. This can be achieved by using mixtures of solvents in the different steps of the SPE workflow, adjusting the affinity of these mixtures for the compounds they are aimed to remove. The solvents used in the different steps have to be investigated with regards to their effect in the number of extracted compounds, and if any modifications are needed to improve the protocol.

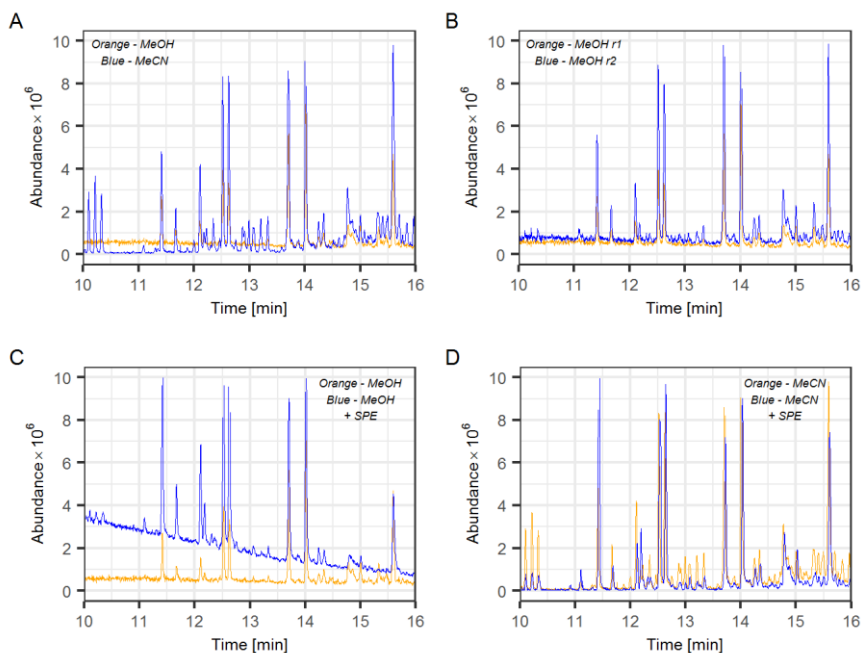


Figure 6: Chromatograms showing **A** - UAE using MeCN and MeOH as solvent. **Paper I**, figure 2A and figure 3A. **B** - Comparing replicates of UAE using MeOH as solvent. **Paper I**, figure 2A and figure 2C. **C** - comparing UAE with MeOH, with and without SPE concentration. **Paper I**, figure 2A and figure 2B. **D** - comparing UAE with MeCN, with and without SPE concentration. **Paper I**, figure 3A and figure 3B.

To be able to compare the results from different iterations of the extraction methods, a metric of comparison needed to be established. Based on previous studies of metabolites extracted from *C. rotundus* from different geographical locations, it was determined that six different metabolites would be used as a metric of comparison as these appeared to be continuously present in the analysis of *C. rotundus*. The six metabolites

were α -pinene[80-86], β -pinene[80-86], limonene[80, 82-86], β -caryophyllene[81, 83, 85], α -humulene[81, 83, 85, 86] and caryophyllene oxide[80-86]. In table 1, the relative peak area percentage of these metabolites are compared between the pre and post SPE-concentrated samples of the initial UAE study. From these results, it can be seen that the relative peak area percentages are higher after the SPE concentration in many of the cases. As the volume has been reduced, due to the SPE protocol, this results in an increased concentration of the metabolites. It can also be seen that the size of the peak areas for all metabolites were similar in the two different extraction solvents.

Even though the metric for comparison was the six metabolites previously described, there was also the incentive to extract as high a number of metabolites as possible. Since the aim of the study was to provide information about which secondary metabolites are produced by a specific plant, the larger number of individual peaks weighed in the favour of MeCN, therefore using this as the solvent of choice for the extraction protocol.

Table 1: Mean values for relative peak area percentages with standard error for extractions using UAE performed with methanol or acetonitrile as solvent. Area MeOH pre SPE - Mean area percentage for samples using methanol as extraction solvent with no SPE (n=2), Area MeOH post SPE - Mean area percentage for samples using methanol as extraction solvent with SPE (n=2), Area MeCN pre SPE - Mean area percentage for samples using acetonitrile as extraction solvent with no SPE (n=2), Area MeCN post SPE - Mean area percentage for samples using acetonitrile as extraction solvent with SPE (n=2), SE - Standard error of the mean. **Paper I**, table 1.

Compound	Area MeOH \pm SE [%] pre SPE	Area MeOH \pm SE [%] post SPE
<i>α-pinene</i>	1.05 \pm 0.43	2.13 \pm 0.37
<i>β-pinene</i>	2.86 \pm 0.59	4.32 \pm 0.99
<i>Limonene</i>	0.77 \pm 0.08	1.56 \pm 0.12
<i>β-caryophyllene</i>	0.68 \pm 0.13	1.94 \pm 0.16
<i>α-humulene</i>	1.35 \pm 0.16	3.33 \pm 0.35
<i>Caryophyllene oxide</i>	6.13 \pm 0.48	6.19 \pm 0.58

Compound	Area MeCN \pm SE [%] pre SPE	Area MeCN \pm SE [%] post SPE
<i>α-pinene</i>	1.04 \pm 0.24	1.37 \pm 0.27
<i>β-pinene</i>	2.15 \pm 0.55	2.58 \pm 0.53
<i>Limonene</i>	0.55 \pm 0.2	0.76 \pm 0.13
<i>β-caryophyllene</i>	0.66 \pm 0.27	1.7 \pm 0.52
<i>α-humulene</i>	1.78 \pm 0.4	2.8 \pm 0.91
<i>Caryophyllene oxide</i>	6.05 \pm 0.67	6.11 \pm 0.17

2.2 Ultrasound Assisted Extraction comparing roots and shoots of *Cyperus rotundus*

In the analyses in section 2.1, the use of both roots and shoots in a mixture constituted the samples. A point of interest was to investigate whether any metabolites originated from one plant source or if all metabolites were present in both the roots and the shoots of the plants. As it has been shown, semiochemicals can originate from different types of plant tissue, and the isolation of the source is important of the understanding of the behaviour of the vectors[31, 32]. Thus, extractions on both of the different plant tissues were performed using acetonitrile as extraction solvent. As the extraction solvent was determined, the SPE protocol could be altered to suit the extraction solvent better. Changes in solvents used in the second washing step as well as elution step were investigated and adapted to suit

the extraction with MeCN. Comparing the relative peak areas of the identified metabolites would show if they were extracted in a higher extent from one of the two plant tissues. In table 2 the comparison of the SPE-concentrated MeCN extractions from the preliminary study is compared with the extractions from the different plant tissues.

Table 2; Relative peak area percentages for samples extracted from roots (n=4) and shoot parts (n=4) using the adapted SPE method SE – Standard error. Values from acetonitrile samples that have been concentrated with the initial SPE method are included for comparison (n=2), * - detected once, no SE available. **Paper I**, table 2.

Compound	Initial trial MeCN UAE Area \pm SE [%]	MeCN UAE roots Area \pm SE [%]	MeCN UAE shoot Area \pm SE [%]
<i>α-pinene</i>	1.37 \pm 0.27	0	0
<i>β-pinene</i>	2.58 \pm 0.53	0	0
<i>Limonene</i>	0.76 \pm 0.13	0	0
<i>β-caryophyllene</i>	1.7 \pm 0.52	0	0
<i>α-humulene</i>	2.8 \pm 0.91	0.17*	0
<i>Caryophyllene oxide</i>	6.11 \pm 0.17	1.47 \pm 0.74	0

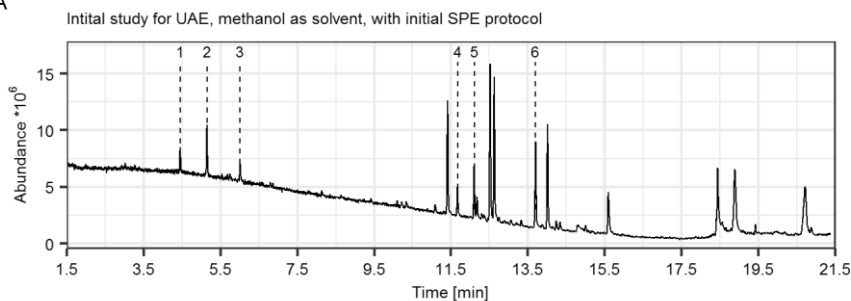
This second method for extraction of the target metabolites showed poor results, that did not align with the results of the initial study. Results obtained in the initial study, and with the UAE method with the adapted SPE protocol, are compared in figure 7. In order to identify the peaks with as small variation as possible, and to remove operator bias, the use of integration methods was applied. The parameters for the integration method used for the samples of the initial UAE study was the *ICIS* present in the *Xcalibur* software. The integration parameters for the UAE method described in this section, with the adapted SPE method are shown in table 3. The two methods in table 3 are used for peak detection, named “Peak Detection Method”, and for determining total area in the chromatogram, named “Total Area Method”. The peak detection method was also used to determine the individual area values of the detected metabolites, so that the relative peak area percentage values could be calculated with the total area of the respective analysis.

Table 3: Method parameters for peak detection and total area

Parameter	Peak Detection Method	Total Area Method
Initial Area Reject	1500000	1
Initial Peak Width	0.02	0.02
Shoulder Detection	Off	Off
Initial Threshold	16	15

The peaks representing the target compounds were not sufficiently large to be identified as peaks using the integration method shown in table 3, thus zero-values are reported. However, in figure 7, traces of the metabolites from the root samples can still be seen. It should be noted that even though a sample could contain trace amounts of metabolites, these were not sufficiently high in abundance to be identified as peaks.

A



B

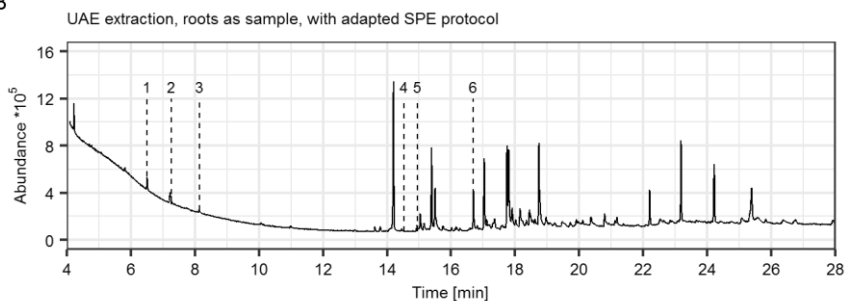


Figure 7: Comparison of analysis of UAE samples concentrated with SPE from the initial trial and UAE samples concentrated with SPE using roots as sample. **Paper I**, figure 1B (A) and figure 4A (B).

