A new sampling method for human skin volatile analysis by comprehensive gas chromatography and mass spectrometry

By

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Declaration

I, Alexis Pieter Roodt, declare that the dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Summary

Volatile organic compounds (VOCs) emanating from the surfaces of human skin have been of great interest to researchers in medical and forensic fields, as well as to biologists studying the ecology of blood-feeding insect vectors of human disease. Applications of skin volatile analyses often require the relative abundances of analytes to be compared between samples in addition to the qualitative identification of the variety of compounds present. Current methodologies used for the non-targeted collection of a broad range of volatile analytes from the skin surface have limited quantitative capacity and there is a need to develop new methods for the collection and pre-concentration of skin volatiles which can facilitate such applications.

The use of in-house developed passive sampling devices constructed in the forms of bracelets and anklets was investigated to address this need. The samplers were employed as non-invasive passive sampling devices for the non-targeted collection and concentration of volatile human skin emissions prior to comprehensive gas chromatographic time-of-flight mass spectrometric (GC × GC-ToFMS) analysis.

The effects of experimental parameters, such as the orientation of the sampler relative to the skin surface and the duration of sampling, as well as data processing procedures, such as peak alignment and normalization, were investigated for their effect on the validity of the results obtained. Sampling of the compounds emanating from the skin surface of an individual has resulted in the tentative identification of hundreds of analytes based on mass spectra library matches and linear retention index (LRI) values. Compounds collected were from a wide range of compound classes some of which have not previously been reported as volatile skin emissions. Comparison of normalized unique mass peak areas between an individual's ankles and wrists has revealed relative quantitative differences and similarities in the amounts of potential human semiochemicals affecting the behaviour of anthropophilic mosquito species. The results highlight the feasibility of the new sampling method for further expanding the current knowledge on human skin volatile emissions.

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List of abbreviations

ΔH_S – enthalpy of sorption

σ – Standard deviation

$\bar{u}$ – Mobile phase linear velocity

$\bar{x}$ – mean

A – Area

ANOVA – Analysis of variance

B – Longitudinal diffusion

c – Normalization constant

$C_{\#}$ – Carbon chain length

CAR – Carboxen

CAS – Chemical Abstract Service

C_e – external concentration

C_i – internal concentration

C_M – Mass transfer coefficient in a column mobile phase

C_S – Mass transfer coefficient in a column stationary phase

COW – Correlation optimized warping

D – Diffusion coefficient

^1D – Primary chromatographic dimension

^2D – Secondary chromatographic dimension

DNPH – Dinitrophenylhydrazine

DVB – Divinylbenzene

EAG – Electroantennography

E_d – activation energy of diffusion

EI – Electron ionization

EPA – Environmental Protection Agency

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FID – Flame Ionization Detector

FR – Fisher Ratio

GC – Gas Chromatography

GC×GC – Comprehensive two-dimensional gas chromatography

GC×GC-ToFMS – GC×GC hyphenated with time of flight mass spectrometry

GC-GC – Heart cut gas chromatography

GC-MS – Gas chromatography hyphenated with mass spectrometry

H – Column length

HETP – Height Equivalent to Theoretical Plate

ID – Internal diameter

J – Flux

K – Partition coefficient

L – Length

LDA – Linear Discriminant Analysis

LRI – Linear Retention Index

LTPRI – Linear temperature programmed retention index

M_R – Modulation Ratio

MS – Mass spectrometry

MSTUS – Mass spectral total useful signal

n – Number of samples

n_T – Total number of samples

N – Number of theoretical plates

NA – Not available

NIH – National Institute of Health

NIST – National Institute of Standards and Technology

NQ – Not quantifiable

P – Permeability

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PCA – Principal Components Analysis

PCA-DA – Principal Components Discriminant Analysis

PDMS – Polydimethylsiloxane

PTR-MS – Proton Transfer Reaction Mass Spectrometry

QC – Quality Control

qMS – Quadrupole mass spectrometry

RSD – Relative Standard Deviation

S/N – Signal-to-noise ratio

SESI-MS – Secondary electrospray ionization mass spectrometry

SIC – Select ion chromatogram

SIFT-MS – Selected ion flow tube mass spectrometry

SPME – Solid phase micro extraction

SPME-HS – Head space solid phase micro extraction

T – Temperature

t – Time

TD – Thermal desorption

TDS – Thermal desorption system

TIC – Total ion chromatogram

$^{1}t_{R}$ – Analyte peak retention time on the primary column

$^{2}t_{R}$ – Analyte peak retention time on the secondary column

VOC – Volatile organic compound

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Chapter One

Introduction

Contents

1.1 Background

The human volatilome includes volatile organic compounds (VOCs) emanating from a range of human body sources such as exhaled breath, urine and blood. Those volatiles which originate from the surfaces of human skin are of great interest to researchers in many fields. Applications include medical and forensic diagnostics [1] as well as studying the ecology of blood-feeding insect vectors of human disease. The latter application being of great interest in the ongoing search for new ways to efficiently control malaria around the world [2].

Whether investigating sources of human malodour, searching for new biomarkers of disease or insect attracting semiochemicals, human skin is a very complicated sampling matrix to investigate. This is due to a large number of variables at play. Human skin is the largest organ of the human body encompassing a surface area of approximately two square meters for the average adult. The surface of human skin is, however, very far from uniform in composition. The topography and composition of the epidermis layer which constitutes the interface between the human body and the outside environment varies depending on the body region under investigation. The skin surface can be viewed as an ecosystem consisting of several smaller

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interconnected biomes. The different distributions of glands and hair follicles across the skin surface dictate the local skin chemistry. Skin secretions in combination with the amount of moisture and light present give rise to skin microbiomes. Each of which is populated by its own community of micro-organisms. Sweat secreted by eccrine and apocrine glands is both sterile and odourless [3]. These fluids along with the sebum secreted by sebaceous glands contain a wide variety of cellular metabolites such as fatty acids, squalene, triglycerides, sugars and other large molecules which serve as a substrate for bacterial colonies on the skin surface. Many volatile chemical compounds emanating from human skin form as a result of the metabolic action of the endogenous skin bacteria. The species composition and diversity of skin bacteria therefore play a role in an individual's body odour [4] and attractiveness to malaria vector mosquito species such as *Anopheles gambiae sensu stricto* [5]. Resident skin bacteria may come from a broad range of bacteria phyla [6]. Inter-individual differences in species abundance, composition and distribution may be affected by internal factors such as age [7] and gender [8]. External factors such as illness [9] or antibiotic usage [10] can also disrupt the normal species composition and abundance of an individual's skin microflora.

Many studies probing the human skin volatilome have been published in recent years. A wide range of sample collection approaches have been reported in combination with hyphenated separation and detection techniques such as gas chromatography-mass spectrometry (GC-MS) [11]. Both from skin surfaces [12] as well as from lab based skin cell cultures [13-14]. These studies have resulted in the qualitative identification of hundreds of compounds. However, performing research into the composition and cause/effect relationships with regard to changes in volatile skin emissions is currently still limited by the methodology used for sample collection and pre-concentration [11].

There is a need for novel methods to facilitate quantitative and semi-quantitative investigations into skin volatile emissions. Passive sampling is an ideal approach to address this need as such techniques can provide qualitative as well as quantitative information in the form of time weighted average amounts of analytes present in a sampled environment. This information can be used to construct representative profiles of compounds emanating from the surfaces of a human subject's skin. These

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profiles when collected under different circumstances and compared may reveal differences and trends in human skin volatile composition.

1.2 Approach

Polydimethylsiloxane (PDMS) silicone rubber in the form of patches has previously shown promising results when used to analyze VOCs on the surfaces of human skin [15]. This project proposes to investigate the use of PDMS silicone rubber tubes formed into closed loops which can be worn on sampled subjects' wrists or ankles for the collection and concentration of volatile human skin emissions prior to gas chromatographic analysis. These devices have previously been applied successfully in many in-house developed analytical sampling methods. Such applications include their use in water sampling for the detection of urban pollutants [16] as well as atmospheric sampling of polycyclic aromatic hydrocarbons [17] and assessment of milk volatiles [18]. Detailed evaluation of the applicability and operational parameters of these tubes is required with regard to their ability to collect and pre-concentrate low concentrations of volatile organic compounds emanating from the surface of human skin in combination with Thermal desorption – Two Dimensional Gas Chromatography – Electron Impact – Time of Flight Mass Spectrometry (TD – GC×GC – EI –ToFMS) analysis. The overall objective is the development of a new method which will allow for the comparison of volatile organic compound profiles collected from different samples. This will facilitate investigations into the differences in skin volatiles collected from different skin regions or under different circumstances.

The new method of sampling and pre-concentration of skin VOCs is required to provide a competitive alternative to current sampling techniques by mitigating disadvantages associated with sample collection, solvent extraction and/or sample preparation techniques. Additional factors taken into consideration are the cost and time required for analyses as well as the simplicity of use. The simplicity of the method is of great advantage in order to reduce sources of error and to facilitate large scale sampling experiments.

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1.3 Project aims

The aims of this research include:

1) An investigation of an optimal sampler set up during skin volatile collection. This includes investigation of parameters such as direct skin contact vs. non-contact sampler arrangements, the duration of sampling, as well as investigating the number of analytes collected and effects on subsequent data analysis.
2) An investigation into the diversity of compounds collected by the proposed passive samplers and comparison of these results to the compounds currently reported in the literature using a variety of sampling methods.
3) Assessment of passive sampling results in order to demonstrate the real world functionality and application of the new passive sampling method.

1.4 Arrangement and presentation

The remainder of this manuscript is presented as follows; Chapter Two includes a review of published methods for skin volatile sampling as well as a detailed review of physical and chemical characteristics of PDMS in a passive sampling context. A detailed discussion of the analytical instrumentation used is provided in Chapter Three. In Chapter Four some of the problems and solutions with regard to the analysis of large data sets and software tools used for untargeted analyses are addressed. The experimental set up is presented in Chapter Five. The results and discussion of experimentation undertaken to develop and validate the sampling method are presented in Chapter Six. The results and discussion relating to the real world application of the new method are covered in Chapter Seven. Finally, conclusions, recommendations and future work are discussed in Chapter Eight.

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1.5 References

[1] Curran AM, Prada PA, Furton KG. The differentiation of the volatile organic signatures of individuals through SPME-GC/MS of characteristic human scent compounds. J Forensic Sci. 2010;55(1):50–7.

[2] Zwiebel LJ, Takken W. Olfactory regulation of mosquito-host interactions. Insect Biochem Mol Biol. 2004;34(7):645–52.

[3] Shelley WB, Hurley HJ, Nicholas AC. Axillary Odor: Experimental study of the role of bacteria apocrine sweat and deodorants. Arch Dermatol Syph. 68:430, 1953.

[4] Xu Y, Dixon SJ, Brereton RG, Soini HA, Novotny M V, Trebesius K, et al. Comparison of human axillary odour profiles obtained by gas chromatography/mass spectrometry and skin microbial profiles obtained by denaturing gradient gel electrophoresis using multivariate pattern recognition. Metabolomics. 2007;3(4):427–37.

[5] Verhulst NO, Qiu YT, Beijleveld H, Maliepaard C, Knights D, Schulz S, et al. Composition of human skin microbiota affects attractiveness to malaria mosquitoes. PLoS One. 2011;6(12):1-7.

[6] Muszer M, Noszczynska M, Kasperkiewicz K, Skurnik M. Human Microbiome: When a Friend Becomes an Enemy. Arch Immunol Ther Exp (Warsz). 2015;63(4):287–98.

[7] Somerville DA. The normal flora of the skin in different age groups. Br J Dermatol. 1969;81(4):248–58.

[8] Grice EA, Segre JA. The skin microbiome. Nat Rev Microbiol. 2013;9(4):244–53.

[9] Prugnolle F, Lefèvre T, Renaud F, Møller AP, Missé D, Thomas F. Infection and body odours: Evolutionary and medical perspectives. Infect Genet Evol. 2009;9(5):1006–9.

[10] Ferrer M, Méndez-García C, Rojo D, Barbas C, Moya A. Antibiotic use and microbiome function. Biochem Pharmacol. 2017;134:114-126.

[11] Pandey SK, Kim KH. Human body-odor components and their determination. TrAC - Trends Anal Chem. 2011;30(5):784–96.

[12] de Lacy Costello B, Amann A, Al-Kateb H, Flynn C, Filipiak W, Khalid T, et al. A review of the volatiles from the healthy human body. J Breath Res. 2014;8(1):014001.

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[13] Filipiak W, Mochalski P, Troppmair J, Unterkofler K, Agapiou A, Davis C, et al. A compendium of volatile organic compounds (VOCs) released by human cell lines. Curr Med Chem. 2016;23(20):2112-2131.

[14] Acevedo CA, Sanchez EY, Reyes JG, Young ME. Volatile profiles of human skin cell cultures in different degrees of senescence. J Chromatogr B Anal Technol Biomed Life Sci. 2010;878(3-4):449–455.

[15] Jiang R, Cudjoe E, Bojko B, Abaffy T, Pawliszyn J. A non-invasive method for in vivo skin volatile compounds sampling. Anal Chim Acta. 2013;804:111–119.

[16] Naude Y, Gorst-Allman P, Rohwer E. A cheap and simple passive sampler using silicone rubber for the analysis of surface water by gas chromatography-time of flight mass spectrometry. Water SA. 2015;41(2, SI):182–188.

[17] Forbes PBC, Karg EW, Zimmermann R, Rohwer ER. The use of multi-channel silicone rubber traps as denuders for polycyclic aromatic hydrocarbons. Anal Chim Acta. 2012;730:71–79.