3. Olfactometric bioassays

In combination with determining what semiochemicals are available from the different graminoid plants that have been associated with the egg-laying sites of the malaria vector[15], it is also important to determine which plants elicit the strongest attraction. In **paper II**, a two-port olfactometric setup was used to determine the attraction of gravid *An. gambiae* s.s female mosquitoes to the different graminoid plants when compared to different substrates. A semi-field test setup was also used to establish if these attractions are persistent at longer ranges than that of the two-port olfactometric test setup.

3.1 Two-port olfactometric bioassays

The two-port olfactometric bioassay was used to test the odour-orientation of the malaria vector. The experiments were carried out by releasing 100 gravid *An. gambiae* s.s female mosquitoes into a small chamber with two openings, leading to one of two equal-size chambers. In one of the chambers, a bucket of lake water was placed, while a bucket with lake water, containing approximately 350 grams of a graminoid plant, was placed in the second chamber. A small fan was used to pump air from both chambers to the chamber in which the mosquitoes were released. The mosquitoes were left in the setup between 18:00 p.m. to 8:00 a.m. the following day to make their decision of which chamber they would move towards. The number of mosquitoes choosing either of the chambers was recorded. The replicate was discarded and repeated when mortality was $\geq 20\%$ in the release chamber or when less than 50% of the released mosquitoes responded, meaning that they remained in the release chamber for the duration of the time they were given to make a choice. A full description of the replicates can be found in **paper II**, table 1. In figure 8, the two-port olfactometric bioassay is visualized.

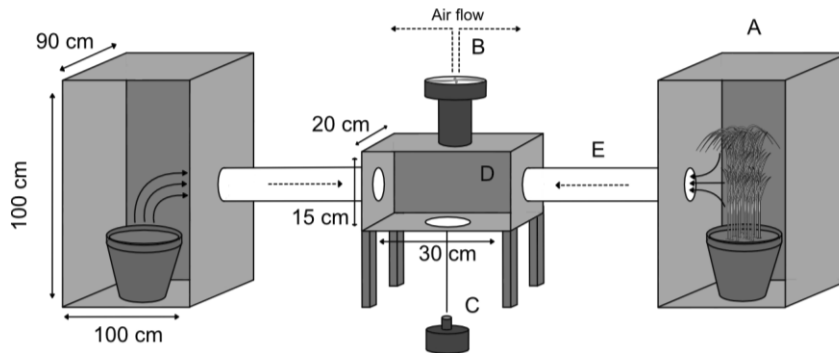


Figure 8: The olfactometer bioassay experimental setup. The substrates were placed in the chambers (A) from which a fan (B) drew air from the substrate chambers to the outside. The fan was mounted on the top side of the release chamber (D) and the mosquito release cup (C) was mounted on the bottom side of the chamber. The mosquitoes that made a directional choice were trapped in either of the two trapping chambers (E) and data were recorded every morning by removing the fan pipe and the trapping chambers. Adapted from **Paper II**, figure 1.

The results from these trials gave important insight into the choices made by the malaria vector. Firstly, the results showed that the mosquitoes made an equally distributed choice of between the two chambers when lake water was compared to lake water or when both chambers were empty. However, in the choice between an empty chamber and a chamber containing lake water, the vector showed a preference towards the lake water. This observation was in line with earlier results showing that the vector prefers to move from lower to higher humidity[87]. Furthermore, it was observed that using any of the graminoid plants mentioned in section 1.2 as substrate gave higher odd ratios of finding mosquitoes in the corresponding test chamber when compared to lake water. This was also true for the ornamental plant *C. setaceum*, even though it does not occur naturally at the egg-laying sites. It was also seen that *C. rotundus* showed a slightly higher attraction of the vector when tested against the *C. dactylon* and *P. repens* plants.

3.2 Semi-field bioassays

In order to test the results from the two-port olfactometric bioassays in a setup that more resembled the natural environment of the vector, semi-field bioassays were tested using BG-sentinel™ mosquito traps (Biogents AG, Regensburg, Germany). This was also done to obtain more information on attraction at longer ranges from the plants, while using free-flying mosquitoes. The experiments were setup in an enclosed field, whose dimensions were 11.8 m long × 6.8 m wide × 2.4 m high, under ambient environmental conditions, and using the traps to expose the

vectors to the substrates[88]. The traps and the release points of the mosquitoes were placed as far apart as possible. Substrates were placed randomly in the positions available, and the position of the traps were exchanged in consecutive nights. During the experiments, 200 gravid *An. gambiae s.s* mosquitoes were released at 18:00 p.m. and allowed to navigate in the field until 8:00 a.m. the following morning. The traps were then collected and the number of mosquitoes in the traps were counted. A full description of the replicates can be found in **paper II**, table 1. A schematic representation of semi-field bioassays is shown in figure 9.

From these experiments it could be further determined that when given the choice between plants and lake water versus just lake water, there is higher preference for the former. It was also determined that the attraction effectiveness between plant species was not as clear as it had been when the bioassay was performed in the two-port olfactometric setup. It could be seen that *C. rotundus* still showed higher attraction than *P. repens* in this setup, albeit the difference was slight. From these bioassay results, it can be concluded that there are volatile cues that help the mosquitoes to find the location of the graminoid plants, but that determining which plant is the most effective attractant is not straight-forward under the circumstances that more resemble the natural environment.

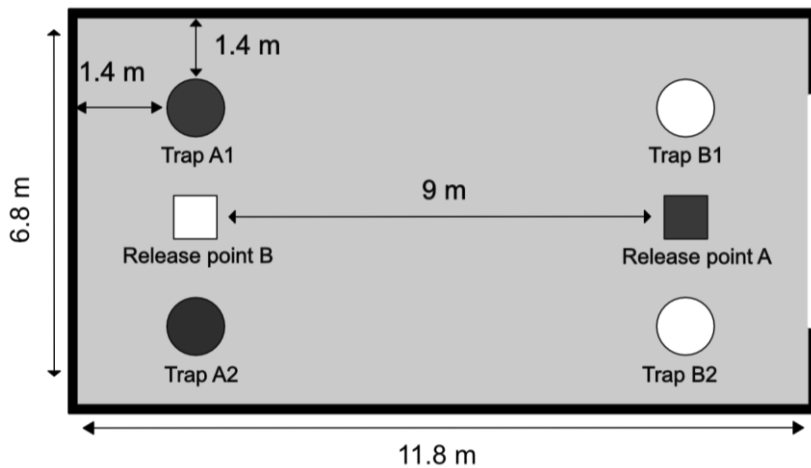


Figure 9: Schematic overview of mosquito release points and BG-sentinel trap positions for semi-field bioassays. When releasing the mosquitoes from point A, the traps were placed in location A1 and A2, and likewise when mosquitoes were released from point B. Adapted from **Paper II**, figure 2B.

4. Solid-phase microextraction analysis of graminoid plants

Liquid-solid extraction is a valuable tool in the uncovering of the large assortment of metabolites that can be present in plants. There is, however, also a need to understand which of these metabolites are emitted into the headspace above the plant. In order to determine this, **paper I** describes the method used for analysing the headspace of *C. rotundus* in a laboratory setting using SPME. This method was then applied in **paper III** to analyse the headspace of the remaining three graminoid plants, *C. dactylon*, *C. exaltatus* and *P. repens* in a laboratory setting. In **paper I**, the results from the laboratory setting were also compared to that of sample collections performed in Kenya, on live plants of *C. rotundus* in a field setting.

4.1 Chemical profile of *Cyperus rotundus* using SPME in a laboratory setting

The results of the olfactometric bioassays, shown in section 3.1, showed that there was a behavioural response of the gravid *An. gambiae s.s* females to the different graminoid plants described in section 1.2, when compared to lake water. A SPME method in a laboratory setting was developed to investigate what chemicals could be detected in the headspace of the plants under the circumstances of the two-port olfactometric bioassay[89]. Aspects of the static headspace collections were modelled after the two-port olfactometric setup described in section 3.1 and in **paper II**. Examples of such aspects were the ratio between the mass of the plants and the volume of water present, and equilibration time of the headspace. Other aspects were investigated, such as extraction time and desorption time[89], until a method for analysis was determined. Exploring the static headspace of the plant mass could provide information and context to both the results of the bioassays and the ultrasound extractions, showing the value of analysis performed in a laboratory setting.

The method for static headspace sampling, using Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) SPME fibres, was developed using plant mass of *C. rotundus*. In figure 10, the difference in the results from the method using UAE and the adapted SPE protocol are compared to the SPME method, both for root samples. Furthermore, the comparison of the relative peak area percentages is presented in table 4. From this table, the values from the SPME analysis show that it is detecting the metabolites continuously, much like the initial trials of UAE samples, from section 2.1. In table 4, the results of the UAE analysis of the shoot samples have been omitted since, as reported in table 2, they were too low to be recognized by the integration method or not detected, and thus given a zero value. From table 4, it can be seen that the relative area of the metabolites is still, in

most cases, higher in the results from the initial method from section 2.1. It can also be seen that the reported relative area percentages of limonene are considerably higher in the shoot samples than in the root samples of the SPME method. It can also be seen that caryophyllene oxide gave higher relative peak areas in the root samples of the SPME analysis. Caryophyllene oxide is the only metabolite that was detected more than once in the UAE analysis where the roots and shoots were separated.

Table 4: Comparing results between the two UAE methods for extracting metabolites and comparing it to the SPME method for metabolite collection in the headspace of roots (n=4) and shoots (n=4). SE – Standard error, * - detected once, no SE reported. **Paper I**, table 3.

Compound	Initial trial MeCN Area ± SE [%]	UAE MeCN roots Area ± SE [%]	SPME roots Area ± SE [%]	SPME shoot Area ± SE [%]
<i>α-pinene</i>	1.37 ± 0.27	0	0.04 ± 0.01	0.09 ± 0.01
<i>β-pinene</i>	2.58 ± 0.53	0	0.11 ± 0.03	0.51 ± 0.05
<i>Limonene</i>	0.76 ± 0.13	0	0.68 ± 0.13	9.01 ± 0.97
<i>β-caryophyllene</i>	1.7 ± 0.52	0	1.62 ± 0.09	4.73 ± 0.33
<i>α-humulene</i>	2.8 ± 0.91	0.17*	2.07 ± 0.12	3.81 ± 0.18
<i>Caryophyllene oxide</i>	6.11 ± 0.17	1.47 ± 0.74	2.25 ± 0.23	0.27 ± 0.07

Based on the results from the SPME samples, as well as comparing them with those of the UAE samples from section 2.2, it was determined that the SPME method would be used for the analysis of the other three graminoid plants growing in the wetlands of Kenya. This decision was based on the results of the SPME study, which showed the detection of all six metabolites continuously. The SPME method was also designed with the olfactometric bioassays in mind, thus it would be more just to use this method to profile the three graminoid plants. This would provide information regarding the resulting relative peak area from metabolites in the other plants, and provide a basis for comparison between the plants, to further explain the results discussed in section 3.1.

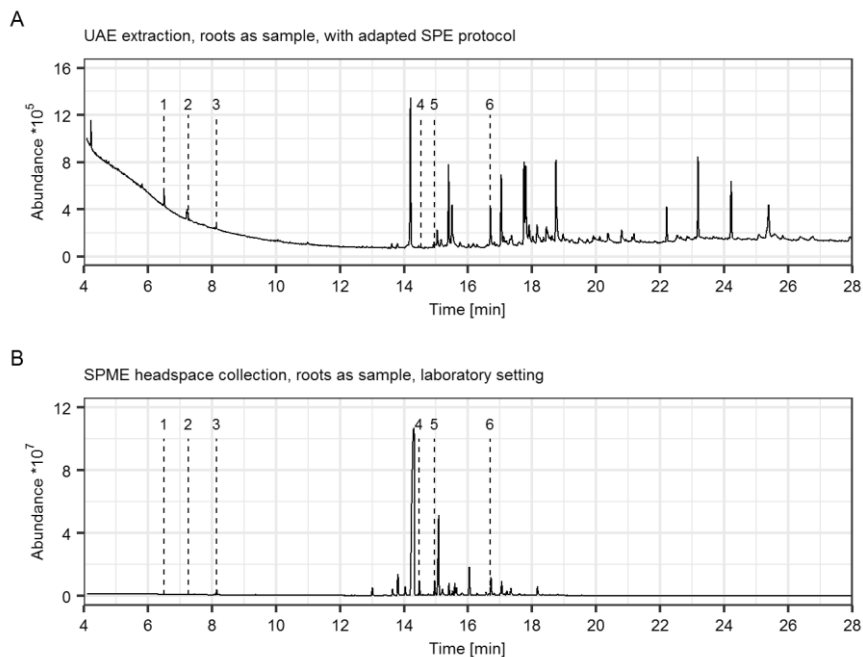


Figure 10: Comparison of analysis of UAE samples concentrated with SPE using roots as sample and Headspace-SPME sample collection using roots as sample. **Paper I**, figure 4A (A) and figure 5A (B).

4.2 Chemical profile of three graminoid plants using SPME in a laboratory setting

In continuation, the SPME method from **paper I** was used to analyse the headspace of the remaining three graminoid plants. Root and shoot tissue of *C. dactylon*, *C. exaltatus* and *P. repens* were analysed and the detected relative peak area percentage representing the compounds were compared. This section relates the results to A3, described in the introduction. In figure 11, the number of compounds, the class to which they belong as well as how many compounds that were unique or detected from more than one plant is shown.

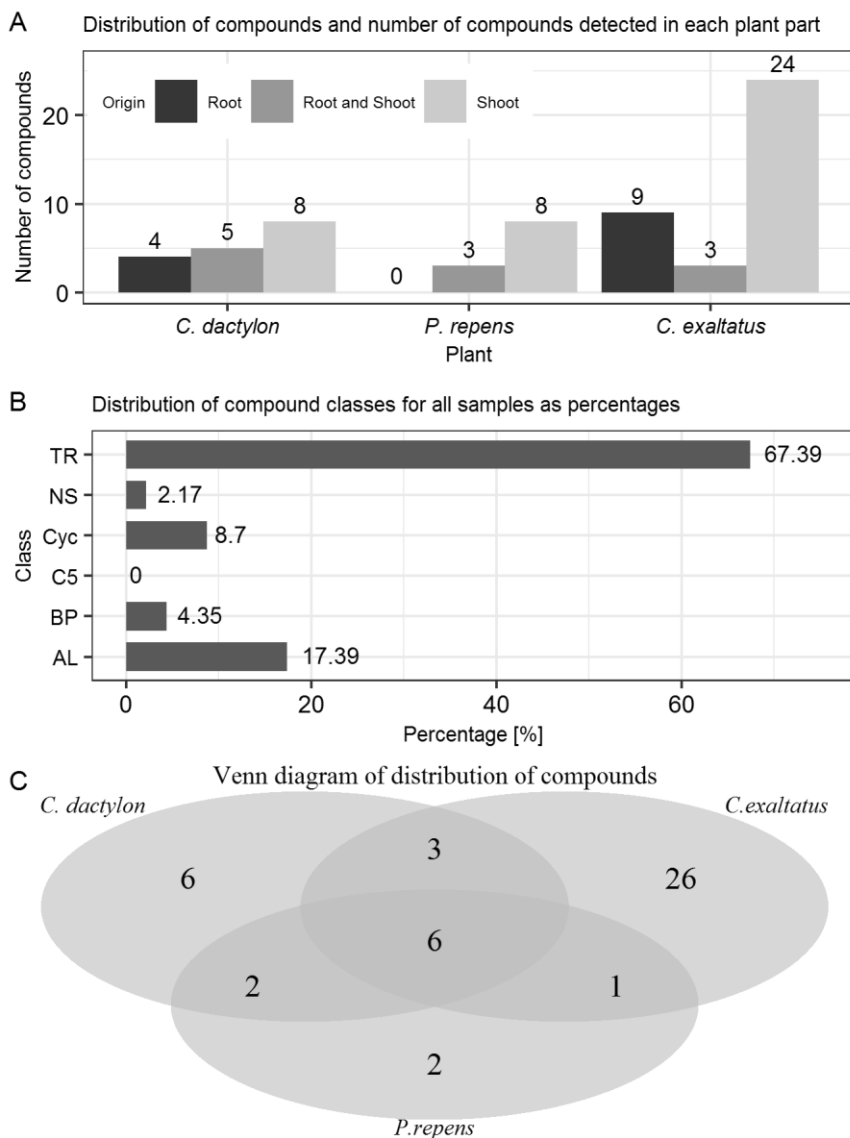


Figure 11: **A**—Shows the distribution of compounds detected in the roots, shoots, and both plant parts for each of the graminoid plants. **B**—Shows the percentage distribution of the classes for the identified compounds for all samples analysed. **C**—Shows a Venn diagram of the number of unique compounds detected for each of the three plants as well as the number of compounds found in two or all three plant species. TR—Terpenoid, AL—Aliphatics, BP - Benzenoid /Phenylpropanoids, C5- C5-branched compounds, NS—Nitrogen- or sulphur-containing compounds, Cyc—Cyclic miscellaneous compounds. Adapted from **Paper III**, figure 1.

From the results reported in **paper III**, it can be seen that compounds such as α -pinene, β -pinene, 3-octanone and limonene are found in each plant, in either the root and/or the shoots. In **paper III**, one-way ANOVA was used to determine if there were significant differences in the relative peak area peaks reported for the compounds from different analyses. This statistical analysis was used to highlight if there was a reoccurring compound that could add context to the results from the bioassays. The one-way ANOVA analysis used was calculated at a 95 % confidence interval and assumed homoscedasticity and normality of the data. The results of the one-way ANOVA were then further evaluated with post-hoc test to see which peak areas were significantly different. In table 5, the results of the post-hoc Tukey analysis for comparison of overlapping compounds detected in the plants are shown and in table 6, the comparison of compounds found in both tissue samples of the individual plants are shown.

Table 5. Post-hoc Tukey analysis of five compounds found in the headspace of at least two of the three graminoid plants, either in roots or shoot samples. If p-value is < 0.05, then there is a significant difference between the area of the compounds for the plants specified. Adapted from **Paper III**, table 2.

Compound	(I) Plant	(II) Plant	Mean difference	p-Value
<i>α-pinene</i>	<i>P. repens</i>	<i>C. dactylon</i>	6.058835	0.161362
<i>β-pinene</i>	<i>P. repens</i>	<i>C. dactylon</i>	12.830001	0.0414622
	<i>P. repens</i>	<i>C. exaltatus</i>	16.122119	0.0134851
	<i>C. exaltatus</i>	<i>C. dactylon</i>	-3.292118	0.8048685
<i>3-octanone</i>	<i>P. repens</i>	<i>C. dactylon</i>	3.0250021	0.5777641
	<i>P. repens</i>	<i>C. exaltatus</i>	0.2862853	0.9964308
	<i>C. exaltatus</i>	<i>C. dactylon</i>	2.7387168	0.7014438
<i>β-myrcene</i>	<i>P. repens</i>	<i>C. dactylon</i>	3.447685	0.250943
<i>citronellol</i>	<i>P. repens</i>	<i>C. dactylon</i>	3.7893436	0.2009792

Table 6: Post-hoc Tukey analysis of six compounds found in the headspace samples of both the root and shoot samples of each of the three graminoid plants. If p-value is < 0.05, then there is a significant difference between the area of the compounds detected in the two tissues for the plant specified. Adapted from **Paper III**, table 3.

Plant	Compound	(I) Part	(II) Part	Mean difference	p-Value
<i>C. dactylon</i>					
	<i>α-pinene</i>	Shoot	Root	-4.265665	0.3140036
	<i>β-pinene</i>	Shoot	Root	7.038193	0.113681
	<i>3-octanone</i>	Shoot	Root	-3.63632	0.1490245
	<i>decane</i>	Shoot	Root	2.052726	0.1791336
	<i>limonene</i>	Shoot	Root	5.093832	0.0724245
<i>C. exaltatus</i>					
	<i>β-pinene</i>	Shoot	Root	-16.27886	5.88e-05
	<i>cyperene</i>	Shoot	Root	-5.619737	0.0012586
	<i>β-selinene</i>	Shoot	Root	-10.47628	0.050796
<i>P. repens</i>					
	<i>β-pinene</i>	Shoot	Root	-22.34587	0.0005098
	<i>3-octanone</i>	Shoot	Root	3.887919	0.6062301

In figure 11C, it is seen that there is a total of six compounds overlapping between all the plants, but only five compounds are compared. Due to the fact that the sixth overlapping compound found in the comparison was not detected in more than one sample, the assumptions made for the one-way ANOVA analysis are not applicable. Similar omissions were made for compounds in table 6. The compounds that were not included in table 5 and 6 are described in **paper III**. Based on the results from the one-way ANOVA analysis performed in **paper III**, a few observations could be made. The compounds that were detected across the three graminoid plants was β -pinene and 3-octanone, in a manner that allowed for the one-way ANOVA analysis to be performed. It could also be seen that β -pinene gave a significantly different relative peak area percentage in the headspace of *P. repens* when compared to the other two plants, shown in table 5. Furthermore, the statistical analysis was performed for compounds detected in both the root and shoot parts of each plant. Based on this analysis, it could be observed that the compound β -pinene showed a significant difference in relative peak area percentage when comparing the detected signal from roots and shoot samples from both *C. exaltatus* and *P. repens*. The same observation was shown for cyperene for *C. exaltatus*

while no common compounds for the different plant tissues showed a significant difference at a 95% confidence interval for *C. dactylon*. The significant differences could be used to motivate the results shown in **paper II**, for example that the odds ratio of *P. repens* could be a result of the significantly different amount of β -pinene expressed by the plant. Furthermore, observations such as the performance of *P. repens* in the semi-field bioassays could be related to the significantly different percentage β -pinene detected in the roots and the shoots of the plants. However, the results from the semi-field assays would require a headspace collection under similar conditions for a more accurate relation to be established.