[18] Naude Y, Van Aardt M, Rohwer ER. Multi-channel open tubular traps for headspace sampling, gas chromatographic fraction collection and olfactory assessment of milk volatiles. J Chromatogr A. 2009;1216(14):1804–2798.

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Chapter Two

Human skin volatile analysis and passive sampling using polydimethylsiloxane

Contents

2.1 Introduction to skin volatile organic compound sampling

The investigation of volatile organic compounds (VOCs) originating from human skin surfaces has been of interest to researchers for several decades. Much early work was focused on the collection and qualitative analysis of odorous samples collected from the axillary region of human subjects. Later investigations involved other body regions and included diagnostic and forensic applications [1]. Analytical instrumentation and techniques of separation and detection have advanced significantly during the period of these skin volatile investigations. However, even when sophisticated comprehensive separations and accurate mass measurements are available, the method of sample collection is still crucial to ensure that the results which are obtained are truly representative of the skin volatilome. It is therefore

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important that sampling methods which are used for the collection of skin VOCs are sufficiently rugged and should preferably have been tested and validated under field conditions [1].

Qualitative reports of human skin volatiles collected from different body regions are abundant in academic literature. Quantitative or semi-quantitative methodologies and results are, however, few and far between. Quantitative investigation of skin emanations is of great relevance in many applications. In the health field, changes in normal skin emanations are of interest for diagnostic applications as biomarkers and evidence of changes in metabolic function. To date, several diseases have been investigated for their link to changes in abundances of volatile compounds emitted in breath or from skin, for example, changes in volatile profiles associated with skin cancer melanoma cells [2]. In the forensic field quantitative detection of human skin emanations is of interest for locating humans in search and rescue applications [3]. Differences in relative abundances of skin volatiles have also been linked to the differing attractiveness of human blood hosts to anthropophilic *Aedes aegypti* mosquitoes [4].

A review article by de Lacy Costello *et al.* (2014) which included a literature survey of all the volatile organic compounds reported to originate from various skin surfaces of human subjects listed a total of 532 compounds [5]. This review concluded that the large number and diversity of compounds collected, as well as discrepancies between the compounds identified in different studies covered in this review can be attributed to external, internal and methodology factors. Some degree of variation between studies is to be expected based on internal factors relating to the sampled subjects. These factors include gender [6] or skin age [7]. External factors such as the widespread use of fragrances in hygiene and cosmetic products make compounds from these sources common contaminants in all skin volatile studies. While the majority of skin volatile studies make use of hyphenated gas chromatography mass spectrometry (GC-MS) as an analysis technique, there are a wide array of different sampling approaches used by different researchers [1]. A large source of variation between studies can thus be attributed to sample collection and pre-analysis methodologies. Most studies include pre-concentration steps in their sampling methodology although online methods have also been reported.

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Sampling methods have made use of adsorbent or absorbent materials which are used in direct contact with human skin or used to sample the atmosphere above the skin surface. Among these PDMS plays a prominent role being used both in its standard form as a patch placed on the skin surface [8] or, often with modified functionality, as the coating on solid phase micro extraction (SPME) fibers [9]. The rest of this chapter includes review and discussion of different sampling approaches used for collecting VOCs emanating from human skin surfaces as well as more detailed look into the properties and use of PDMS for passive absorption and quantification of organic molecules.

2.1.1 Direct contact sampling methods

Sampling techniques which involve direct contact with human skin are common in academic literature and have been extensively used for non-targeted analysis of volatile compounds [10]. Commonly employed sampling techniques in this category involve the use of adsorptive materials like glass and textile materials or absorptive polymer materials placed in direct contact with the skin surface. These techniques, while relatively uncomplicated to use, often suffer from difficulties associated with background subtraction, quantification and differentiation between volatile and semi-volatile components in a sample.

A technique for collecting skin volatiles which was developed by researchers interested in identifying olfactory cues involved in blood host identification by anthropophilic mosquito species makes use of small glass beads. Sampling using this technique involves a subject handling and rolling the beads across the surface of the skin for a set amount of time. The residual skin compounds which remain on the surface of the glass are then analyzed by solvent extraction prior to GC analysis [11] or directly by thermal desorption of the glass beads into a GC system [12]. The use of this method was spurred on by the observation that anthropophilic mosquitoes were attracted to recently handled glass beads in behavioural studies [13]. The attraction, however, quickly waned as the volatile compounds triggering the response evaporated from the glass surface. This method had several favourable

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aspects such as the possibility for convenient and solvent free sample introduction into gas chromatography instruments by thermal desorption. The technique is however limited to sampling major components of the skin volatile profile and suffers from its inability to retain and concentrate trace and highly volatile analytes. This is a major disadvantage when trying to piece together the complex relationships involved in human host finding and identification of potential human semiochemicals. The technique has been used in semi-quantitative application to compare relative abundances of analytes collected from different individuals [4]. Difficulty with regard to accurate normalization of analyte peak areas before comparison limits this technique to mainly qualitative analysis of skin volatiles.

Another approach commonly employed for the collection of skin VOCs involves the use of an adsorbent material which is placed directly on the skin surface of a subject being sampled. This is often done in conjunction with physical activity to induce sweating. Solid phase micro extraction head space (SPME-HS) collection of volatiles from sterilized gauze pads [14-17] or textile materials [18, 19] which have been in contact with the surface of a subject's skin have been reported. Head space extraction of the collection medium provides a viable method for collection of strictly volatile components originating from the surface of the human skin. This technique retains the attributes favourable to the glass bead technique such as simplicity and ease of sample collection and introduction into analytical instrumentation by thermal desorption of the SPME sampler fiber. This approach has the added advantage of possible sample storage prior to analysis. However, it suffers from more complicated background originating from the adsorbent material which results in a more complicated chromatographic separation and data analysis. Quantitative work making use of cotton pads for sample collection has been reported. Comparison of relative signal areas was made possible by normalization to the mass of cotton pad analyzed [20].

Recently, polydimethylsiloxane (PDMS) coated stirrer bars placed directly on the surface of a human subject's skin has been used for large scale skin VOC sampling experiments [6]. A simpler alternative involves using PDMS patches which have been applied directly to the surface of a subject's skin [8], [21-23]. Techniques involving PDMS have the advantage of simple sample introduction prior to GC

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analysis by thermal desorption of the PDMS material. Solvent extraction and thermal conditioning of PDMS samplers results in a low background. Reports of quantitative analysis of skin volatiles using PDMS could not be found in the literature.

2.1.2 Non-contact sampling methods

Several non-contact sampling techniques have been developed for human skin volatile analysis [10]. These techniques are used to collect VOCs in the space around the surface of human skin and can involve the construction of sampling apparatus of varying complexity.

The apparatus may be as simple as a steel petri dish containing 2,4-dinitrophenylhydrazine (DNPH) soaked filter paper placed over the surface of the skin for the quantitative targeted collection of aldehydes and ketones [24]. More complicated apparatus may include a sampling chamber constructed from glass and stainless steel [25] or simply bags made from nalophan [26] or other synthetic materials [27]. An individual's hand or arm is sealed inside the vessel allowing the atmosphere surrounding the subject's skin to be replaced by an analytically pure gas such as nitrogen or helium. The skin volatile containing atmosphere inside the apparatus is subsequently sampled using a built–in cryogenic trap or SPME fiber inserted into the headspace. These apparatus have the advantage of easy background correction and accurate quantification through the incorporation of calibrated gas standards.

Direct online analysis of skin volatiles has been performed using secondary electrospray ionization mass spectrometry (SESI-MS) [28], selected ion flow tube mass spectrometry (SIFT-MS) [29] and proton transfer reaction mass spectrometry (PTR-MS) [30]. These approaches rely on constant monitoring of background signals for background removal. The detection of analytes is dependent on the ionization efficiency and sensitivity of the instrument used. Quantitative measurement is limited to real time fluctuations in analyte signals.

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2.2 Passive sampling using PDMS

Polydimethylsiloxane (PDMS) is a popular and widely used material in analytical and separation science applications [31]. The material has found application as stationary phases in GC columns, as a sorbent for solid phase micro-extraction (SPME) fibers [32] and packed bed samplers [33]. The application of this material in the form of patches for the collection of skin volatiles has been previously mentioned (Section 2.1.1). In addition, commercially available silicone rubber wristbands have also been applied as passive samplers in order to investigate work place exposure to potentially harmful volatile compounds over long sampling periods, up to 30 days, with large numbers of sampled subjects [34].

2.2.1 Physical and chemical properties of PDMS

The physical and chemical properties of PDMS make this an ideal material for use in passive sampler applications. PDMS has a low glass transition temperature (T_g) of approximately 150 K [35]. The glass transition temperature of a material is the range of temperature at which the amorphous form of the material transforms to a glassy solid. Having a glass transition point below the temperature of operation gives several advantages. It allows the material to be used in the form of a flexible solid gum during sampling. This is ideal as it results in the material behaving as a solvent into which analytes can diffuse rather than acting as an adsorptive surface onto which analytes can form a finite number of temporary bonds [36]. This is complemented by the material's stability and inertness at temperatures high enough to facilitate thermal desorption of analytes absorbed in the polymer matrix. In addition, PDMS exhibits low diffusive selectivity typical of rubbery polymers in contrast to glassy polymers which have a more rigid polymer backbone that may hinder the absorption of certain analytes [37]. The interaction between analytes and the predominantly non-polar silicone backbone of PDMS is the major parameter affecting the uptake of external molecules by the material. The PDMS material can, therefore, be thought of as a non-polar solvent into which analytes will partition from

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a surrounding matrix with partition coefficients being comparable to analyte – octanol partition coefficients [38].

2.2.2 Theory of analyte uptake by PDMS

The widespread introduction of polymer materials during the twentieth century lead to great interest and effort being directed towards the investigation of the mechanisms of sorption and diffusion of gaseous species through these materials. This research has benefited the field of analytical chemistry by providing a basis for the development of new stationary phases for chromatographic columns as well as for passive sampler applications.

Theory describing analyte uptake during the general passive sampling experiment using PDMS is based on several assumptions [38, 39] namely:

(i) The sorption volume of the sampler will remain constant during sampling.

(ii) The majority of gaseous molecules undergoing diffusion are of relatively low molecular weight and are present at low concentration in the surrounding atmosphere during sampling.

(iii) The diffusion of analytes in PDMS is independent of the direction of movement of analytes and is described by Fick's law of diffusion.

(iv) Interactions between analyte molecules do not influence the diffusion coefficients of analytes in the polymer.

(v) Sorption of analytes from the surrounding atmosphere does not remove sufficient analyte to alter the surrounding concentration such that the diffusion coefficient would change during the course of sampling.

Models describing analyte diffusion in polymer materials have been based on a theory developed by Fick in the mid nineteenth century [39]. Fick's model was proposed based on the hypothesis that diffusion of gaseous molecules into a

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polymer was related to the concentration gradient of the molecules in the system perpendicular to the surface of the polymer.

Fick's First law can be used to describe the flux of molecules traveling in the opposite direction to their concentration gradient through the interface between a sorbent material and the external environment. The flux, J (kg/s), is calculated as a function of the diffusion coefficient, D (m^2/s), of the particular analyte in the specific sorbent material with surface area, A (m^2), down the analyte's concentration gradient, dC/dx, perpendicular to the sorbent surface (Eqn. 1) [40].

$$J = -DA\frac{dC}{dx} \text{ ...Eqn. 1}$$

This equation can be expressed in a more intuitive form which relates the mass of analyte diffusing through a sorbent material in a specific amount of time to its concentration gradient across the membrane surface (Eqn. 2).

$$\frac{m}{t} = \frac{DA}{l}(c_e - c_i) \text{ ... Eqn. 2}$$

Where, m (kg) is the mass of analyte diffusing through a sorbent of thickness l (m) during time, t (s). The concentrations of the analyte at the external and internal surfaces of the polymer membrane are denoted by c_e and c_i (kg/cm^3) respectively [41].

2.2.3 External parameters affecting analyte uptake by PDMS

The physiochemical properties of the non-polar backbone of the PDMS polymer material dominate the absorption process during passive sampling. The non-polar characteristic of this material aids its application as a passive sampler due to its ability to exclude water which is abundant in most real world sampling environments. The diffusion coefficient, D of an analyte is a reflection of the ability of the analyte to migrate through a polymer at a particular concentration. Diffusion and partition coefficients are a function of the ambient temperature during sampling. The permeability of a polymer material towards a specific analyte is defined as the

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product of the analytes' diffusion coefficient, D, (Eqn 3.) and the partition coefficient, K, (Eqn 4.) of the analyte between the external environment and the membrane.

Diffusivity can be expressed according to the Arrhenius equation (Eqn. 3).

$$D = D_0 exp[-E_d \left(\frac{1}{RT} - \frac{1}{RT_0}\right)] \ldots \text{Eqn. 3}$$

The partition coefficient can be expressed using Van't Hoff's equation (Eqn. 4).

$$K = K_0 exp[-\Delta H_s \left(\frac{1}{RT} - \frac{1}{RT_0}\right)] \ldots \text{Eqn. 4}$$

The product of equations 3 and 4 gives an expression for the polymer's permeability to a specific analyte (Eqn. 5).

$$P = P_0 exp[-(E_d + \Delta H_s) \left(\frac{1}{RT} - \frac{1}{RT_0}\right)] \ldots \text{Eqn. 5}$$

Where P (m^2/s) is the permeability of the polymer; D_0, K_0 and P_0 are the diffusion coefficient, partition coefficient and permeability constant at T_0 (K); R is the ideal gas constant and T (K) is the temperature; E_d (kJ/mol) is the activation energy of diffusion and ΔH_s (J) is the enthalpy of sorption of the analyte into the polymer matrix [42].