In figure 12, the comparison of the SPME analysis of *C. rotundus* from section 4.1 is shown together with analysis of the other three graminoid plants. Here it can be seen that results from the *C. rotundus* analysis showed an abundance that is a hundredfold larger than the other plants. Another observation that can be made is that the two *cyperaceae* plants gave a larger number of individual peaks than that of the plants of the *poaceae* family. This is also seen in the comparisons in figure 11, however in that figure, *C. rotundus* is not included in the comparison. The chromatograms shown are the third replicate of the respective analyses.

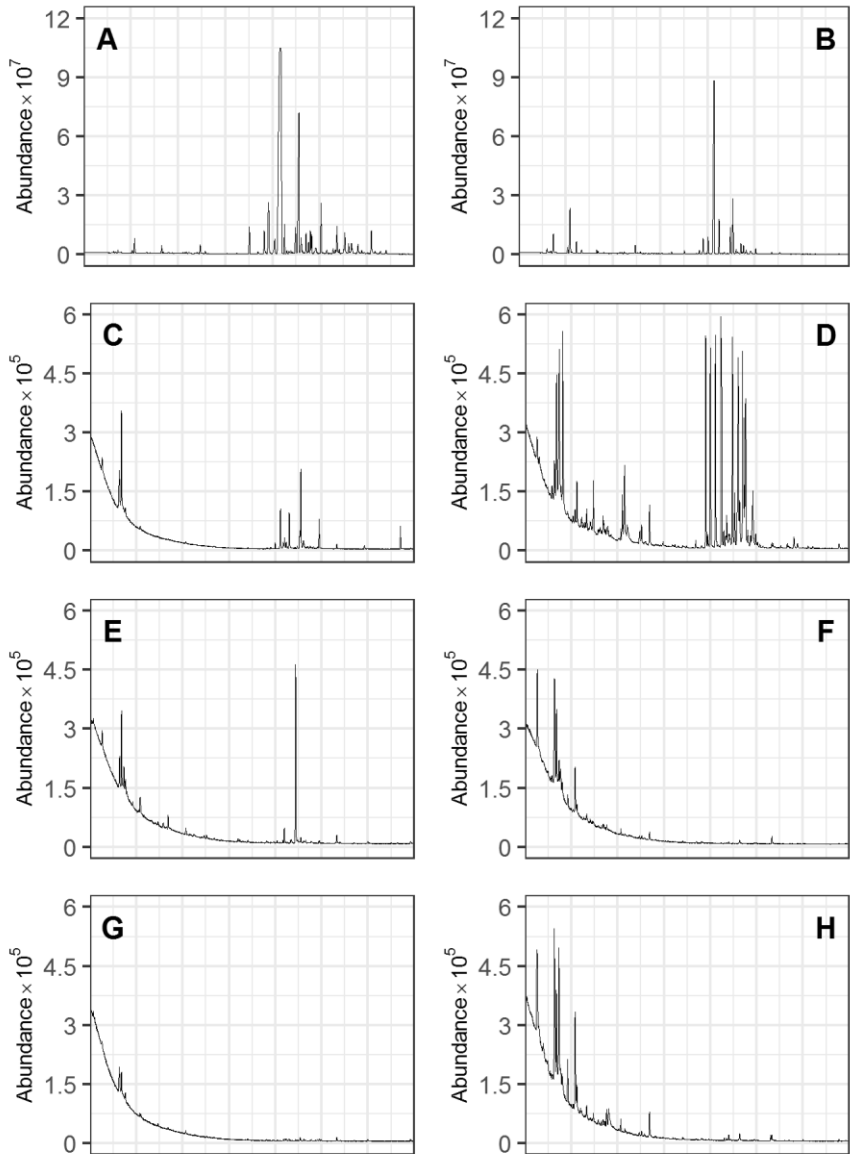


Figure 12: SPME analysis replicate number 3 in laboratory setting for the four graminoid plants. **A** – roots of *C. rotundus*, **B** – shoots of *C. rotundus*, **C** – roots of *C. exaltatus*, **D** – shoots of *C. exaltatus*, **E** – roots of *C. dactylon*, **F** – shoots of *C. dactylon*, **G** – roots of *P. repens*, **H** – shoots of *P. repens*. The x-axis is in minutes and shows the interval between 6–20 minutes of the total analysis time. Figure is produced using data from **Paper I** and **Paper III**

5. Headspace sampling in a field setting

In **paper I and II**, sampling of headspace of the intact, live plants was performed using both static and dynamic headspace (DHS) sampling. The samples were collected on PDMS/DVB SPME fibres for the static headspace and onto Porapak Q stationary phase filled tubes for the DHS. Both types of samples were analysed using GC-MS.

5.1 Static headspace sampling of *Cyperus rotundus*

For the static collection of headspace from *C. rotundus* samples, the SPME fibres were sent to Mbita, Kenya. Here, the fibres were used to collect the headspace from uprooted, live plants that had their shoots enclosed in heat resisting, roasting bags. The headspace was collected for 6 hours, before the fibres were sealed in polytetrafluoroethylene (PTFE) tape and stored in a freezer, at -71 °C, until they could be transported to Nairobi for analysis.

The relative area of the peaks obtained in the analysis of these headspace collections were compared with the results obtained in the previous studies, described in section 2.1, 2.2 and 4.1. This was done in accordance with A2, stating that laboratory and field analysis should be compared. In table 7, a comparison of the initial study with UAE, the UAE analysis of two plant tissues and the SPME analysis of the two plant tissues in a laboratory setting are compared to the analysis of the live plants in field setting using SPME.

Table 7: Comparing results between the initial study for UAE, the UAE analysis of roots and shoot parts, SPME analysis of roots and shoots parts in laboratory setting and SPME analysis of shoot parts in a field setting (n=5). SE – Standard error, * - detected once, no SE reported. Adapted from **Paper I**, table 4.

Compound	Initial trial MeCN Area \pm SE [%]	MeCN UAE roots Area \pm SE [%]	MeCN UAE shoots Area \pm SE [%]
<i>α-pinene</i>	1.37 \pm 0.27	0	0
<i>β-pinene</i>	2.58 \pm 0.53	0	0
<i>Limonene</i>	0.76 \pm 0.13	0	0
<i>Caryophyllene</i>	1.7 \pm 0.52	0	0
<i>Humulene</i>	2.8 \pm 0.91	0.17*	0
<i>Caryophyllene oxide</i>	6.11 \pm 0.17	1.47 \pm 0.74	0

Compound	SPME roots Area \pm SE [%]	SPME shoots Area \pm SE [%]	SPME Kenya shoot Area \pm SE [%]
<i>α-pinene</i>	0.04 \pm 0.01	0.09 \pm 0.01	0.01 \pm 0
<i>β-pinene</i>	0.11 \pm 0.03	0.51 \pm 0.05	0
<i>Limonene</i>	0.68 \pm 0.13	9.01 \pm 0.97	0
<i>Caryophyllene</i>	1.62 \pm 0.09	4.73 \pm 0.33	0.61 \pm 0.4
<i>Humulene</i>	2.07 \pm 0.12	3.81 \pm 0.18	0.24 \pm 0.16
<i>Caryophyllene oxide</i>	2.25 \pm 0.23	0.27 \pm 0.07	not detected

From the results presented, it can be seen that the SPME method can be used to collect metabolites that are present in conditions similar to a more accurate representation of the environment of the plant. What is also clear when comparing the results of the methods is that there is a large variation in the relative peak area percentage with which the metabolites are detected. This variation could be a product of the sensitivity of the sample collection method, and that performance of the sample techniques are a cause of the variation. There are also several auxiliary factors that can contribute to variation in amount of detected metabolites, such as the role of abiotic stress on the plants when they are removed from the naturally environment[90, 91], and the freshness of the sample at the time of sample collection/extraction.

5.2 Dynamic headspace sampling of graminoid plants

Plants, with a weight of approximately 350 grams, were placed in buckets along with some soil in order to perform the DHS collections. The DHS collections were performed in Mbita, Kenya. The shoot parts were enclosed in heat resisting, roasting bags, and the collections were made on Porapak Q (50 mg, 50/80 mesh) sorbent material that was packed on glass liners with glass wool at both ends. Headspace was collected onto the Porapak Q using field portable pumps to feed the headspace inside the bags onto the stationary phase. The pumps were set to have an in-flow of charcoal-filtered air to the bags at a rate of 500 ml/min, while the out-flow was at a rate of 300 ml/min to pass through the sorbent[92]. This was done for 48 hours, after which the liners were sealed with PTFE tape and kept in a freezer at $-71\text{ }^{\circ}\text{C}$. The sorbent tubes were then shipped to KTH Royal Institute of Technology for analysis. The analytes trapped on the sorbent material were first eluted using 3 ml hexane, and then concentrated to $\approx 250\text{ }\mu\text{l}$ using a desiccator connected to a duo rotary vane pump. In addition to analysing the DHS samples for *C. rotundus*, the three other graminoid plants as well as the ornamental plant, were analysed.