An investigation using a PDMS passive sampler conducted by Seethapathy and Górecki found that $E_d > 0$ and $\Delta H_s < 0$ for small organic molecules from a range of compound classes. Thus it can be concluded that the change in permeability of a polymer with changing temperature is the result of these two opposing contributions. The study found that in the case of PDMS ΔH_s is usually the dominating factor, however, the effect of temperature on the permeability of PDMS is minor [42]. This is in agreement with predicted changes in uptake rate with temperature of 0.2 – 0.4 %/K [43].

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2.3 Quantitative passive sampling

Apart from qualitative work which yields only a positive or negative result for the presence of a specific analyte in a sample, a quantitative analysis goes further to give more detailed information regarding the amounts of the analyte present in the sampled environment. The approach to quantification used for passive sampling may be categorised based on the level of measurement applied and can be either fully-quantitative or semi-quantitative.

2.3.1 Absolute-quantification for PDMS passive sampling

Fully quantitative work aims to provide absolute measurements with regard to the amount of an analyte present in a passive sampler post deployment and may be further related to the concentration of the analyte in the sampled environment by application of validated uptake models to the specific sampler and system in question.

Two main calibration approaches exist for the determination of absolute amounts of analyte in a sample, namely, external and internal calibration strategies, each of which have specific strengths and weaknesses.

External calibration makes use of a concentration - response curve which is set up by running a series of analyses of reference standards over a range of concentrations. The results of which are used to generate an instrument response or calibration curve. By comparing the instrument response generated by a sample to this curve the absolute amount of analyte in the sample may be determined. External concentration response curves have been used for the quantification of volatile plant analytes collected by PDMS passive samplers. Calibration curves were obtained in this study by placing PDMS passive samplers in the headspace of vials containing standard solutions of known concentrations [44].

An alternative strategy for absolute quantification is that of standard addition, this involves the direct spiking of a series of replicate samples with an internal standard

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over a range of concentrations. Thus, allowing for an internal concentration - response curve for the spiked analyte to be set up which upon extrapolation reveals the concentration of the relevant analyte. This standard addition method is preferred for samples having complex matrices in order to take the effect of the matrix on analyte detection into account. This is however done at the expense of larger volumes of a sample which must be collected in order to have sufficient points on the generated calibration curve for accurate quantitation. In this case the internal standard used should have very similar physiochemical properties to the analyte of interest – generally, an isotopically labeled surrogate is preferred. Spiking of isotopically labeled internal reference standards directly into the PDMS material prior to use in passive sampling is covered in the literature [45].

Efforts to quantify the absolute amount of analyte in a passive sampler are not useful if the information cannot be related to the concentration in the external environment. Therefore analyte uptake by passive samplers is required to be well defined under the sampling conditions. This can be done by pre-calibration of samplers which involves the determination of partition coefficients between individual analytes in the external environment and the PDMS material under relevant conditions. In the case of small scale targeted analyses, such partition coefficients are generally not difficult to measure experimentally for a range of conditions. Pre-calibration of PDMS passive samplers can, however, be susceptible to errors introduced by the experimental set-up used. Partition coefficients measured for an air-PDMS system are different under saturated vapour conditions when compared to values measured with lower external concentrations [38]. Untargeted sampling experiments require the collection of a range of analytes by multiple samplers. Pre-calibration of each individual sampler for each individual analyte of interest after their identification may hinder these sampling efforts and has driven the development of alternative methods of relating external analyte concentration to passive sampling results [46]. A method, which arose in response to this problem, involves the application of linear temperature programmed retention indexes (LTPRI) to estimate experimental partition coefficients between gaseous compounds and PDMS [41]. This method was successfully applied for quantitative analysis of a wide range of analytes including aromatic and non-aromatic hydrocarbons, alcohols and esters [41]. The process involves the separation of analytes on a GC capillary column coated with 100%

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PDMS under linear temperature programmed conditions. The method takes advantage of the similarity in the analyte transfer mechanism occurring during GC separation and diffusion of analytes into PDMS passive samplers. This method allows for the estimation of analyte partition coefficients prior to identification of the analyte.

2.3.2 Semi-quantification for PDMS passive sampling

Semi-quantitative measurement, while not absolute with regard to the amounts of analytes in the sampled environment, may provide additional insight to qualitative results by providing information with regard to the relative ratios of analyte signals between samples. Such an approach is applicable when searching for deviations from normal/abnormal thresholds in systems under study where patterns or profiles are correlated with certain conditions and absolute concentrations are not required. Analyte abundances may be compared to a reference sample or statistical approaches can be used to highlight differences between samples. This approach is heavily influenced by the method used for normalization of signals prior to comparison of different samples. For untargeted investigations, it would be practically impossible to provide absolute concentrations for all compounds identified in a range of similar samples. This approach has the advantage of not requiring calibration curves to be set up for individual analytes and thus is more applicable to untargeted analyses.

2.4 Conclusion

The review presented in this chapter began by looking at the main approaches which have been used to sample VOCs originating from the surface of human skin. This revealed that there is a need for new sampling methods which will provide quantitative results in this field. The properties of PDMS which make it an ideal material for use in passive sampler devices were highlighted. Factors affecting

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passive sampling using PDMS which are crucial to experimental design were described. Finally, different quantitation strategies were presented in the context of PDMS passive sampling.

2.5 References

[1] Dormont L, Bessière JM, Cohuet A. Human Skin Volatiles: A Review. J Chem Ecol. 2013;39(5):569–78.

[2] Kwak J, Gallagher M, Ozdener MH, Wysocki CJ, Goldsmith BR, Isamah A, et al. Volatile biomarkers from human melanoma cells. J Chromatogr B Anal Technol Biomed Life Sci.; 2013;931:90–96.

[3] Agapiou A, Amann A, Mochalski P, Statheropoulos M, Thomas CLP. Trace detection of endogenous human volatile organic compounds for search, rescue and emergency applications. TrAC - Trends Anal Chem.; 2015;66:158–75.

[4] Bernier UR, Kline DL, Schreck CE, Yost RA, Barnard DR. Chemical analysis of human skin emanations: comparison of volatiles from humans that differ in attraction of *Aedes aegypti* (Diptera: Culicidae). J Am Mosq Control Assoc. 2002;18(3):186–95.

[5] de Lacy Costello B, Amann A, Al-Kateb H, Flynn C, Filipiak W, Khalid T, et al. A review of the volatiles from the healthy human body. J Breath Res. 2014;8(1):014001.

[6] Penn DJ, Oberzaucher E, Grammer K, Fischer G, Soini HA, Wiesler D, et al. Individual and gender fingerprints in human body odour. J R Soc Interface. 2007;4(13):331–40.

[7] Acevedo CA, Sanchez EY, Reyes JG, Young ME. Volatile profiles of human skin cell cultures in different degrees of senescence. J Chromatogr B Anal Technol Biomed Life Sci. 2010;878(3-4):449–55.

[8] Martin HJ, Turner MA, Bandelow S, Edwards L, Riazanskaia S, Thomas CLP. Volatile organic compound markers of psychological stress in skin: a pilot study. J Breath Res. 2016;10(4):046012.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

[9] Dormont L, Bessiere J-M, McKey D, Cohuet A. New methods for field collection of human skin volatiles and perspectives for their application in the chemical ecology of human-pathogen-vector interactions. J Exp Biol. 2013;216(15):2783–2788.

[10] Pandey SK, Kim KH. Human body-odor components and their determination. TrAC - Trends Anal Chem.; 2011;30(5):784–796.

[11] Schreck CE, Smith N, Carlson DA, Price GD, Haile D, Godwin DR. A material isolated from human hands that attracts female mosquitoes. J Chem Ecol. 1982;8(2):429–38.

[12] Bernier UR, Kline DL, Barnard D, Schreck C, Yost RA, Barnard DR, et al. Analysis of Human Skin Emanations by Gas Chromatography/Mass Spectrometry. 2. Identification of Volatile Compounds That Are Candidate Attractants for the Yellow Fever Mosquito (*Aedes aegypti*) Analysis of Human Skin Emanations by Gas Identification of Volatile Compounds That Are Candidate A. Anal Chem. 2000;72(4):747–56.

[13] Bar-Zeev M, Maibach HI, Khan AA. Studies on the Attraction of *Aedes Aegypti* (Diptera: Culicidae) to Man. J Med Entomol. 1977;14(1):113–20.

[14] Curran AM, Rabin SI, Prada P a., Furton KG. Comparison of the volatile organic compounds present in human odor using SPME-GC/MS. J Chem Ecol. 2005;31(7):1607–19.

[15] Curran AM, Ramirez CF, Schoon AA, Furton KG. The frequency of occurrence and discriminatory power of compounds found in human scent across a population determined by SPME-GC/MS. J Chromatogr B Anal Technol Biomed Life Sci. 2007;846(1-2):86–97.

[16] Curran AM, Prada PA, Furton KG. The differentiation of the volatile organic signatures of individuals through SPME-GC/ms of characteristic human scent compounds. J Forensic Sci. 2010;55(1):50–7.

[17] Mebazaa R, Rega B, Camel V. Analysis of human male armpit sweat after fenugreek ingestion: Characterisation of odour active compounds by gas chromatography coupled to mass spectrometry and olfactometry. Food Chem.; 2011;128(1):227–35.

[18] Prada PA, Curran AM, Furton KG. The evaluation of human hand odor volatiles on various textiles: A comparison between contact and noncontact sampling methods. J Forensic Sci. 2011;56(4):866–81.

[19] de la Mata AP, McQueen RH, Nam SL, Harynuk JJ. Comprehensive two-dimensional gas chromatographic profiling and chemometric interpretation of

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

the volatile profiles of sweat in knit fabrics. Anal Bioanal Chem. Analytical and Bioanalytical Chemistry; 2016;1905–1913.

[20] Verhulst NO, Weldegergis BT, Menger D, Takken W. Attractiveness of volatiles from different body parts to the malaria mosquito *Anopheles coluzzii* is affected by deodorant compounds. Sci Rep. Nature Publishing Group; 2016;6:27141.

[21] Riazanskaia S, Blackburn G, Harker M, Taylor D, Thomas CLP. The analytical utility of thermally desorbed polydimethylsilicone membranes for in-vivo sampling of volatile organic compounds in and on human skin. Analyst. 2008;133(8):1020–7.

[22] Jiang R, Cudjoe E, Bojko B, Abaffy T, Pawliszyn J. A non-invasive method for in vivo skin volatile compounds sampling. Anal Chim Acta.; 2013;804:111–119.

[23] Martin HJ, Reynolds JC, Riazanskaia S, Thomas CLP. High throughput volatile fatty acid skin metabolite profiling by thermal desorption secondary electrospray ionisation mass spectrometry. Analyst. Royal Society of Chemistry; 2014;139(17):4279–4286.

[24] Sekine Y, Toyooka S, Watts SF. Determination of acetaldehyde and acetone emanating from human skin using a passive flux sampler-HPLC system. J Chromatogr B Anal Technol Biomed Life Sci. 2007;859(2):201–7.

[25] Zhang ZM, Cai JJ, Ruan GH, Li GK. The study of fingerprint characteristics of the emanations from human arm skin using the original sampling system by SPME-GC/MS. J Chromatogr B Anal Technol Biomed Life Sci. 2005;822(1-2):244–52.

[26] Mochalski P, King J, Unterkofler K, Hinterhuber H, Amann A. Emission rates of selected volatile organic compounds from skin of healthy volunteers. J Chromatogr B Anal Technol Biomed Life Sci.; 2014;959:62–70.

[27] Nose K, Nunome Y, Kondo T, Araki S, Tsuda T. Identification of gas emanated from human skin: methane, ethylene, and ethane. Anal Sci. 2005;21(6):625–8.

[28] Martínez-Lozano P. Mass spectrometric study of cutaneous volatiles by secondary electrospray ionization. Int J Mass Spectrom. 2009;282(3):128–32.

[29] Jackson P, Attalla MI. N-Nitrosopiperazines form at high pH in post-combustion capture solutions containing piperazine: a low-energy collisional behaviour study. Rapid Commun Mass Spectrom. 2010;24(24):3567–77.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

[30] Steeghs MML, Moeskops BWM, van Swam K, Cristescu SM, Scheepers PTJ, Harren FJM. On-line monitoring of UV-induced lipid peroxidation products from human skin in vivo using proton-transfer reaction mass spectrometry. Int J Mass Spectrom. 2006;253:58–64.

[31] Seethapathy S, Górecki T. Applications of polydimethylsiloxane in analytical chemistry: A review. Anal Chim Acta; 2012;750:48–62.

[32] Musteata FM, Pawliszyn J. In vivo sampling with solid phase microextraction. J Biochem Biophys Methods. 2007;70(2):181–93.

[33] Baltussen E, David F, Sandra P, Janssen HG, Cramers C. Equilibrium sorptive enrichment on poly(dimethylsiloxane) particles for trace analysis of volatile compounds in gaseous samples. Anal Chem. 1999;71(22):5193–8.

[34] O'Connell SG, Kincl LD, Anderson KA. Silicone wristbands as personal passive samplers. Environ Sci Technol. 2014;48(6):3327–3335.

[35] Beck KR, Korsmeyer R, Kunz RJ, Company H, Ave WJ, Il N. An Overview of the Glass Transition Temperature of Synthetic Polymers. J Chem Educ. 1984;61(16):1–3.

[36] Baltussen E, Cramers CA, Sandra PJF. Sorptive sample preparation - A review. Anal Bioanal Chem. 2002;373(1-2):3–22.

[37] Alexander Stern S. Polymers for gas separations: the next decade. J Memb Sci. 1994;94(1):1–65.

[38] De Coensel N, Desmet K, Górecki T, Sandra P. Determination of polydimethylsiloxane-air partition coefficients using headspace sorptive extraction. J Chromatogr A. 2007;1150(1-2):183–189.

[39] Williams MMR. The mathematics of diffusion. Ann Nucl Energy. 1977;4(4-5):205–6.

[40] Bartkow ME, Booij K, Kennedy KE, Müller JF, Hawker DW. Passive air sampling theory for semivolatile organic compounds. Chemosphere. 2005;60(2):170–6.

[41] Seethapathy S, Górecki T. Polydimethylsiloxane-based permeation passive air sampler. Part I: Calibration constants and their relation to retention indices of the analytes. J Chromatogr A.; 2011;1218(1):143–55.

[42] Seethapathy S, Górecki T. Polydimethylsiloxane-based permeation passive air sampler. Part II: Effect of temperature and humidity on the calibration constants. J Chromatogr A.; 2010;1217(50):7907–13.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

[43] Brown RH. Monitoring the ambient environment with diffusive samplers: Theory and Practical Considerations. J Environ Monit. 2000;2:1–9.

[44] Kallenbach M, Oh Y, Eilers EJ, Veit D, Baldwin IT, Schuman MC. A robust, simple, high-throughput technique for time-resolved plant volatile analysis in field experiments. Plant J. 2014;78(6):1060–72.

[45] Booij K, Smedes F, Van Weerlee EM. Spiking of performance reference compounds in low density polyethylene and silicone passive water samplers. Chemosphere. 2002;46(8):1157–61.

[46] Zabiegała B, Górecki T, Namieśnik J. Calibration of permeation passive samplers with silicone membranes based on physicochemical properties of the analytes. Anal Chem. 2003;75(13):3182–92.

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Chapter Three

Instrumentation

Contents

3.1 Gas chromatography

Gas chromatography (GC) is a separation technique which has become the laboratory workhorse for the separation and analysis of mixtures of volatile analytes. Analyses using this technique have reached an extremely high level of quality with several different combinations of column stationary phase chemistry, mobile phase carrier gas, column dimensions and detector being commercially available. The optimization of these and other instrument parameters such as the temperature program during a separation can solve a large proportion of separation problems routinely encountered in research and industry settings. However, when extremely complex samples are encountered which include large numbers of compounds with very similar properties it may not be possible to achieve acceptable resolution for all peaks in a single GC analysis. Such complex samples are often encountered in petrochemical [1], biological [2], forensic [3] and food analysis [4] applications. The separation of analytes in complex mixtures can be addressed by multidimensional

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separation techniques. This was first applied in the form of heart-cutting, GC-GC, separations. A process which subjects only selected fractions containing co-eluting analytes to a subsequent separation dimension [5]. Separations which subject all analytes eluting from the primary separation to a second separation without losing any resolution achieved in the first dimension are termed comprehensive techniques. The combination of two gas chromatography separations fulfilling these criteria gives rise to comprehensive two-dimensional gas chromatography or GC×GC [5].

3.1.1 Fundamental principles

The fundamental principles of performing separations by gas chromatography are based on two competing processes which take place during the course of a separation. These processes are that of chromatographic band separation and band broadening. The theory of gas chromatographic separations has historically been built on the theory of fractional distillation [6]. The efficiency of a chromatographic column is quantified by calculating the number of theoretical plates, N, in the column. Each individual plate corresponds to a hypothetical equilibrium of an analyte between the stationary and mobile phases during a separation. The number of theoretical plates in a column can be experimentally determined based on a calculation involving the isothermal retention time, T_R, and peak width standard deviation, σ, of a test analyte subjected to separation on the column (Eqn. 3.1).

$$N = ({}^{T_R}/_{\sigma})^2 \quad \ldots \text{Eqn. 3.1}$$

The height equivalent to a theoretical plate, $HETP$, relates the theoretical number of plates to the length of the column, H, (Eqn. 3.2).

$$HETP = {}^{H}/_{N} \quad \ldots \text{Eqn. 3.2}$$

The maximum number of theoretical plates and corresponding minimum plate height is desired to improve the performance of a separation. Analyte peak broadening during a separation is affected by a range of physical, kinetic and thermodynamic

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properties of a chromatographic separation. The rate theory model developed by van Deemter separates these parameters into independent mass transfer and diffusion process terms which have an effect on the optimization of separations performed on gas-liquid packed columns under isothermal conditions [7]. This theory was developed to suit open tubular columns with a liquid stationary phase by Golay [8]. The Golay equation (Eqn. 3.3) can be used to describe the relationship between the theoretical plate height and linear velocity during a separation.

$$HETP = \frac{B}{\bar{u}} + (C_S + C_M)\bar{u} \ldots \text{Eqn. 3.3}$$

Where; $\bar{u}$ is the average linear velocity of the mobile phase; *B* is a term describing the longitudinal diffusion; C_S and C_M describe mass transfer in the stationary and mobile phases respectively.

A typical plot describing the relationship between theoretical plate height and average linear flow velocity of the mobile phase has a hyperbolic form characterized by a steep descent, as a result of the first term in Eqn. 3.3, followed by a slow rise in plate height with increasing linear flow rate, the result of an increasing contribution from the second term in Eqn. 3.3 (Fig. 3.1).

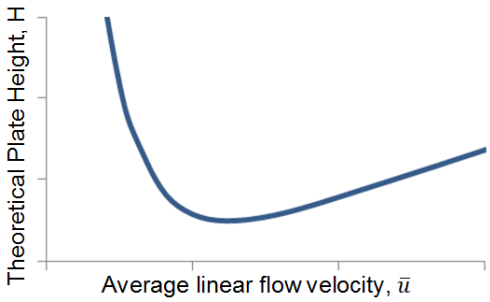

Figure 3.1) Characteristic plot describing the relationship between theoretical plate height and average linear flow velocity of the mobile phase in an open tubular column separation with a liquid stationary phase [9].

Optimized separation conditions seek to find a situation where the linear flow rate corresponds to the minimum plate height which occurs at the expense of analysis speed. Separation parameters such as the stationary phase thickness, the carrier

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gas used or temperature during the separation can be manipulated in order to optimise separations [9].

3.2 Comprehensive two dimensional gas chromatography

The introduction and development of comprehensive two-dimensional gas chromatography (GC×GC), which is a technique that subjects an entire sample to separation on two chromatographic columns simultaneously, has provided a solution for the separation of very complex mixtures. The increased peak capacity obtained using separations in two dimensions has shown added benefits, when compared to traditional single dimension separations, for complex mixtures including those of biological origins such as mouse liver extracts [10], bacterial volatiles [11] and exhaled human breath [12].

3.2.1 Instrument

Comprehensive two-dimensional chromatography (GC×GC) involves the coupling of two capillary columns of different stationary phase chemistry. All the analytes in a sample are subject to separation on both columns giving rise to the term “comprehensive” as opposed to heart-cutting which is a technique that involves the selection, collection and reinjection of analyte fractions of interest from the primary column into the secondary column [5]. Coupling of two capillary columns in GC×GC instruments is done through a modulator which systematically collects and injects fractions of the mobile phase exiting the primary column into the secondary column. A general schematic of a GC×GC instrument is shown in Figure 3.2.

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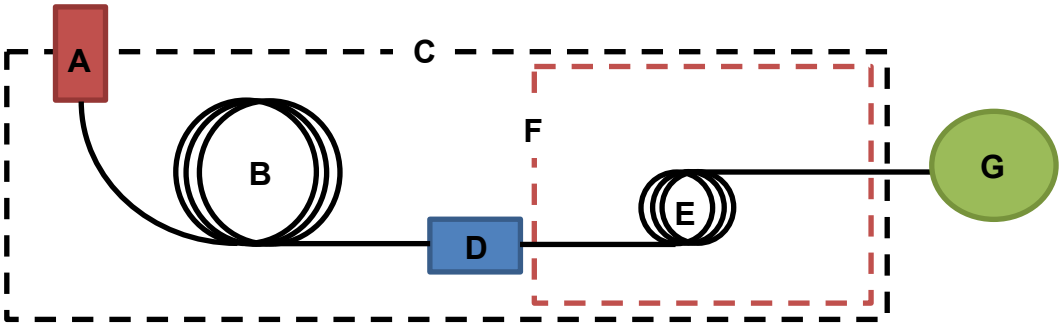

Figure 3.2) Schematic of a GC×GC instrument. The instrument consists of an inlet **(A)**, primary column **(B)**, oven **(C)**, modulator **(D)**, secondary column **(E)**, secondary oven **(F)** and a detector **(G)** [13].

3.2.2 Comprehensive separations and orthogonality

Column selection is an important aspect for consideration when undertaking the separation of complex mixtures by GC×GC. Generally, separations make use of a long (15-60 m) first dimension column (D^1) and a much shorter (0.5-2 m) and narrower (0.1-0.25 mm ID), second dimension column (D^2) [4]. The much shorter and narrower second dimension column is necessary in order to allow rapid separations in the second dimension between injections by the modulator. The compromise which occurs as a result of this requirement is that separations in the second dimension are often not performed under optimal flow rates (Fig. 3.1). The short time available for these separations results in them taking place under near isothermal conditions [14]. While not essential it is common to employ a non-polar primary column coupled to a polar or functionalized secondary column [4]. The advantage to this setup is that structured separations, where peaks are grouped by class or chemical properties, may be attained [4]. For orthogonality to be achieved the mechanisms governing the separation of analytes on each of the columns employed should be the result of different physiochemical interactions between the analytes and the column stationary phase. In a completely non-polar primary column the separation of analytes is related to the boiling point of each analyte while

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separations on columns containing functional groups result from the addition of transient interactions such as dipole or π-bonding interactions between individual analytes and functional groups on the column stationary phase [4].

3.2.3 Modulation

The mechanical process by which analyte bands, which have been separated on the first column, are introduced onto the second column is achieved by the continuous systematic collection of small fractions of the mobile phase leaving the primary column. The small fractions are then subject to refocusing and reinjection onto the secondary column within set time intervals termed the modulation period [15]. These periods are typically in the order of 3-8 seconds depending on the peak widths expected during the separation on the first dimension column [16]. This process is termed modulation and is performed by a modulator which may come in several different designs [4]. The instrumentation used in this study made use of a popular modulator design involving a quad-jet system. The modulator consists of four jets (two hot and two cold) which act in sequence to cryogenically trap small fractions of mobile phase before thermally desorbing and releasing the fraction onto the secondary column. The process is summarised in Figure 3.3.

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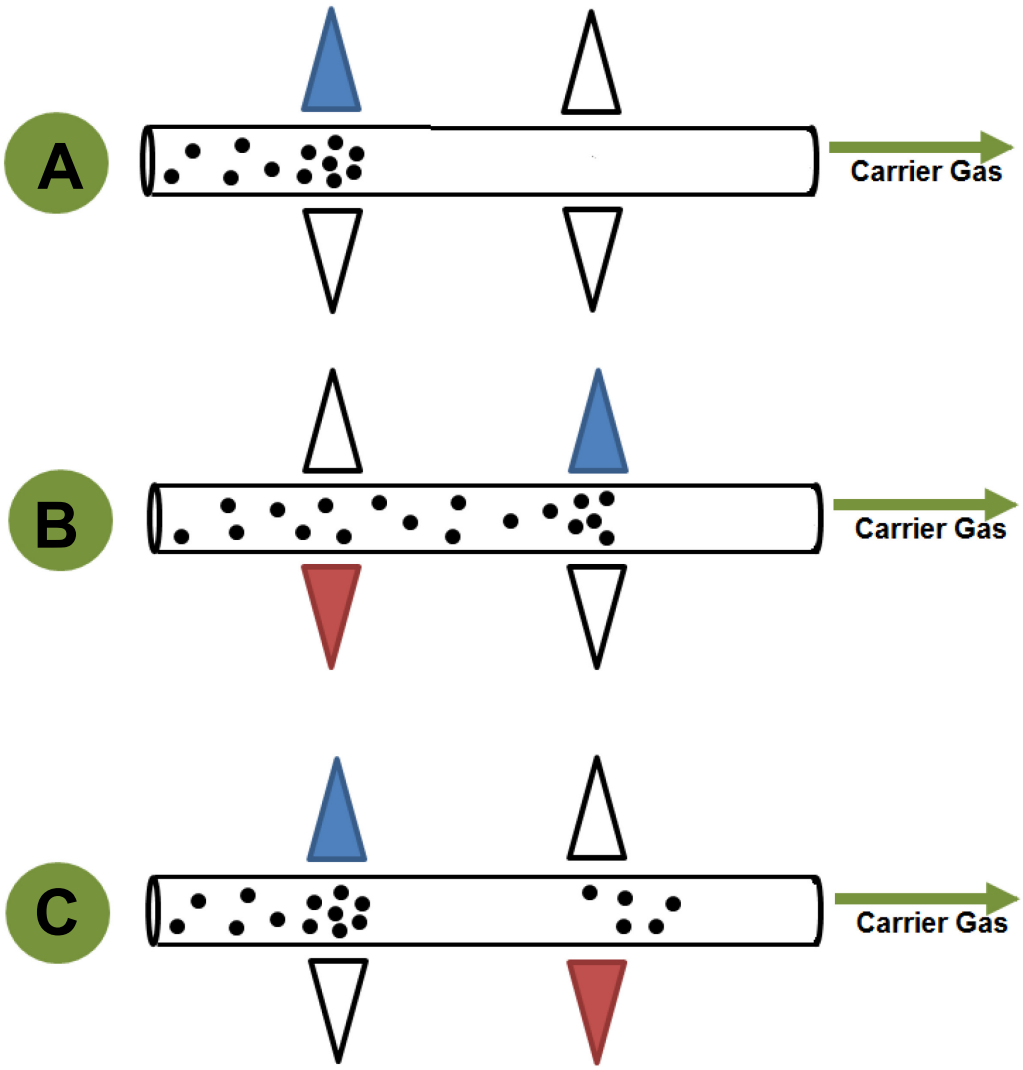

Figure 3.3) Schematic depicting the process of modulation using a quad-jet system. Analytes are first trapped by the first cold jet **(A)**, heating by the first hot jet then releases analytes which are captured by the second cold jet **(B)**, analytes trapped by the second cold jet are released while later eluting analytes are trapped by the first cold jet **(C)**. The process is then repeated [17].