Chemical analyses were done for 21 headspace samples: *C. rotundus* (n = 5), *C. dactylon* (n = 4), *C. exaltatus* (n = 4), *P. repens* (n = 4) and *C. setaceus* (n = 4). A total of 43 VOCs was reported for DHS samples using the previously described method, adding to aim A3. Certain compounds reported from these samples have been reported to elicit electrophysiological responses in gravid and unfed female *Anopheles* mosquitoes. These compounds are; α -pinene[32, 93, 94], β -pinene[32, 95, 96], limonene[32, 93, 95, 96], p-cymene[93, 94, 96], sulcatone[32], α -humulene[97], β -myrcene[98], and β -caryophyllene[32, 96, 99]. Based on the results here, there are not only compounds that have been tested previously, but there are also compounds, such as cyperene, that has not been tested for ovipositional effects. Furthermore, there is an overlap in the compounds identified in the DHS samples, with that of the SPME samples analysed in the laboratory setting. This gives an indication of the value of collecting the headspace in both the static, laboratory setting as well as the DHS in a field setting, which relates to aim 2, described in section 1 of this thesis. Even though there are compounds reported here that are of interest for evaluation of ovipositional strength, the task of identifying said compounds using external standards remains. This task itself poses certain difficulties based on availability and cost of synthesizing the pure standards of certain compounds. The second challenge is the need for a quantitative analysis of target compounds, as to obtain the naturally occurring blend of the different compounds.

6. Ultrasound used in inquiry-based learning

As it was shown in section 2, the use of ultrasound can be used as a method to extract secondary metabolites from plant mass. In addition, the use of SPE showed that it lends itself as a beneficial complement to UAE. Finally, the investigation of semiochemicals' ability to act as allomonones is an interesting aspect for the understanding plant-plant interactions. Therefore, an inquiry-based project was designed in order to develop a methodology that could be implemented to deepen high school students understanding of chemical ecology and analytical chemistry, which works towards aim A4 in this thesis. The project was performed by three high school students from Blackebergs Gymnasium.

The project was based around the inquiry of whether the plant *A. podagraria* had an allelopathic effect on *P. pratense* seeds by extracting its secondary metabolites using UAE. The project therefore included four steps.

- (i) Extraction of metabolites using UAE from roots of *A. podagraria* at two mass-to-solvent ratios, namely 0.5 g/mL and 1 g/mL
- (ii) GC-MS analysis of the extracts for identification of secondary metabolites.
- (iii) Allelopathic trials on seeds of *P. pratense* using the extracts.
- (iv) Allelopathic trials of pure standards based on the metabolites identified in the extracts.

In this project, the methods were predetermined as the extraction for secondary metabolites with UAE, as well as the SPE protocol, had been established. The procedure for the allelopathic trials had also been determined based on previous students' work. This project was the first to merge the two parts together with a predetermined method allowing for the students to not only look at the practical part of the laboratory work, but also to use statistical metrics on a dataset that they produced. The students were also tasked with writing a report detailing their findings and conclusions.

6.1 Analysis of extracts from *Aegopodium podagraria*

The first step involved the preparation of the extracts and their analysis with GC-MS. The extractions were done using the same method as the one discussed in section 2.2. In the work of **paper IV**, the students prepared five replicates of the extraction, referred to as one round of samples, and during the project the students produced three rounds of samples. In total, 15 extracts were analysed with GC-MS and tested for allelopathic effects on *P. pratense* seeds. This was done for two different mass-to-solvent ratios, and these extractions were performed in parallel. In figure 13, the

comparison of extract replicate 2 from all three rounds are shown, for both mass-to-solvent ratios. The figure shows the timeframe 4-16 minutes, but the total time of the analysis was 28 minutes. In the analysis of the extracts, the two secondary metabolites of α -pinene and β -caryophyllene could be detected with MS and identified with both RI and external standards. Both α -pinene and β -caryophyllene have been detected in extracts of *A. podagraria* previously[100-102], thus these compounds were selected to be used as individual pure compounds for investigation of their allelopathic effects on *P. pratense* seeds in step four of the project.

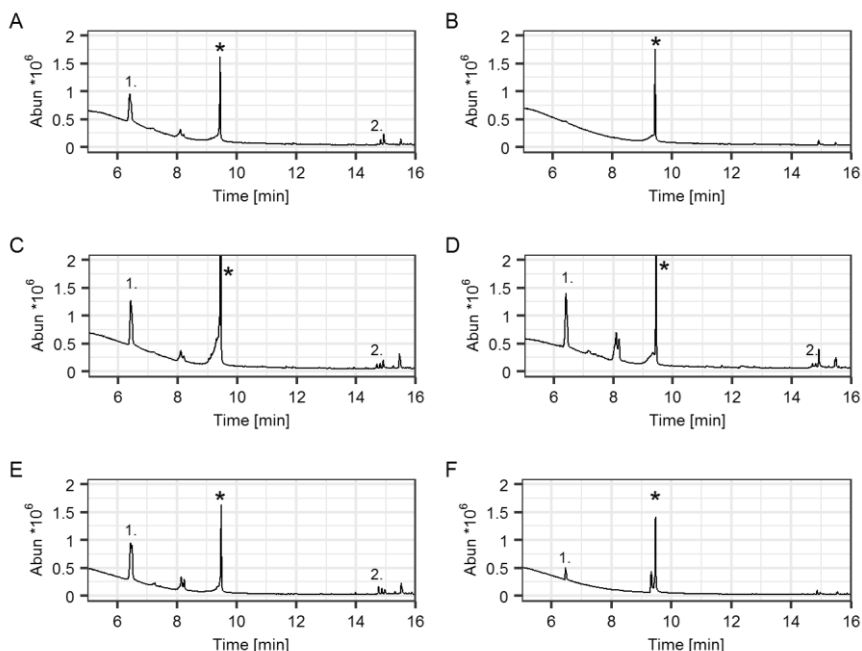


Figure 13: Chromatograms for extracts of *A. podagraria*. **A** – 0.5 g/mL elution I from round 1, **B** – 1 g/mL elution I from round 1, **C** – 0.5 g/mL elution I from round 2, **D** – 1 g/mL elution I from round 2, **E** – 0.5 g/mL elution I from round 3, **F** – 1 g/mL elution I from round 3. All shown chromatograms are the analysis of replicate 2 from respective round of each mass-to-solvent ratio. Heptyl acetate was used as an internal standard and its peak is labelled with an (*). Numbers 1 and 2 show the peak for α -pinene and β -caryophyllene respectively. **Paper IV**, figure 1.

It can be seen from the figure that there is some variation in the signal intensity in the detection of the two terpenoids. The results of the area ratios are shown in figure 14, where the boxplots show the mean and standard deviation of the two metabolites as well as a bar graph comparing the two, based on the quotient between the area of each metabolite and the internal standard. In figure 14, it can be seen that α -pinene was present

with higher regularity, whereas β -caryophyllene was not detected in round 1. The only detection of β -caryophyllene in round 1 is shown as the dot in figure 14B, which is marked as an outlier in the box-and-whiskers plot. However, in round 2 and round 3, both metabolites are detected with some consistency. The inconsistency that is observed in figure 14 can be attributed to sample quality and the sensitivity of the sample preparation method, as mentioned in section 5.1, but it could also be a product of students being inexperienced of working the sample preparation protocol. This would explain why the detection of metabolites becomes more consistent in the two later rounds than the first round of extractions made by the students.

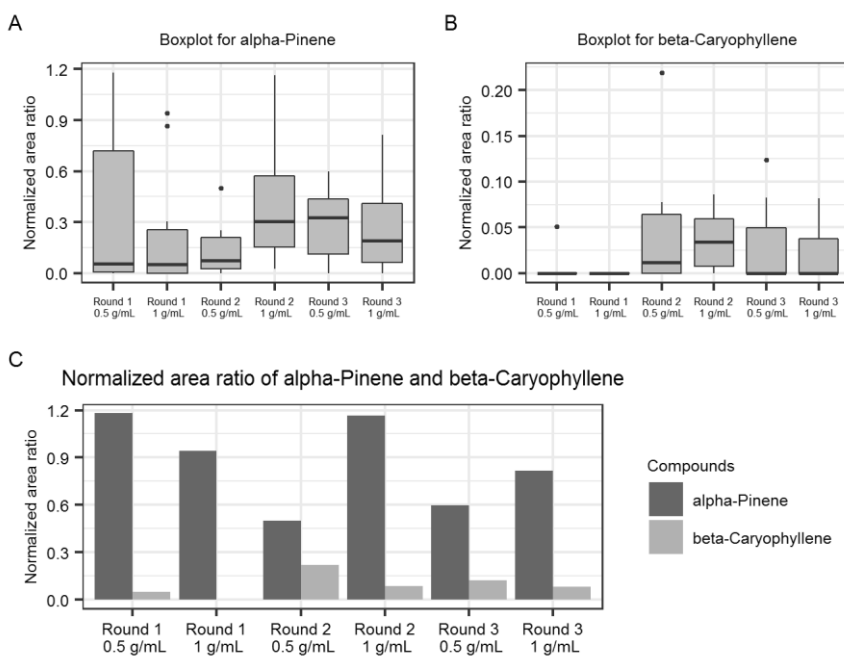


Figure 14: **A** - Boxplot describing the mean and standard deviation of the peak area ratio of α -pinene and internal standard in all 6 sample rounds, **B** - Boxplot describing the mean and standard deviation of the peak area ratio of β -caryophyllene and internal standard in all 6 sample rounds, **C** - bar graph comparing the mean area ratio of α -pinene and β -caryophyllene respectively and internal standard for all 6 sample rounds. **Paper IV**, figure 2.

6.2 Allelopathic study of extracts

After the analysis of the extracts had been performed, the next step was to test the allelopathic effect on the *P. pratense* seeds. Here, the number of seeds sprouting as well as the length of the sprouts were used as a metric to evaluate the effect of the extracts. For each extract, 3 petri-dishes with 20 seeds were tested for allelopathic effects by adding 50 μL of extract to each of the petri-dishes. Since each round consisted of 5 extractions, this resulted in 300 seeds per mass-to-solvent ratio per round. A more detailed description of protocol of the growth trials is available in **paper IV**. In figure 15, the histograms for the growth studies are shown. This includes the results from all rounds and replicates of the extracts of the seeds. It should be noted that all results showing seeds that did not spring any sprouts have been omitted in the figure in order to give a clearer overview of the distribution of the seeds that did germinate.