The amount of time taken for each modulation cycle is an important parameter to be considered while setting up a GC×GC method. Setting a long collection period will result in a loss of the resolution already gained from separation on the primary column as several close eluting peaks may be collected together. In contrast, an extremely rapid sampling period will not allow sufficient time for peaks which have already entered the secondary column to elute before the next injection takes place. This occurrence, “termed wrap around” has the potential to decrease the

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separation's resolution due to the overlap of components of two separate injections in the secondary column [18]. Thus it is necessary to select an optimal modulation period which slices each first dimension peak several times but is also longer than the time required for analytes to elute from the secondary column. Theoretical investigation of the effect of modulation time on the total peak area and the accuracy with which the primary retention time of a peak can be calculated indicates that each peak should be sampled at least four times in order to retain the relevant information. However, Gaussian peaks corrupted by noise in the signal may require a higher sampling frequency to better reconstruct the original peak signal [16]. The sampling rate during modulation can be described by the modulation ratio, M_R, which is defined as the ratio of four times the standard deviation of a peak divided by the modulation period [19]. When successfully applied the modulation process produces a series of sharp peaks which are received by the detector. The sum of these peak areas gives the total area of the original peak eluting from the first dimension. The modulation process benefits the separation not only by facilitating the orthogonal separation and resulting increase in separation space but also gives rise to increased sensitivity of the technique by generating high intensity narrow peaks at the detector [13].

3.2.4 Detectors

Very fast detection systems with small internal volume are required for GC×GC separations due to the narrow analyte peaks which elute from the second dimension column [13]. Non-selective detection systems such as flame ionization detectors (FID) and time-of-flight mass spectrometers (ToF-MS) as well as several element selective systems have been successfully coupled to GC×GC instrumentation [5]. However, time-of-flight mass spectrometry has become the gold standard for metabolic research involving GC×GC separations [2]. Several qualities make these detectors well suited for non-targeted investigations of biological systems. This includes rapid signal collection (5-30 kHz) simultaneously across all mass channels, as well as a wide mass range which encompasses a broad range of organic

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molecules [15]. In addition, non-skewed fragmentation patterns acquired using these detection systems can provide an additional separation dimension through the process of signal deconvolution and by comparison to published library spectra can aid in the tentative identification of unknown compounds present in a sample [2]. Further discussion of the steps involved in the processing of data collected by GC×GC ToF-MS analyses in a metabolomics context is provided in Chapter Four.

3.3 References

[1] Potgieter H, Bekker R, Govender A, Rohwer E. Two-dimensional gas chromatography-online hydrogenation for improved characterization of petrochemical samples. J Chromatogr A.; 2016;1445:118–125.

[2] Almstetter MF, Oefner PJ, Dettmer K. Comprehensive two-dimensional gas chromatography in metabolomics. Anal Bioanal Chem. 2012;402(6):1993–2013.

[3] Sampat A, Lopatka M, Sjerps M, Vivo-truyols G. Forensic potential of comprehensive two-dimensional gas chromatography. TRaC - Trends Anal Chem.; 2016;80:345–363.

[4] Adahchour M, Beens J, Brinkman UAT. Recent developments in the application of comprehensive two-dimensional gas chromatography. J Chromatogr A.; 2008;1186:67–108.

[5] Marriott PJ, Chin S, Maikhunthod B, Schmarr H, Bieri S. Multidimensional gas chromatography. TRaC - Trends Anal Chem.; 2012;34:1–21.

[6] Martin AJP, Synge RLM. A new form of chromatogram employing two liquid phases. Biochem J. 1941;35(12):1358–68.

[7] van Deemter JJ, Zuiderweg FJ, Klinkenberg A. Longitudinal diffusion and resistance to mass transfer as causes of nonideality in chromatography. Chem Engng Sci. 1956;5:271-289.

[8] Golay M. Theory of chromatography in open and coated tubular columns with round and rectangular cross-sections. In: Desty DH (ed) Gas chromatography

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

(Amsterdam symposium proceedings 1958). London: Butterworths; 1958. p. 139–143.

[9] Dettmer-Wilde K, Engewald W. Practical Gas Chromatography. London:Springer; 2014. p. 21-57.

[10] Koek MM, van der Kloet FM, Kleemann R, Kooistra T, Verheij ER, Hankemeier T. Semi-automated non-target processing in GC × GC-MS metabolomics analysis: Applicability for biomedical studies. Metabolomics. 2011;7(1):1–14.

[11] Caldeira M, Perestrelo R, Barros AS, Bilelo MJ, Morête A, Câmara JS, et al. Allergic asthma exhaled breath metabolome : A challenge for comprehensive two-dimensional gas chromatography. J Chromatogr A.; 2012;1254:87–97.

[12] Bean HD, Dimandja JD, Hill JE. Bacterial volatile discovery using solid phase microextraction and comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry. J Chromatogr B.; 2012;901:41-46.

[13] Dallüge J, Beens J, Brinkman UAT. Comprehensive two-dimensional gas chromatography: a powerful and versatile analytical tool. J Chromatogr A. 2003;1000(1-2):69–108.

[14] Mühlen C Von, Marriott PJ. Retention indices in comprehensive two-dimensional gas chromatography. Anal Bioanal Chem. 2011;401:2351–2360.

[15] Mondello L, Tranchida PQ, Dugo P, Dugo G, Comprehensive two-dimensional gas chromatography-mass spectrometry: A review. Mass Spectrometry Reviews.; 2008;27:101–24.

[16] Seeley J V. Theoretical study of incomplete sampling of the first dimension in comprehensive two-dimensional chromatography. J Chromatogr A.; 2002;962:21–27.

[17] Anonymous. Pegasus HT Hardware Instruction Manual. LECO Corperation. 2011. p. 5-25.

[18] Harynuk J, Górecki T, Campbell C. On the Interpretation of GC×GC Data. LC-GC North America. 2002;20(9):876-892.

[19] Khummueng W, Harynuk J, Marriott PJ. Modulation ratio in comprehensive two-dimensional gas chromatography. Anal Chem. 2006;78(13):4578–87.

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Chapter Four

Chemometrics and metabolomics

Contents

4.1 Introduction

In recent decades growth of the "*omics*" field has been fuelled by advances in laboratory instrumentation and methodologies [1]. This field encompasses the targeted and non-targeted analysis of components of biological systems and

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includes the study of several areas that reflect the physiological status of a cell or organism. Examples include proteomics, genomics and metabolomics. Research is performed with the goal of identifying metabolites which are linked to a specific pathological or physiological process [2]. Metabolomics is the study of low molecular weight (<1500 Da) organic compounds which form part of the various catabolic and anabolic biosynthetic pathways in a cell. Due to the broad range of cellular metabolites encountered several sub-fields of metabolomics exist based on the metabolite class or origin. Examples include “Lipidomics” [2], which is the study of the lipid profile of a biological system, or “Volatolomics” [3], the study of VOCs originating from a biological system. Non-targeted analyses involve the simultaneous sampling of a wide range of components making up a biological system. The non-targeted analysis of a biological system provides an instantaneous picture of a broad range of metabolites present in a sample. These metabolome “snapshots” may be accumulated and compared in order to monitor changes in a system under certain conditions [4]. This approach may be used without prior knowledge of the components of the system in order to guide subsequent targeted analyses which are generally more absolute with regards to quantification. Chemometrics is defined as the science of relating measurements performed on a system or chemical process to the current state of the system by application of mathematical or statistical procedures [5]. This process, in the context of metabolomics research making use of GC×GC-ToFMS, includes pre-processing of the raw mass spectrometry (MS) instrument signal, initial data processing, peak alignment, data validation and feature detection. It is important to take each step of the data processing workflow into account in order to ensure that valid results are obtained. A summary of the non-targeted metabolomics workflow involving GC×GC-ToFMS is given in Figure 4.1. Non-targeted analyses require a similar systematic approach to validation as is expected for targeted analyses [6]. The remainder of this chapter includes discussion of the factors affecting processing and non-targeted analysis of GC×GC-ToFMS data sets post sample acquisition.

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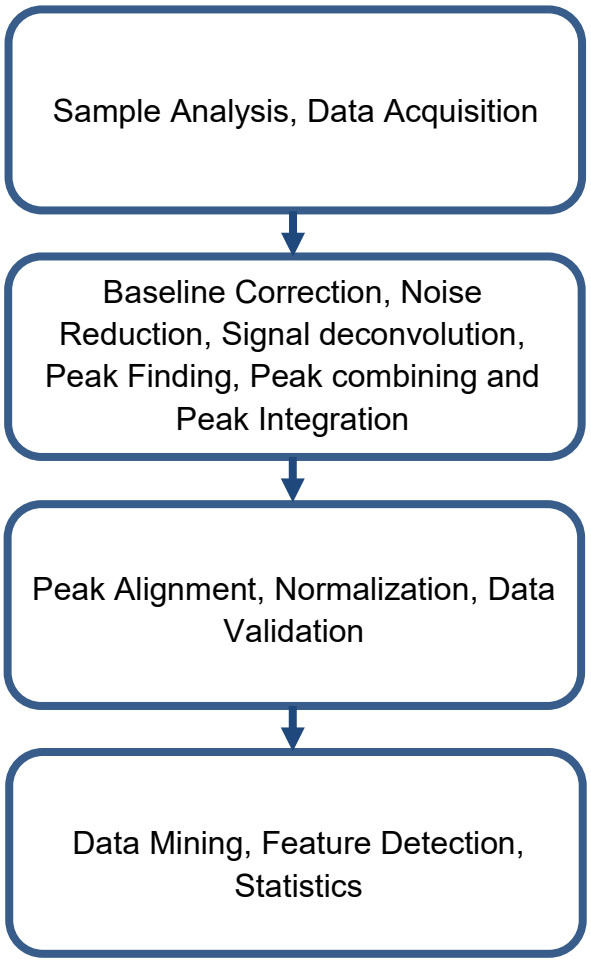

Figure 4.1) Non-targeted data processing workflow post sample acquisition for metabolomics research involving comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-ToFMS).

4.2 Initial GC×GC-ToFMS data processing

The processing of the signal recorded at the detector during a GC×GC-ToF separation includes baseline correction, noise reduction, signal deconvolution, peak finding, peak recombination and peak integration. This culminates in the generation of a three-dimensional contour plot of the separation space and a peak table where each entry corresponds to a chromatographic peak recorded by the software along with, among others, corresponding retention times, peak intensities and signal-to-noise ratios.

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4.2.1 Visualizing comprehensive separations in three dimensions

In Section 3.2.3 the process of modulation by which analytes are transferred from the primary column to the secondary column in a comprehensive GC×GC separation was described. This process results in a continuous series of short chromatographic separations being recorded at the detector. Side by side rearrangement of the continuous signal at the detector based on the modulation period is performed in order to construct a chromatogram in three dimensions (Fig 4.2) [7].

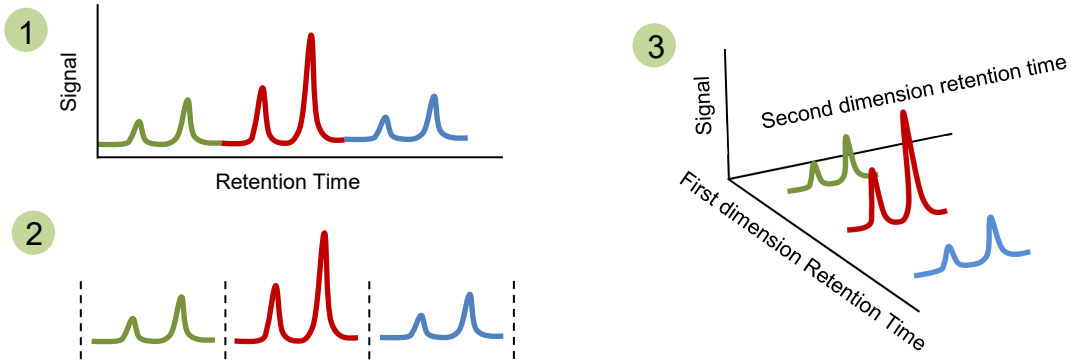

Figure 4.2) Construction of a three-dimensional GC×GC Chromatogram. **(1)** A continuous signal is recorded at the detector. **(2)** The signal is segmented based on the modulation period. **(3)** Segments are arranged side by side to generate a contour plot.

The narrow sub-peaks produced during the modulation process give the added advantage of increasing the sensitivity of the technique. However, the sub-peaks in different modulation periods which correspond to the same analyte peak need to be correctly recombined in order to obtain an accurate representation of the original peak. The recombination of sub-peaks is dependent on the matching of mass spectra from each sub-peak. Factors such as detector saturation by high intensity peaks may result in a poor match for sub-peaks and failure to combine all sub-peaks of the original analyte peak [8].