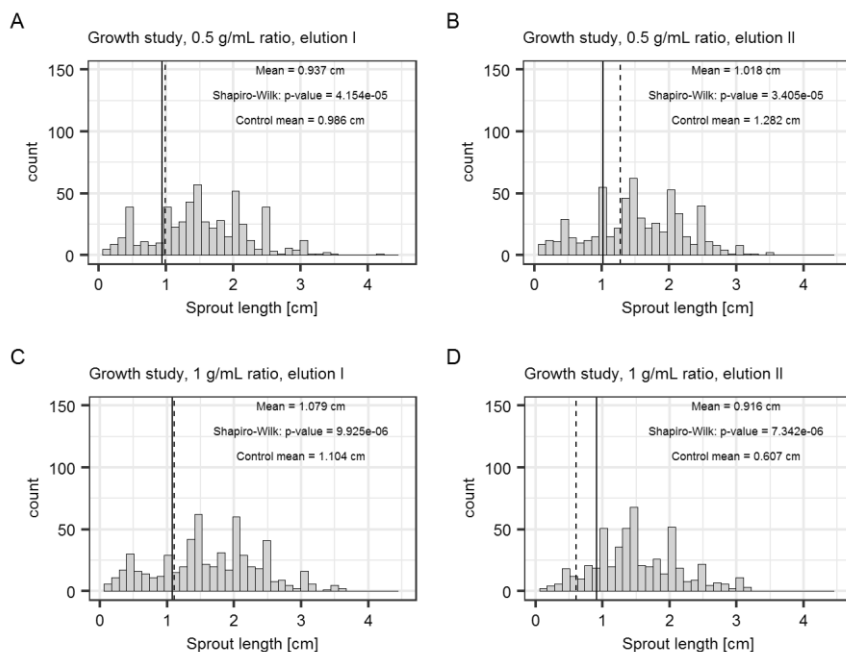


Figure 15: **A** - Growth histograms for Elution I with a 0.5 g/mL ratio, marked with mean, mean of control and metric of normality, **B** - Growth histograms for Elution II with a 0.5 g/mL ratio, marked with mean, mean of control and metric of normality, **C**- Growth histograms for Elution I with a 1g/mL ratio, marked with mean, mean of control and metric of normality, **D** - Growth histograms for Elution II with a 1 g/mL ratio, marked with mean, mean of control and metric of normality. **Paper IV**, figure 3.

From the allelopathic trial results, the students had a large dataset to work with, and used this to compute some basic statistical metrics of the dataset. In their report, the students described the mean and standard deviation of the subsets of data. It should be noted that these metrics are based on the premise of normality, which we can see from the Shapiro-Wilk test in figure 15, is not quite appropriate. However, due to the academic level of the students, it was determined that the metrics of standard deviation would still be calculated as for normally distributed data, as alternative approaches fall outside the scope of the academic level. Based on the percentage of inhibition, *i.e.* number of seeds that did not sprout vs the total number of seeds treated, there was not a clear trend of allelopathy from the extracts. Inhibition could be seen for the extracts, but this was also apparent in the controls. The controls were samples that had undergone the same procedure, but having any plant mass added to the solvent during the UAE step. Even though the inhibition from the extract was not clear, the inclination was sufficient for the project to pursue testing of individual compounds detected in the extracts.

6.3 Allelopathic study of pure standards

Based on the data extrapolated from the GC-MS analysis of the extracts, an approximation of concentration could be made for the compounds α -pinene and β -caryophyllene. As it has been discussed in section 6.1, these compounds have been reported as present in *A. podagraria* previously, and both α -pinene[103-105] and β -caryophyllene[106, 107] have displayed allelochemical properties. A solution of α -pinene with a concentration of 50.05 $\mu\text{g/mL}$ in hexane, and one of β -caryophyllene with a concentration of 9.86 $\mu\text{g/mL}$ in hexane were prepared based on the results of the GC-MS analysis. A blend of the two terpenoids was prepared in hexane with the same concentrations as for the individual solutions. Finally, 3 solutions were prepared with concentrations 100-fold higher than the previously described solutions. Hexane blanks were also prepared to be used as controls in the allelopathic study of the pure compounds. The allelopathic study of the pure compounds was setup in the same way as for the extracts and consisted of 5 replicate dishes with 20 seeds per solution, the results of which are shown in figure 16. From the results it can be concluded that solutions of the terpenoids with a concentration based on the GC-MS results, did not inhibit the seeds to sprout as much as their concentrated counterpart. This is seen in figure 16A, where the results are visualized, showing the increased effect of inhibition between the two concentrations of individual terpenoids and the unaffected inhibition of the blend, despite concentration differences. The results from the blends shows that not only does it inhibit the seeds from sprouting, regardless of concentration, but also show the same amount of inhibition on the length of the sprouts. This

inhibition of length on sprouts is shown in figure 16B, which also shows that the solutions of the pure, individual terpenoids were less allelopathic than their concentrated counterpart with regards to sprout length. These results lead to two conclusions, the first being that the allelopathic effect of the individual terpenes is concentration dependant. The second one is that there is a blend effect that pertains to the allelopathic strength of *A. podagraria*, but that this one does not appear to be concentration dependant.

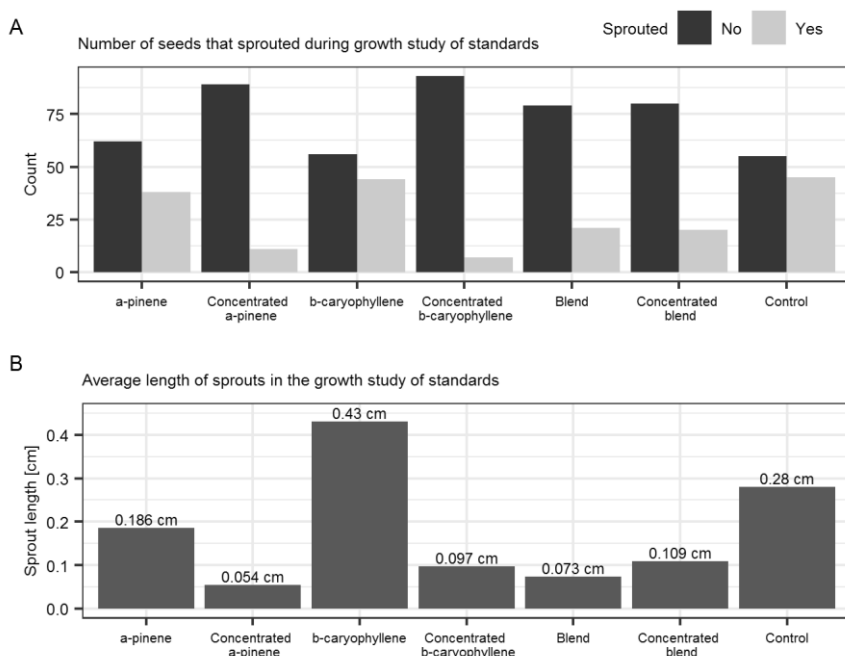


Figure 16: **A**-Number of seeds that shot sprouts during the growth study of the pure compounds and blends, **B** - The average length of sprouts that shot sprouts during the same growth study. **Paper IV**, figure 4.

6.4 Student evaluation

The students were asked to answer questions regarding their perceived view of the learning content and how they had experienced the project. The survey was anonymous, and the consensus from all participating students was that they had a chance to practice scientific methods, and to apply them to a research topic. They also felt that they received help from the supervisors when they needed it but were also given ample chance to explore solutions on their own. The students stated that they would have

liked more scheduled time for guided literature searches to get a better understanding of the different techniques used in the research work, and stated that the one piece of advice they would give to students performing this project in the future would be to read more literature regarding the subjects.

Based on the feedback of the students, adaptations of the project can be made with more time spent on reading and understanding the different aspects of the project. It could be necessary for the supervisors to adapt a clearer syllabus, with more defined intended learning outcomes, as to adjust the expectations of the students. The expectations of the students to understanding every aspect of the project has to be moderated to suit their academic level as to not overwork students at lower academic levels. Therefore, the project could be adapted to suit bachelor and master-levels students as well, as the project can be formulated to put more emphasizes on method development and statistical analysis. It is with these tools and the addition of a more defined syllabus that the project can be improved.

7. Conclusions and future prospects

It has been shown that there is valuable information to gain from analysing the graminoid plants included in this work in a laboratory setting. Information regarding the presence of secondary metabolites is available, albeit the fact that selection of sample collection method impacts the relative peak area percentages with which the metabolites are detected. This allows the work to contribute to aim A1, defined in section 1. Even though the UAE method, combined with SPE, showed promise as a method for producing samples containing the metabolites of the plants, there were also inconsistencies in the performance of the method. In order to further improve the method of UAE/SPE, and continue striving for A1 in section 1, a factorial design to investigate the UAE parameters for extraction of secondary metabolites from the plant materials could be employed. Using a model, such as a Box-Behnken model[108], for parameters such as extraction time and mass-to-solvent ratio could reveal what parameters play the largest role in the extraction, and can help improve the method. Further optimisation of the SPE-protocol could further improve the performance of the method.

The results of the qualitative analysis of the graminoid plants using the laboratory and the live sample collection methods give a grand overview of available metabolites. With the aim of determining novel metabolites for testing as oviposition attractants, there are several compounds presented in the analysis that one can choose from, which connects back to both aim A2 and A3. To further confirm the identity of certain compounds, analysis using external standards must be performed, as to verify the presented results. After this has been done, quantitative analysis of compounds detected in the headspace of the graminoid plants is also an important body of work. This should be done predominantly with the DHS sampling as to elucidate the quantities expressed by live plants.

Continuing from the quantitative analysis of the compounds reported from the DHS samples, electroantennogram for the reported compounds of interest could be performed to test for activity. This would provide insight to what compounds elicits a response from the mosquitoes, what blend of compounds elicits the strongest effects and evaluate potential dose dependencies. This future work would further explore aim A3.

Finally, it was shown that the UAE method also could be used for the identification of metabolites that are allelopathic to *P. pratense* seeds from the *A. podagraria* plant, which ties into A4. The UAE method and the accompanying GC-MS analysis allow students to get into contact with analytical chemistry, to understand what this entails. It has been suggested that this method can be adopted to suit a bachelor, or master, thesis project by demanding more method optimisation, adapted statistical analysis as

well as testing more metabolites for the allelopathic strengths, both individually and in blends.

8. Acknowledgements

I would like to express my deepest gratitude to my supervisor Åsa Emmer, for giving me the opportunity to perform this work as well as guiding and supporting me through it. I would also like to thank my co-supervisor Jenny Lindh for her valuable input and revision of the work and for the rewarding discussions regarding different topics of the work.

I want to thank my colleague, Getachew Eticha Bokore, for without him this work would not be what it is. I also want to express thanks to Getachew's supervisor, Ulrike Fillingner, for her invaluable input and her help driving the joint work. A sincere thanks should also be given to Richard Tamre, Patrick Onyango and Tullu Bukhari.

A special thank you to Lovisa Malm, Narin Abdollahzadeh, Negar Gohari, Maria Almöv and Annika Norin for a great collaboration between KTH and Blacquebergs Gymnasium.

I would like to thank all my colleagues in our research group, both to those who are still at KTH and those who have concluded their work at KTH – Leila Josefsson, Joakim Romson, Saara Mikkonen, Yuye Zhou, Debbie Van Der Burg and Adilijiang Wumaierjiang.