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4.2.2 Mass spectrum signal deconvolution

The use of a mass spectrometer hyphenated to a comprehensive separation has the advantage of providing an additional dimension of separation to the chromatographic separations. Monitoring the rise and fall of individual masses in a series of mass spectra at extremely high repetition rates allows software algorithms to identify and separate the mass spectra corresponding to overlapping analyte peaks. Vendor provided software is able to do this provided the analytes do not perfectly co-elute and can separate analytes with peak apexes differing by as little as 0.05s. The results allow the software to reconstruct the chromatogram providing peak information for many overlapping analytes. Time-of-Flight mass spectrometry (ToFMS) facilitates the deconvolution process by collecting a range of masses simultaneously. Deconvolution of mass spectra from a scanning detector would be inaccurate due to the fact that the rise and fall of individual masses are skewed across time [9]. Factors which affect the deconvolution process include the uniqueness of the masses in the mass spectra of overlapping peaks [10]. For this reason, spectrum deconvolution is not well suited for the separation of peaks corresponding to co-eluting stereoisomers.

4.3 Chromatographic peak alignment

The next step in the metabolomics workflow, after initial data processing, is the alignment of the signal peaks detected across multiple samples. Subtle variations in temperature, pressure and flow rate between runs can cause retention time variations for the same analyte peak in different samples. The alignment of analyte peaks in different samples forms part of the process to consolidate the data into a usable format for subsequent statistical analysis and data mining procedures. Numerous approaches have been used to perform this alignment [11]. Koek *et al*. used the calibration feature in ChromaToF software to perform peak table alignment of a set of mouse liver samples which had been subject to GC×GC-MS analysis. This involved the inclusion of all the analytes which were detected in a pooled quality

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control sample of the mouse livers in a calibration table. All other samples were then processed against this calibration table in order to align the peaks across all the chromatograms collected [12]. This approach, however, proved to be very time consuming and requires the sampled material to be in a liquid form which can be mixed to obtain pooled samples. Other methods which have been developed to address the challenge of accurate and repeatable peak alignment have followed two main approaches. These are profile based alignment of chromatograms and peak table based alignment.

4.3.1 Profile based alignment

Hantao *et al*. used a correlation optimized warping (COW) algorithm to align total ion chromatograms collected during a GC×GC—qMS separation of plant metabolites [13]. Versions of this approach were originally developed for single column GC separation data sets [14] and were later adapted for GC×GC peak alignment [15]. The algorithm is used to optimize the correlation between characteristic vectors in local regions of two chromatograms by stretching and compressing regions of the time axes. The process requires a reference chromatogram to be selected and is most applicable to homogeneous samples with similar numbers and distributions of chromatographic peaks. Homogeneous in this sense referring to the conditions under which experimental data was acquired. This approach is most applicable when a detection system which only provides an intensity signal, such as a Flame Ionization Detector (FID), is used. Its application to MS data sets does not take into account mass spectrum data of each peak during alignment and is therefore suited for alignment of TICs of homogeneous samples [16]. The alignment of non-homogenous samples has, however, been performed by making use of selected ion count (SICs) chromatograms corresponding to well resolved derivatized analytes [4].

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4.3.2 Peak table based alignment

Recent years have seen the development of several software platforms with the aim of aligning data sets generated by two-dimensional separations in combination with MS detectors. These platforms make use of vendor provided software to perform initial data processing such as baseline correction, deconvolution and peak finding. The resulting peak tables are then exported and aligned by the external software. These platforms have the added advantage of taking both the retention times and mass spectral information into account during peak alignment thus lowering the number of false alignments. Many open source platforms have been developed in this field and are compatible with data generated by LECO ChromaToF software for GC×GC-ToFMS analyses. Examples include: MSort [17], DISCO [18], INCA [19], mSPA [20], SWPA [21], Guinau [22], MetPP [23]. The large number of publications in this field represents the evolution of different alignment and data processing strategies [11]. The use of ChromaToF software for initial data analysis and peak finding operations excludes these software platforms from the processes of unique ion selection for each individual peak in each chromatogram to be aligned. The selection of the most unique quantitative ion for each entry in the peak table is performed automatically by the ChromaToF software during the data processing. The resolution and noise in each particular chromatogram affect the selection of the unique ion for each analyte peak [10]. Therefore, the same unique ion corresponding to a specific peak may not always be selected in every chromatogram being aligned and quantitative applications of these software platforms for large numbers of samples in a fast and automated data processing protocol is limited to making use of the areas of TIC signals. ChromaToF Statistical Compare, as the instrument vendor provided alignment software, has access to the data processing and unique ion selection steps performed during initial signal processing by the ChromatToF software used to control the instrument. Quantitative work using this software benefits from the availability of peak area data corresponding to the most unique ion of aligned peaks across samples [10].

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4.3.3 ChromaToF Statistical Compare

ChromaToF Statistical Compare is a commercially available software tool which is able to perform GC×GC chromatogram peak alignment based on a peak table based approach. The alignment is based not only on the retention times of peaks but also a mass spectral similarity parameter which employs an algorithm similar to a mass fragment library search. Factors affecting the alignment of peaks include the chromatographic resolution, peak intensity, resolution and shape as well as the uniqueness of analyte masses [10].

Alignment of chromatograms by ChromaToF Statistical Compare software follows a sequence of eight steps [24]:

1) First, the baseline of each peak's unique mass is defined by the software.

2) Peaks above the baseline are identified.

3) The spectra of peaks within the retention time window set by the user are compared to each other using a similar algorithm to the library search function. For this reason, a similarity threshold must be specified. The mass threshold set removes masses with relative abundance below this limit from the calculation. Values for these parameters must be selected so as to mitigate the effects on selectivity caused by peak intensity.

4) After peaks from different samples are grouped a reference peak is determined. This is the peak with the highest purity. The peak purity represents how well the deconvoluted data fits the actual data at the peak apex. Peak purity is negatively affected by peak overlap and positively affected by the increasing uniqueness of masses in overlapping mass spectra.

5) In order to minimise missing values, the software performs a second peak finding operation. This is set at a lower S/N threshold than the initial peak finding operation but is performed in the same sequence of events (Steps 2 and 3).

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6) A quantification mass is selected from each group of aligned peaks. The most common unique mass from the group is used for this purpose. Peak area and height calculations are performed using this mass.

7) Peaks can be excluded from the final results based on the number of samples which contain them.

8) A mass library search is performed using the mass spectrum of the reference peak determined in step 4.

4.4 Method validation and non-targeted metabolomics

Method validation is a key step in the development and implementation of any new laboratory analysis protocol. Validation is required to ensure that the procedures for sample collection and analysis which are followed give reliable results under the conditions applied. Variability of this type is termed the technical variability of the method and is especially relevant for procedures aimed at metabolomics analyses due to the inherently large amounts of variability within biological systems [25]. The total variability of a measurement and resulting significance of results obtained are affected by both sources of variation. The process of validation aims to identify sources of systematic errors associated with the experimental design and equipment used for analysis. Parameters which are considered during analytical method validation are given in Table 4.1. Assessment of a method based on these parameters is intended to confirm a method is “fit for purpose” whereby results obtained match the criteria of the end-user [26].

The non-targeted approach used in metabolomics research aims to collect as many analyte signals as possible from samples which relate to different situations of the sample under study. Comparison of analyte signals between different sample situations, and not the absolute quantification of analytes, is most relevant to this approach. For this reason, method validation in this field is focused on ensuring that changes in analyte signals recorded accurately reflect changes in the analyte concentrations between samples [27].

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Table 4.1) Parameters used for analytical method validation [27].

Parameter	Description
Accuracy	The accuracy of a measurement is defined as the closeness of fit between a measurement and an accepted reference value.
Precision	The precision of a series of measurements is defined as the closeness of agreement between the values determined under the same conditions. The precision can be considered in the short term (intra-analyses) or long term (inter-analyses).
Specificity	The ability of a method to perform measurements on a specific analyte without interference from other matrix components in the sample being analyzed.
Limit of detection (LOD)	The lowest amount of an analyte which may be qualitatively identified accurately during an analysis.
Limit of quantification (LOQ)	The lowest amount of an analyte which may be quantitatively measured while maintaining suitable levels of accuracy and precision.
Linear range	The range of analyte concentrations over which a method will yield data which maintains suitable levels of accuracy and precision.
Ruggedness	The ability of a method to resist changes in results when minor changes in experimental conditions are experienced.

4.4.1 Instrument quality control

Non-targeted metabolomics studies involve high throughput analyses of large numbers of samples and replicates in order to generate sufficient data to produce statistically significant results. Repeatability of measurement is an important parameter during the course of such analyses. The methodology employed should, therefore, include some quality control aspects to assess gradual change in instrument performance. These changes may include changes in the sensitivity of MS detectors over time. For this reason, many researchers have included the randomization of sample order as part of their methodology [27]. Inclusion of quality control (QC) samples during the course of sample analysis is performed in order to monitor changes in detector responses. Metabolomics studies involving the analysis of bio-fluids or liquid samples generally make use of pooled samples, composed of a small volume of each test sample, as QC samples [27]. An alternative approach involves spiking all test samples with one or several internal standards. Analysing a

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series of diluted QC samples is also used to assess an instrument's linear response range [27].

4.4.2 Replicates and validation

It has been shown that variability in quantitative measurements performed on analytes originating from genetically identical plants grown under identical circumstances can exceed variability due to analytical repeatability by three to four fold [28]. This example highlights one of the main challenges faced by quantitative metabolomics studies performed on biological systems. Validation of the results obtained from a non-targeted analysis is therefore essential to ensure that the differences detected between different biological samples are the result of biological processes and not instrument or methodology factors. The measurement precision is, therefore, an important parameter in non-targeted metabolomics. Analysis of replicate samples is required to investigate this parameter. Validation involves the calculation of basic summary statistics. This includes the calculation of means, standard deviations, variances and standard errors of measurements collected for replicate analyses. In a non-targeted metabolomics context, it has been proposed that measurements which show Relative Standard Deviations (RSDs) for the technical variability of greater than 30% are not considered good candidates for biomarker discovery [27].

4.4.3 Data normalization

Normalization is an important aspect of metabolomics research whenever any form of quantitative insight is desired. Normalization is used to correct for errors introduced due to small differences in volume of individual samples which are analyzed. Other sources of error may include instrument performance such as ionization or detector efficiency when using MS. Sample normalization can be

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performed pre- or post-data acquisition. A range of methods exist for signal normalization but all aim to adjust the signals acquired from different samples such that they are comparable to each other [29].

Common normalization strategies used in metabolomics studies include normalization to a single measured analyte. This may be an internal standard added to the sample prior to the analysis or it may be an endogenous metabolite which is known to be present at consistent levels in the sample being analyzed. Endogenous metabolites in urine, such as creatinine, have been used to compensate for differences in sample concentrations due to biological reasons [29]. Another strategy involving the regular analysis of reference samples has been shown to efficiently correct for batch-to-batch variation in acquired signals especially when long time intervals between analyses are involved [30]. An alternative strategy which can be applied post data acquisition makes use of the total useful mass spectrometry signal acquired during the analysis of an individual sample [31]. This value is then used as a denominator in the scaling of all individual analyte signals. All of the normalization methods which have been mentioned are applied uniformly as a scaling factor to analyte signals in a respective sample. In a non-targeted investigation this does not take into consideration the chemical class diversity of the analytes in the sample. Different chemical classes may be more susceptible to certain sources of error than others. The application of global normalization procedures which are applied to a broad range of analytes in a single sample should therefore be validated [32].

4.5 GC×GC-MS Data mining

The aim of non-targeted metabolomics analyses is to compare instantaneous “snapshots” of the metabolome under investigation in order to identify differences which may reflect changes in metabolic processes. The visualization of the large amounts of data generated by a single experiment is the final step in the metabolomics workflow. The general metabolomics data mining problem involves comparing a large number of measurements from biological samples in order to identify differences while taking into account the biological variability within the

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system being sampled. Processing of the large volumes of data generated by metabolomics experiments to obtain useful insight into the systems under study is a major challenge. Many univariate and multivariate statistical techniques have been applied in order to address this challenge and are usually transferable between *omics* fields. The use and applications of many statistical methodologies have been the subject of recent review articles [11, 33].

4.5.1 Comparison of GC×GC chromatograms

Straight forward visual comparison of chromatograms generated during comprehensive gas chromatograms is not practical due to the large number of peaks and the small variations which may be present. Simple comparisons of chromatograms involving the averaging and subtracting of TIC peak areas have been used to identify analyte peaks which differ between two GC×GC-ToFMS samples. This approach fails to take into account variability in the analysis and may place emphasis on medium to high differences between samples [34]. Parametric statistics, such as the Student's t-test, have been applied to peak table data sets [34]. However, statistical power in the case of small sample size ($n<5$) studies is often a problem faced by exploratory metabolomics research. In addition, statistical methods which include consideration of variation in measurement between sample replicates are preferable for valid feature detection. The Fisher Ratio (FR), which is an analysis of variance (ANOVA) type technique, is capable of addressing these issues and may be used in an exploratory context to compare data sets which contain relatively few samples with replicates [35]. Increasing the sample size is, however, a prerequisite to identify small differences between data sets.

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4.5.2 Fisher ratios

Feature detection using the Fisher ratio method makes use of the ratios of the between-sample variance (Eqn. 4.2) and within-sample variance (Eqn. 4.3) which are compared for every measurement across all the samples (Eqn. 4.1). Large ratios are indicative of differences between samples. The calculated Fisher ratios are used for feature detection after a suitable threshold value has been established.

$$Fisher\ ratio, F = \frac{\sigma^2_{between-sample}}{\sigma^2_{within-sample}} \quad \text{...Eqn. 4.1}$$

$$\sigma^2_{between-sample} = \frac{\sum_{i=1}^{1}(\bar{x}_i - \bar{x})^2 n_i}{(k-1)} \quad \text{... Eqn. 4.2}$$

$$\sigma^2_{within-sample} = \frac{\left(\sum_{i=1}^{i}\sum_{j=1}^{j}(x_{ij} - \bar{x})^2\right) - \left(\sum_{i=1}^{i}(\bar{x}_i - \bar{x})^2 n_i\right)}{(n_T - k)} \quad \text{... Eqn. 4.3}$$

For each of n_T measurements over k samples, the total number of replicate measurements in the ith sample, is given by n_i and the mean value of the measurements is given by $\bar{x}_i$. The overall mean value across all samples is given by $\bar{x}$. The subscript j indexes all replicate measurements across all samples.

Fisher ratios provide a fast method to identify measurements in a large data set which vary between samples. Selection of an appropriate threshold value is dependent on the number of samples included in the comparison [36]. Feature detection using this method is affected by the number of replicates collected for each sample as well as the reproducibility of sample preparation and analysis.

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4.5.3 Multivariate methods

Multivariate statistics may be applied to metabolomics data in order to simplify large volumes of data. Two broad categories of multivariate method exist namely, unsupervised and supervised methods [11].

Unsupervised methods of multivariate data analysis, also referred to as descriptive models, are generally exploratory in nature. Methods which fit into this category make no initial assumptions about the nature of the data and may be used to reveal patterns or evaluate the data for the presence of expected trends [11]. A commonly applied multivariate statistic used in Volatolomics research which falls into this category is the method of principal components analysis (PCA) [37]. Principal components analysis is often used in combination with univariate feature detection methods and involves the reduction of the dimensionality of the data being processed. The reduction in dimensionality makes visualization of the data more convenient and visual classification of groups of data points is possible [38].

Supervised methods, which are also referred to as predictive models, attempt to classify new data based on a prior model data set [11]. Commonly applied techniques include partial least square discriminant analysis (PLS-DA) [39] and linear discriminant analysis (LDA) [11]. Partial least squares discriminant analysis relates a data set to a response vector by a linear regression model. Applications include the identification of variables which may be used to discriminate between classes as well as to predict class affiliation [40]. Linear discriminant analysis reduces the dimensionality of multivariate data while preserving the interclass separation [11].

4.6 Conclusions

Non-targeted metabolomics studies have the great potential to aid in exploratory investigations of biological systems. The differences in samples analyzed have the potential to lead to the discovery of new biomarkers related to specific pathogenic or

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physiological processes. Comprehensive gas chromatography coupled with time-of-flight mass spectrometry and associated signal deconvolution is an extremely powerful technique which is well suited to the collection and non-targeted analysis of complex biological samples. However, it is important to ensure appropriate considerations have been included in the experimental design and post-analysis data treatment in order to ensure the validity of results obtained.

4.7 References

[1] Viant MR, Sommer U. Mass spectrometry based environmental metabolomics: A primer and review. Metabolomics. 2013;9(SUPPL.1):144–158.

[2] Bruggeman FJ, Westerhoff H V. The nature of systems biology. Trends Microbiol. 2007;15(1):45–50.

[3] Broza YY, Zuri L, Haick H. Combined Volatolomics for Monitoring of Human Body Chemistry. Sci Rep. 2015;4(1):4611.

[4] Almstetter MF, Oefner PJ, Dettmer K. Comprehensive two-dimensional gas chromatography in metabolomics. Anal Bioanal Chem. 2012;402(6):1993–2013.

[5] Gemperline P. Practical guide to chemometrics. 2nd ed. Boca Raton : CRC/Taylor & Francis; 2006.

[6] González O, Blanco ME, Iriarte G, Bartolomé L, Maguregui MI, Alonso RM. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. J Chromatogr A. 2014;1353:10–27.

[7] Harynuk J, Górecki T. On the interpretation of GC×GC data. LC-GC North Am. 1999;20(9):247–60.

[8] Bean HD, Hill JE, Dimandja JMD. Improving the quality of biomarker candidates in untargeted metabolomics via peak table-based alignment of comprehensive two-dimensional gas chromatography-mass spectrometry data. J Chromatogr A. 2015;1394:111–117.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

[9] Anonymous. Pegasus HT Hardware Instruction Manual. LECO Corporation. 2011.

[10] Anonymous. ChromaTOF Software Instruction Manual. LECO Corporation. 2012.

[11] Lubes G, Goodarzi M. Analysis of Volatile Compounds by Advanced Analytical Techniques and Multivariate Chemometrics. Chem Rev. 2017;117:6399-6422.

[12] Koek MM, van der Kloet FM, Kleemann R, Kooistra T, Verheij ER, Hankemeier T. Semi-automated non-target processing in GC × GC-MS metabolomics analysis: Applicability for biomedical studies. Metabolomics. 2011;7(1):1–14.

[13] Hantao LW, Aleme HG, Passador MM, Furtado EL, Ribeiro FA de L, Poppi RJ, et al. Determination of disease biomarkers in Eucalyptus by comprehensive two-dimensional gas chromatography and multivariate data analysis. J Chromatogr A. 2013;1279:86–91.

[14] Johnson KJ, Wright BW, Jarman KH, Synovec RE. High-speed peak matching algorithm for retention time alignment of gas chromatographic data for chemometric analysis. J Chromatogr A. 2003;996(1-2):141–55.

[15] Zhang D, Huang X, Regnier FE, Zhang M. Two-dimensional correlation optimized warping algorithm for aligning GCxGC-MS data. Anal Chem. 2008;80(8):2664–71.

[16] Sinha AE, Prazen BJ, Synovec RE. Trends in chemometric analysis of comprehensive two-dimensional separations. Anal Bioanal Chem. 2004;378(8):1948–1951.

[17] Oh C, Huang X, Regnier FE, Buck C, Zhang X. Comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry peak sorting algorithm. J Chromatogr A. 2008;1179(2):205–15.

[18] Bing Wang, Aiqin Fang, John Heim, Bogdan Bogdanov, Scott Pugh ML, Zhang and X. DISCO: Distance and Spectrum Correlation Optimization Alignment for Two Dimensional Gas Chromatography Time-of- Flight Mass Spectrometry-based Metabolomics. 2011;82(12):5069–81.

[19] Almstetter MF, Appel IJ, Gruber MA, Lottaz C, Timischl B, Spang R, et al. Integrative normalization and comparative analysis for metabolic fingerprinting by comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry. Anal Chem. 2009;81(14):5731–9.

[20] Kim S, Fang A, Wang B, Jeong J, Zhang X. An optimal peak alignment for comprehensive two-dimensional gas chromatography mass spectrometry using mixture similarity measure. Bioinformatics. 2011;27(12):1660–1666.

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

[21] Kim S, Koo I, Fang A, Zhang X. Smith-Waterman Peak Alignment for Comprehensive Two-dimensional Gas Chromatography Mass Spectrometry. BMC Bioinformatics. 2011;12:235.

[22] Castillo S, Mattila I, Miettinen J, Orešic M, Hyötyläinen T. Data analysis tool for comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry. Anal Chem. 2011;83(8):3058–3067.

[23] Wei X, Shi X, Koo I, Kim S, Schmidt RH, Arteel GE, et al. MetPP: A computational platform for comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry-based metabolomics. Bioinformatics. 2013;29(14):1786–92.

[24] Anonymous. Statistical Compare User's Guide v1.4. LECO Corporation. 2013.

[25] Noack S, Wiechert W. Quantitative metabolomics: A phantom? Trends Biotechnol. 2014;32(5):238–244.

[26] Thompson M, Ellison SLR, Wood R. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). Pure Appl Chem. 2002;74(5):835–55.

[27] Naz S, Vallejo M, García A, Barbas C. Method validation strategies involved in non-targeted metabolomics. J Chromatogr A. 2014;1353:99–105.

[28] Fiehn O, Kopka J, Dörmann P, Altmann T, Trethewey RN, Willmitzer L. Metabolite profiling for plant functional genomics. Nat Biotechnol. 2000;18(11):1157–1161.

[29] Wu Y, Li L. Sample normalization methods in quantitative metabolomics. J Chromatogr A. 2016;1430:80–95.

[30] Chen M, Rao RSP, Zhang Y, Zhong CX, Thelen JJ. A modified data normalization method for GC-MS-based metabolomics to minimize batch variation. 2014;3:439.

[31] Peralbo-Molina A, Calderón-Santiago M, Priego-Capote F, Jurado-Gámez B, Luque de Castro MD. Development of a method for metabolomic analysis of human exhaled breath condensate by gas chromatography-mass spectrometry in high resolution mode. Anal Chim Acta. 2015;887:118–26.

[32] Reisetter AC, Muehlbauer MJ, Bain JR, Nodzenski M, Stevens RD, Ilkayeva O, Metzeger BE, Newgard CB, Lowe WL Scholtens DM. Mixture model normalization for non-targeted gas chromatography/mass spectrometry metabolomics data. BMC Bioinformatics. 2017;18(1):84.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

[33] Saccenti E, Hoefsloot HCJ, Smilde AK, Westerhuis JA, Hendriks MMWB. Reflections on univariate and multivariate analysis of metabolomics data. Metabolomics. 2014;10(3):361–74.

[34] Shellie RA, Welthagen W, Zrostliková J, Spranger J, Ristow M, Fiehn O, et al. Statistical methods for comparing comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry results: Metabolomic analysis of mouse tissue extracts. J Chromatogr A. 2005;1086(1-2):83–90.

[35] Adam T, Ferge T, Mitschke S, Streibel T, Baker RR, Zimmermann R. Discrimination of three tobacco types (Burley, Virginia and Oriental) by pyrolysis single-photon ionisation-time-of-flight mass spectrometry and advanced statistical methods. Anal Bioanal Chem. 2005;381(2):487–499.

[36] Johnson KJ, Synovec RE. Pattern recognition of jet fuels: comprehensive GC×GC with ANOVA-based feature selection and principal component analysis. Chemom Intell Lab Syst. 2002;60(1-2):225–237.

[37] de la Mata AP, McQueen RH, Nam SL, Harynuk JJ. Comprehensive two-dimensional gas chromatographic profiling and chemometric interpretation of the volatile profiles of sweat in knit fabrics. Anal Bioanal Chem. Analytical and Bioanalytical Chemistry. 2016;1905–1913.

[38] Jolliffe IT. Principal Component Analysis. 2nd Edition. New York. Springer. 2002.

[39] Di Natale C, Paolesse R, D'Arcangelo G, Comandini P *et al.* Identification of schizophrenic patients by examination of body odor using gas chromatography-mass spectrometry and a cross-selective gas sensor array. Med Sci Monit. 2005;11(8):CR366–75.

[40] Bartel J, Krumsiek J, Theis FJ. Statistical Methods for the Analysis of High-Throughput Metabolomics Data. Comput Struct Biotechnol J. 2013;4(5): e201301009

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Chapter Five

Experimental set-up

Contents

5.1 PDMS Passive sampler construction

Passive sampler loops were manufactured by cutting lengths of polydimethylsiloxane (PDMS) tubing (Class VI USP Medical Grade Silicone tubing, 0.305 mm ID, 0.635 mm OD, Technical Products Inc. of Georgia, USA) to pre-determined lengths which would allow for their easy and comfortable use as bracelets and anklets. Wrist and

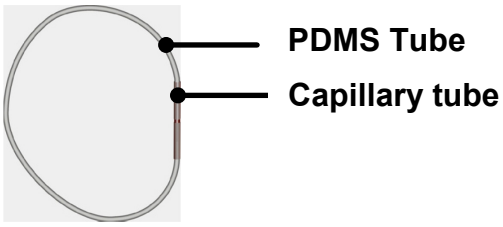

Figure 5.1) Diagram depicting the in-house constructed PDMS passive sampler loop design [1].

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ankle samplers were constructed from tubing of lengths 180 and 240 mm respectively. A small piece of uncoated silica capillary column (0.25 mm ID) (SGE Analytical Science, Separation Scientific (Pty) Ltd, South Africa) was inserted to connect the two ends of the tube in order to form a closed loop (Figure 5.1). The masses of the PDMS material were 0.0573 ± 0.0025 g and 0.0750 ± 0.0024 g for the bracelet and anklet samplers respectively.

5.2 Pre- and post-sampling procedure

Prior to use all samplers were cleaned and conditioned as described by Triñanes *et al*. (2015) [2]. In brief, samplers were subjected to three rounds of solvent extraction using a mixture of (1:1, v/v) acetone (AR 98.0%, Merck Chemical (Pty) Ltd., South Africa) and methanol (AR 99.5%, Merck Chemical (Pty) Ltd., South Africa). Glass vials containing the solvent and samplers were sonicated for five minutes between solvent exchanges. The samplers were placed in 17.8 cm long glass desorption tubes (4 mm ID, 6 mm OD) (Gerstel, Chemetrix, South Africa) and then conditioned with hydrogen gas (Afrox, South Africa) at 100 mL/min for ten hours at 280°C in an off-line tube conditioning system (Gerstel, Chemetrix, South Africa) after which three more solvent extractions were performed using acetonitrile (AR 99.5%, Merck Chemical (Pty) Ltd., South Africa). The samplers were tapped against the inside wall of a glass beaker to remove excess solvent before being sealed in a glass vial until deployment. All glassware was cleaned and dried at 70 °C before use. Handling of the samplers during and after the cleaning procedure was performed in a manner so as to limit exposure of the samplers to skin secretions. After passive sampler deployment the passive samplers were sealed in glass vials and stored at -80 °C until analysis. Storage at this temperature has been reported to preserve PDMS passive samplers for up to twenty-one days without statistically significant changes in absorbed VOC levels [3]. All samplers used in this study were analyzed within seventy-two hours of being collected.