Also, all the students that I have had the pleasure of working with in bachelor thesis works – Charlotte Stjernqvist, Adrian Södergren, Emma Lundell, Filip Friberg, Kid Karjalainen, Leona Clarin, Felicia Karlahag, Amanda Hamrin, Madeleine Lövås and Cagenna Linne Magendran. Also, Daniel Ruotsalainen and Leonard Sjöström for their excellent master thesis work. Thank you for wanting to be involved in my projects and (hopefully) enjoying the work.

This work would not be possible without the financial support from the Swedish Research Council, Carl Trygger Foundation, KTH Opportunities Fund, Linders stiftelse, Klasons stiftelse and Bengt Lundqvists Minne.

Finally, I want to thank my family, the OVEM in LOVEM and all my friends for supporting me and for being there for me during my time as a PhD student. I have all of you to thank for so much.

9. References

1. Wilson, J.A., et al., *What is a vector?* Phil. Trans. R. Soc, 2017. **372**(20160085).
2. Bentley, M.D. and J.F. Day, *Chemical ecology and behavioral aspects of mosquito oviposition*. Annu Rev Entomol, 1989. **34**: p. 401-21.
3. Ferguson, H.M., et al., *Ecology: a prerequisite for malaria elimination and eradication*. PLoS Med, 2010. **7**(8): p. e1000303.
4. Bennett, J.W. and R. Bentley, *What's in a Name?—Microbial Secondary Metabolism*, in *Advances in Applied Microbiology*, S.L. Neidleman, Editor. 1989, Academic Press. p. 1-28.
5. World Health Organization, *WHO Malaria Terminology*. 2019, Geneva, Switzerland.
6. Harbach, R.E., *The phylogeny and classification of Anopheles*. In *Anopheles Mosquitoes—New Insights into Malaria Vectors*. 2013, London, United Kingdom: InTech.
7. Hay, S.I., et al., *Developing Global Maps of the Dominant Anopheles Vectors of Human Malaria*. PLoS Med, 2010. **7**(2).
8. Kyalo, D., et al., *A geo-coded inventory of anophelines in the Afrotropical Region south of the Sahara: 1898-2016*. Wellcome Open Research, 2017. **2**(57).
9. Takken, W. and S.W. Lindsay, *Increased Threat of Urban Malaria from Anopheles stephensi Mosquitoes, Africa*. Africa. Emerg Infect Dis, 2019. **25**(7).
10. Takken, W. and S.W. Lindsay, *Factors affecting the vectorial competence of Anopheles gambiae: a question of scale*. In *Takken W., Scott T.W., eds. Ecological Aspects for Application of Genetically Modified Mosquitoes*. 2003, Dordrecht: Kluwer Academic Publishers.
11. Heuskin, S., et al., *The use of semiochemical slow-release devices in integrated pest management strategies*. Biotechnol. Agron. Soc. Environ., 2011. **15**(3): p. 459-470.
12. Brezolin, A.N., et al., *Tools for detecting insect semiochemicals: a review*. Anal Bioanal Chem, 2018. **410**: p. 4091-4108.
13. Fillinger, U., et al., *The practical importance of permanent and semipermanent habitats for controlling aquatic stages of Anopheles gambiae sensu lato mosquitoes: operational observations from a rural town in western Kenya*. Trop Med Int Health, 2004. **9**(12): p. 1274-1289.
14. Imbahale, S.S., et al., *A longitudinal study on Anopheles mosquito larval abundance in distinct geographical and environmental settings in western Kenya*. Malar J, 2011. **10**: p. 81.

15. Bokore, G.E., et al., *A cross-sectional observational study investigating the association between sedges (swamp grasses, Cyperaceae) and the prevalence of immature malaria vectors in aquatic habitats along the shore of Lake Victoria, western Kenya*. F1000Res, 2020. **9**: p. 1032.
16. Linnaeus, C., *Species Plantarum* Vol. 1: 45. 1753.
17. Jussieu, A.L., *Genera Plantarum*. 1789. **26**.
18. Retzius, A.J., *Observationes Botanicae*. Vol. 5:11. 1789.
19. Persoon, C.H., *Synopsis Plantarum*. Vol. 1:85. 1805.
20. Barnhart, J.H., *Bulletin of the Torrey Botanical Club*. Vol. 22:7. 1895.
21. Linnaeus, C., *Species Plantarum, Editio Secunda*. Vol. 1: 87. 1762.
22. Morrone, O.N., *Annals of Botany*. Vol. 106: 129. 2010, Oxford.
23. Linnaeus, C., *Species Plantarum*. Vol. 1: 265. 1753.
24. Lindley, J., *An Introduction to the Natural System of Botan*. Vol. 21. 1836.
25. Linnaeus, C., *Species Plantarum*. Vol. 1:59. 1753.
26. Eneström, J., S. Andersson, and T. D´Hertefeldt, *Partitioning of genetic variation in the weedy clonal herb Aegopodium podagraria (Apiaceae) in Sweden*. Nordic Journal of Botany, 2009. **27**: p. 437-443.
27. Dicke, M. and M. Sabelis, *Infochemical Terminology: Based on Cost-Benefit Analysis Rather than Origin of Compounds?* Functional Ecology, 1988. **2**(2): p. 131-139.
28. Knudsen, J.T., et al., *Diversity and Distribution of Floral Scent*. Botanical Review, 2006. **72**(1): p. 1-120.
29. Knudsen, J.T., L. Tollstein, and L.G. Bergström, *Floral scents - a checklist of volatile compounds isolated by headspace techniques*. Phytochemistry, 1993. **33**(2): p. 253-280.
30. Wooding, M., et al., *Controlling mosquitoes with semiochemicals: a review*. Parasit Vectors, 2020. **13**(1): p. 80.
31. Eneh, L.K., et al., *Cedrol, a malaria mosquito oviposition attractant is produced by fungi isolated from rhizomes of the grass cyperus rotundus*. Malaria Journal, 2016. **15**(1): p. 478-482.
32. Wondwosen, B., et al., *Rice volatiles lure gravid malaria mosquitoes, Anopheles arabiensis*. Scientific Reports, 2016. **6**: p. 37930.
33. Dahan-Moss, Y., et al., *Member species of the Anopheles gambiae complex can be misidentified as Anopheles lesoni*. Malar J, 2020. **19**(89).
34. Liu, J., et al., *Pressurised hot water extraction in continuous flow mode for thermolabile compounds: extraction of*

- polyphenols in red onions*. Anal Bioanal Chem, 2014. **406**: p. 441-445.
35. Chemat, F., et al., *Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review*. Ultrasonics Sonochemistry, 2016. **34**: p. 540-560.
36. Lavilla, I. and C. Bendicho, in *Water Extraction of Bioactive Compounds*, H. Dominguez González, Jesús González Muñoz, M., Editor. 2017, Elsevier. p. 291-316.
37. Brás, T.N., Luísa A., J.G. Crespo, and M.F. Duarte, *Effect of extraction methodologies and solvent selection upon cyanopicrodin extraction from Cynara cardunculus leaves*. Separation and Purification Technology, 2019. **236**.
38. Zhou, G., et al., *An optimized ultrasound-assisted extraction and simultaneous quantification of 26 characteristic components with four structure types in functional foods from ginkgo seeds*. Food Chemistry, 2014. **158**: p. 177-185.
39. Wu, J.L., Lidong and F.-t. Chau, *Ultrasound-assisted extraction of ginseng saponins from ginseng roots and cultured ginseng cells*. Ultrasonics Sonochemistry, 2001. **8**: p. 347-352.
40. Sajid, M. and J. Plotka-Wasyłka, *Combined extraction and microextraction techniques: Recent trends and future perspectives*. Trends in Analytical Chemistry, 2018. **103**(1): p. 74-86.
41. Azmir, J., et al., *Techniques for extraction of bioactive compounds from plant materials: A review*. Journal of Food Engineering, 2013. **117**(4): p. 426-436.
42. Ormeno, E., A. Goldstein, and U. Niinemets, *Extracting and trapping biogenic volatile organic compounds stored in plant species*. Trends in Analytical Chemistry, 2011. **30**(7): p. 978-989.
43. Camel, V., *Solid phase extraction of trace elements*. Spectrochimica Acta Part B, 2003. **58**: p. 1177-1233.
44. Harris, D.C., in *Quantitative chemical analysis*. 2010, W. H. Freeman and Co.: New York. p. 713-714.
45. Camel, V., *Solid phase extraction of trace elements*. Spectrochimica Acta Part B, 2003. **58**(7): p. 1177-1233.
46. Arthur, C.L. and J. Pawliszyn, *Solid Phase Microextraction with Thermal Desorption Using Fused Silica Optical Fibers*. Analytical Chemistry, 1990. **62**(19).
47. Tholl, D., et al., *Practical approaches to plant volatile analysis*. The Plant Journal, 2006. **45**: p. 540-560.
48. Baek, Y.-S., et al., *Volatiles Profile of the Floral Organs of a New Hybrid Cymbidium, 'Sunny Bell' Using Headspace Solid-Phase*

- Microextraction Gas Chromatography-Mass Spectrometry Analysis*. Plants, 2019. **8**: p. 251-259.
49. Lee, Y.-G., et al., *Volatile Profiles of Five Variants of Abeliophyllum distichum Flowers Using Headspace Solid-Phase Microextraction Gas Chromatography–Mass Spectrometry (HS-SPME-GC-MS) Analysis*. Plants, 2021. **10**: p. 11.
50. Pawliszyn, J., in *Handbook of Solid Phase Microextraction*. 2012, Elsevier. p. 13-59.
51. Liu, S. and G. Ouyang, in *Solid Phase Microextraction*. 2016, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 1-16.
52. Souza-Silva, E.A., et al., *Insights into the Effect of the PDMS-Layer on the Kinetics and Thermodynamics of Analyte Sorption onto the Matrix-Compatible Solid Phase Microextraction Coating*. Anal. Chem., 2017. **89**(5): p. 2978-2985.
53. Souza-Silva, E.A., et al., *Methodical evaluation and improvement of matrix compatible PDMS-overcoated coating for direct immersion solid phase microextraction gas chromatography (DI-SPME-GC)-based applications*. Analytica Chimica Acta, 2016. **920**: p. 54-62.
54. Souza-Silva, E.A. and J. Pawliszyn, *Optimization of Fiber Coating Structure Enables Direct Immersion Solid Phase Microextraction and High-Throughput Determination of Complex Samples*. Anal. Chem., 2012. **84**: p. 6933-6938.
55. Górecki, T. and J. Pawliszyn, *Effect of Sample Volume on Quantitative Analysis by Solid-phase Microextraction*. Analyst, 1997. **122**(10): p. 1079-1086.
56. Kudlejova, L., S. Risticovic, and D. Vuckovic, in *Handbook of Solid Phase Microextraction*. 2012, Elsevier. p. 201-249.
57. Zhu, F., et al., *Applications of in vivo and in vitro solid-phase microextraction techniques in plant analysis: A review*. Analytica Chimica Acta, 2013. **794**: p. 14.
58. Stashenko, E.E. and J.R. Martínez, *Sampling flower scent for chromatographic analysis*. J. Sep. Sci, 2008. **31**: p. 2022-2031.
59. Bartle, K.D. and P. Myers, *History of gas chromatography*. Trends in Analytical Chemistry, 2002. **21**: p. 547-557.
60. James, A.T. and A.J.P. Martin, *Gas-liquid partition chromatography*. Analyst, 1952. **77**(921): p. 915-932.
61. Berkov, S., et al., in *Bioprocessing of Plant In Vitro Systems*, A. Pavlov, S. Angeloff, and T. Bley, Editors. 2018, Springer.
62. Cagliero, C., et al., *Gas chromatography of essential oil: State-of-the-art, recent advances, and perspectives*. J Sep Sci, 2021. **45**(1): p. 94-112.
63. Desai, N. and D. Alexander, in *Plant Genomics to Plant Biotechnology*. 2013. p. 49-65.

References

64. Rockwood, A.L., M.M. Kushnir, and N.J. Clarke, in *Principles and applications of clinical mass spectrometry: Small molecules, peptides, and pathogens*. 2018, Elsevier: Amsterdam, Netherlands. p. 33-65.
65. Gruber, B., F. David, and P. Sandra, *Capillary gas chromatography-mass spectrometry: Current trends and perspectives*. Trends in Analytical Chemistry, 2020. **124**.
66. Marriott, P.J., in *Encyclopedia of Analytical Science (Second Edition)*, P. Worsfold, A. Townshend, and C. Poole, Editors. 2005, Elsevier. p. 7-18.
67. Van Den Dool, H. and P.D. Kratz, *A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography*. Journal of Chromatography A, 1963. **11**: p. 463-471.
68. United Nations, *Transforming our World: the 2030 Agenda for Sustainable Development*. 2015.
69. Žlabur, J.S., et al., *Ultrasound as a Promising Tool for the Green Extraction of Specialized Metabolites from Some Culinary Spices*. Molecules, 2021. **26**(1866): p. 15.
70. Tungmunnithum, D.G., L., et al., *Green ultrasound assisted extraction of trans rosmarinic acid from Plectranthus scutellarioides (L.) R. Br. leaves*. Plants, 2019. **8**(3).
71. United Nations. *The 17 Goals*. 20 Jan 2022]; Available from: sdgs.un.org/goals.
72. United Nations. *Transforming our world: the 2030 Agenda for Sustainable Development*. 20 Jan. 2022]; Available from: sdgs.un.org/2030agenda.
73. Asenso-Okyere, K., et al., *A review of the economic impact of malaria in agricultural development*. Agricultural Economics, 2011(42): p. 293-304.
74. Gallup, J.L. and J.D. Sachs, *The economic burden of Malaria*. Am J Trop Med Hyg, 2001. **64**: p. 85-96.
75. Willis, D.W. and N. Hamon, *Impact of eliminating malaria by 2040 on poverty rates among agricultural households in Africa*. Gates Open Research, 2018. **2**(69): p. 20.
76. World Health Organization, *World Malaria Report 2017*. 2017: Geneva.
77. Tabetando, R. and A.M. Njong, *Impact of malarai control on late and early infant mortality in Senegal*. South African Journal of Economics, 2017. **85**(1): p. 15.
78. Pathania, V., *The Impact of Malaria Control on Infant Mortality in Kenya*. The University of Chicago Press Journals, 2014. **62**(3): p. 459-487.

79. Poole, C.F., A.D. Gunatilleka, and R. Sethuraman, *Contributions of theory to method development in solid-phase extraction*. J. Chromatogr. A, 2000. **885**: p. 17-39.
80. Poyraz, I.E., B. Demirci, and S. Kucuk, *Volatiles of Turkish Cyperus rotundus L. Roots*. Records of Natural Products, 2017. **12**(3): p. 222-228.
81. Kilani, S., et al., *Chemical Composition, Antibacterial and Antimutagenic Activities of Essential Oil from (Tunisian) Cyperus rotundus*. Journal of Essential Oil Research, 2005. **17**(6): p. 695-700.
82. Kilani, S., et al., *Comparative Study of Cyperus rotundus Essential Oil by a Modified GC/MS Analysis Method. Evaluation of Its Antioxidant, Cytotoxic, and Apoptotic Effects*. Chemistry and Biodiversity, 2008. **5**(5): p. 729-742.
83. Jirovetz, L., et al., *Comparative Analysis of the Essential Oil and SPME-Headspace Aroma Compounds of Cyperus rotundus L. Roots/Tubers from South-India Using GC, GC-MS and Olfactometry*. Journal of Essential Oil Bearing Plants, 2004. **7**(2): p. 100-106.
84. Zhang, L.-L., et al., *Chemical composition, antibacterial activity of Cyperus rotundus rhizomes essential oil against Staphylococcus aureus via membrane disruption and apoptosis pathway*. Food Control, 2017. **80**: p. 290-296.
85. Lawal, O.A. and A.O. Oyediji, *Chemical Composition of the Essential Oils of Cyperus rotundus L. from South Africa*. Molecules, 2009. **14**(8): p. 2909-2917.
86. Essaidi, I., et al., *Chemical Composition of Cyperus rotundus L. Tubers Essential Oil from the South of Tunisia, Antioxidant Potentiality and Antibacterial Activity against Foodborne Pathogens*. Journal of Essential Oil Bearing Plants, 2014. **17**(3): p. 522-532.
87. Okal, M.N., et al., *Water vapour is a pre-oviposition attractant for the malaria vector Anopheles gambiae sensu stricto*. Malar J, 2013. **12**: p. 365.
88. Okal, M.N., et al., *Analysing chemical attraction of gravid Anopheles gambiae sensu stricto with modified BG-sentinel traps*. Parasites and Vectors, 2015. **8**(301).
89. Sjöström, L. *Chemical Volatile Species from Cyperus Rotundus, Cyperus Involukratus and Panicum Repens using HS-SPME-GC-MS*. 2020 [cited 2022; Available from: urn:nbn:se:kth:diva-273932].
90. Gouinguene, S.P. and T.C. Turlings, *The effects of abiotic factors on induced volatile emissions in corn plants*. Plant Physiol, 2002. **129**(3): p. 1296-307.

References

91. Holopainen, J.K. and J. Gershenzon, *Multiple stress factors and the emission of plant VOCs*. Trends Plant Sci, 2010. **15**(3): p. 176-84.
92. Raguso, R.A. and O. Pellmyr, *Dynamic Headspace Analysis of Floral Volatiles: A Comparison of Methods*. JSTOR, 1998. **81**(2): p. 238-254.
93. Wondwosen, B., et al., *A(maize)ing attraction: gravid Anopheles arabiensis are attracted and oviposit in response to maize pollen odours*. Malar J, 2017. **16**(1): p. 39.
94. Wondwosen, B., et al., *Sweet attraction: sugarcane pollen-associated volatiles attract gravid Anopheles arabiensis*. Malar J, 2018. **17**(1): p. 90.
95. Nyasembe, V.O., et al., *Behavioural response of the malaria vector Anopheles gambiae to host plant volatiles and synthetic blends*. Parasit Vectors, 2012. **5**: p. 234.
96. Deletre, E., et al., *Electrophysiological and behavioral characterization of bioactive compounds of the Thymus vulgaris, Cymbopogon winterianus, Cuminum cyminum and Cinnamomum zeylanicum essential oils against Anopheles gambiae and prospects for their use as bednet treatments*. Parasit Vectors, 2015. **8**(316).
97. Meza, F.C., et al., *Behavioural and Electrophysiological Responses of Female Anopheles gambiae Mosquitoes to Volatiles from a Mango Bait*. J Chem Ecol, 2020. **46**(4): p. 387-396.
98. Nyasembe, V.O., et al., *Host plant forensics and olfactory-based detection in Afro-tropical mosquito disease vectors*. Plos Negl Trop Dis, 2018. **12**(2).
99. Janaki, S., et al., *Chemical composition and insecticidal efficacy of Cyperus rotundus essential oil against three stored product pests*. International Biodeterioration & Biodegradation, 2018. **133**: p. 93-98.
100. Orav, A., A. Viitak, and M. Vaher, *Identification of bioactive compounds in the leaves and stems of Aegopodium podagraria by various analytical techniques*. Procedia Chemistry, 2010. **2**: p. 152-160.
101. Kapetanios, C., et al., *Chemical and Principal-Component Analyses of the Essential Oils of Apioideae Taxa (Apiaceae) from Central Balkan*. Chemistry and Biodiversity, 2008. **5**: p. 101-118.
102. Borg-Karlson, A.-K., I.-. Valterova, and A.L. Nilsson, *Volatile compounds from flowers of six species in the family Apiaceae: Bouquets for different pollinators?* Phytochemistry, 1994. **35**(1): p. 111-119.

103. Abraham, D., et al., *Effects of four monoterpenes on germination, primary root growth and mitochondrial respiration of maize*. Journal of Chemical Ecology, 2000. **26**(3): p. 611-624.
104. Nishida, N., et al., *Allelopathic effects of volatile monoterpenoids produced by Salvia leucophylla: Inhibition of cell proliferation and DNA synthesis in the root apical meristem of Brassica campestris seedlings*. Journal of Chemical Ecology, 2005. **31**(5): p. 1187-1203.
105. Singh, P.H., et al., *α -Pinene Inhibits Growth and Induces Oxidative Stress in Roots*. Annals of Botany, 2006. **98**(6): p. 1261-1269.
106. Wang, R., et al., *Cloning, expression and wounding induction of β -caryophyllene synthase gene from Mikania micrantha H.B.K. and allelopathic potential of β -caryophyllene*. Allelopathy J., 2009. **24**(1): p. 35-44.
107. Kil, B.S.D., et al., *Allelopathic effects of Artemisia lavandulaefolia*. Korean J. Ecol., 2000. **23**(2): p. 149-155.
108. Box, G.E.P. and D.W. Behnken, *Some New Three Level Designs for the Study of Quantitative Variables*. Technometrics, 1960. **2**(4): p. 455-475.