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5.3 Instrument and software parameters

Chemical compounds collected by the passive samplers were thermally desorbed (TD) in a thermal desorption system (TDS) (Gerstel, Chemetrix, South Africa) and focused using a cooled injection system (CIS) before being injected onto the primary column. The operational parameters used during the thermal desorption and cryo-focusing procedures are summarized in table 5.1.

Table 5.1) Operational parameters used during thermal desorption of PDMS passive sampler loops.

Thermal Desorption Conditions	
Desorption mode:	Splitless
Initial temperature:	30 °C
Initial time:	3 min
Temperature ramp:	60 °C/min
End temperature:	280 °C
Hold time:	10 min
Transfer line temperature:	350 °C
Cooled Injection System Conditions	
Initial temperature:	-100 °C
Temperature ramp:	10.0 °C/s
End temperature:	250 °C

Analyte bands were separated by comprehensive two-dimensional gas chromatography (GC×GC) hyphenated with a time-of-flight mass spectrometer (ToFMS). The operational parameters used during the separation and detection of analytes are provided in Table 5.2.

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Table 5.2) Instrument parameters used during the separation and analysis of volatile analytes by GC×GC-ToFMS.

GC×GC Conditions	
GC instrument:	Agilent GC 7890A
Inlet conditions:	Solvent vent mode (purge on 90 s), Desorption flow 100 mL/min
Column 1 (D^1):	Rxi-1 MS, 30 m x 0.25 mm ID x 0.25 µm film thickness
Column 2 (D^2):	Rxi-17Sil MS 1 m x 0.25 mm x 0.25 µm film thickness
Carrier gas:	Helium (Ultra high purity, Afrox, South Africa), 1.4 ml/min, constant flow mode
Primary oven temperature programming:	40 °C for 1.5 min, to 300 °C at 10 °C/min, hold 8 min.
Secondary oven offset:	5 °C from the primary oven
Modulator offset:	15 °C from the secondary oven
Modulation period:	3 s (hot pulse 0.8 s)
Transfer line temperature:	300 °C
ToFMS Conditions:	
Detector:	LECO Pegasus 4D Time-of-Flight Mass Spectrometer
Acquisition rate:	100 spectra/s
Source temperature:	230 °C
Detector voltage:	1750 V
Stored mass range:	35 – 500 Da

Initial processing of the data generated by the mass spectrometer was processed using ChromaToF (Version 4.50.8.0) software. The software parameters used for all data processing are summarized in Table 5.3.

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Table 5.3) Data Processing parameters used by ChromaToF Software.

ChromaToF Data Processing Method	
Baseline offset:	1.0
Peak find S/N:	100.0
Match required to combine sub peaks:	650
Similarity match required for library identification:	750
Library:	NIST/EPA/NIH Mass Spectral Library (EI) (2014): mainlib and replib

The peak tables generated by the initial data processing during each analysis were manually inspected for entries flagged as having saturated masses or poor deconvolution purity prior to alignment. This was performed in order to identify peaks which may not be aligned correctly between samples due to multiple peak markers being placed for a single saturated peak by the software. The alignment of peak table entries across samples was performed using the Statistical Compare function of the ChromaToF software. Software settings used for the alignment process are given in Table 5.4.

Table 5.4) ChromaToF Statistical Compare settings used during peak alignment.

ChromaToF Statistical Compare Data Processing Method	
Mass threshold:	5
Minimum similarity match:	600
Maximum number of modulations apart:	1
Maximum second dimension retention time difference:	0.1 s
Initial peak finding S/N:	100
Second peak finding S/N:	20

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5.4 Nomenclature

An explanation of the nomenclature used when describing the chromatographic analysis of individual PDMS sampler loops and subsequent alignment of the chromatographic data is described visually in Figure 5.2. In the context of this and all remaining chapters, the term sample is used to refer to an individual passive sampler loop or corresponding chromatographic data. The term sample set is used to refer to all samples collected from a specific skin region. In addition, the term peak table entry refers to an individual analyte peak detected in the chromatographic data of a particular sample while the term peak marker is used to refer to peak table entries which have been aligned across samples and sample sets by the peak alignment software.

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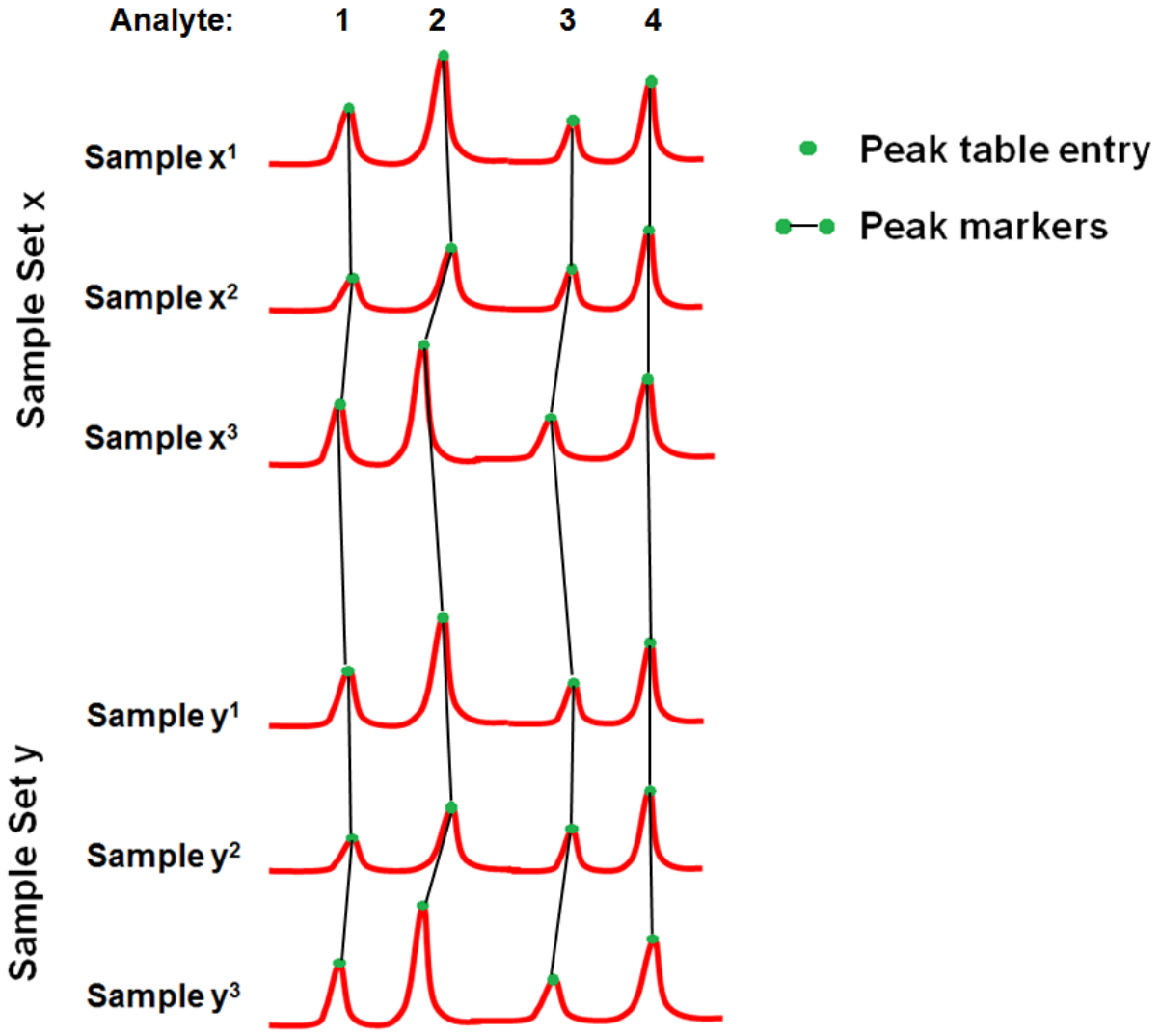

Figure 5.2) Visual portrayal of the nomenclature used to describe the chromatographic data obtained from the analysis of individual samples which form part of sample sets that include replicate skin samples and aligned quality control samples. Alignment of peak table entries corresponding to individual analyte peaks detected in individual samples results in the placement of peak markers by the alignment software.

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5.5 Sampling duration and sampler positioning experiment procedure

5.5.1 Context

Factors affecting the absorption of organic compounds by PDMS material were discussed in detail in Section 2.2. From this review, it was concluded that there are two main external parameters which affect the amount of analyte absorbed during passive sampling using PDMS. These are the duration of sampling and external temperature. In addition, a review of factors affecting software performance during peak table alignment in Section 4.3.3 highlighted that good chromatographic separation and large signal-to-noise of analyte peaks will enhance the results of software alignment. The skin's surface temperature fluctuates only minimally with the body's circadian rhythm and other physiological factors [4], thus changes in the temperature of the external environment during sampling are the major source of temperature variation during sampling. An experiment was designed in order to compare the performance of contact and non-contact arrangements of samplers on a subject's wrist over varying lengths of time. These experiments involved the manufacture of an aluminium arm band which could be worn by an individual and onto which PDMS samplers could be placed. Deploying the samplers in this way limited the direct contact between the skin and the PDMS sampler itself during sampling. This set up was compared to the results obtained when PDMS samplers which were worn in direct contact with the subject's skin.

The application of PDMS passive samplers mounted on an aluminium bracelet above the surface of a subject's skin was hypothesized to contribute to the method in two ways:

1) Mounting the samplers above the surface of skin would limit the diffusion of compounds of low volatility into the PDMS material. Thus providing a more representative profile of the volatile compounds present in the headspace above a subject's skin.

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2) Adjusting the uptake rates and number of large molecular weight compounds collected in the PDMS samplers would reduce the total number of peaks and peak overlap in the two dimensional GC chromatograms obtained. This would simplify the large amount of data collected in each sample and facilitate the automated procedures such as peak finding and peak alignment carried out by the ChromaToF software.

It was hypothesized that the non-contact samplers would require more time to collect similar amounts of analytes compared to the contact samplers. An optimum duration of sampling was therefore sought for exploratory work. This optimum duration would be judged by the performance of the alignment software results. This performance was assessed based on two criteria:

1) The number of peaks detected after different durations of sampling.

2) The number of missing peak table entries in the data sets generated.

5.5.2 Non-contact sampler holder design

The aluminium bracelet used to hold the PDMS passive samplers above the surface of a subject's skin was constructed from a single piece of aluminium which was cut and formed into the bracelet shape (length: 195 mm, width: 18 mm, thickness: 1.5 mm). The design included a channel into which the PDMS bracelets could be placed (Fig 5.3a) which held them in place during sampling. The floor of this channel was covered in regularly spaced holes, 2 mm diameter and 1.0 mm apart, which facilitated the ambient movement of gas across the surface of the samplers (Fig. 5.3b).

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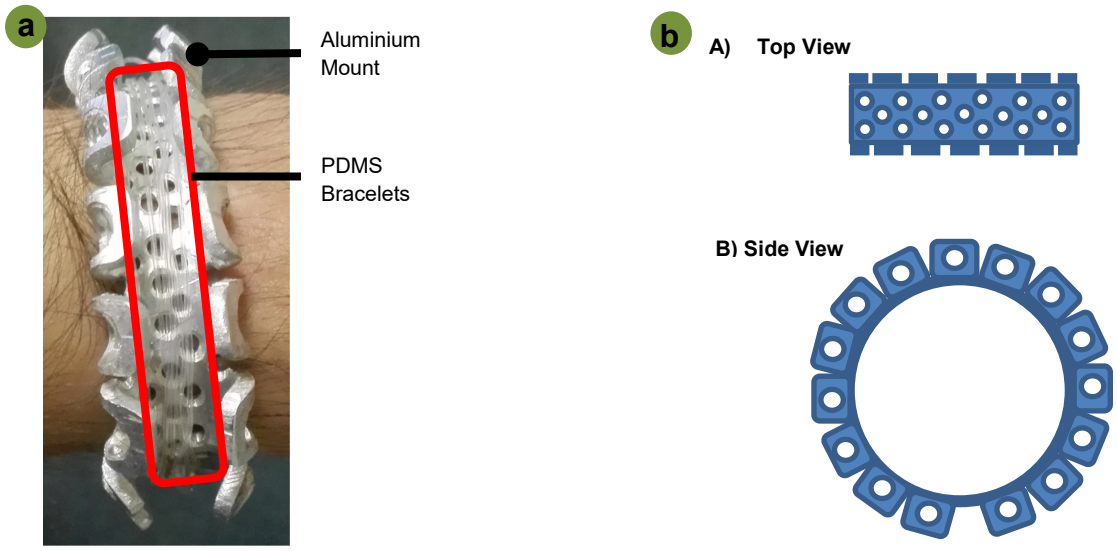

Fig 5.3a) Aluminium bracelet holding PDMS bracelets during sampling. **b)** Schematic top view **(A)** and side view **(B)** of the aluminium bracelet sampler holder.

The aluminium bracelet sampler holder was cleaned by ultrasonication for 10 minutes in a mixture of acetone:methanol (1:1) followed by 10 minutes ultrasonication in acetonitrile.

5.5.3 Method

Sample Collection: Two rounds of sample collection were carried out with six replicate samplers being placed either in the aluminium sampler holder or in direct contact with the skin surface. Samplers worn in direct contact with the skin surface were allowed to move freely during sampling. All other aspects of the two rounds of sample collection followed an identical procedure. Bracelets were worn on the non-dominant wrist of a single Caucasian male, aged 26. The single sampled subject remained in a laboratory environment which was maintained at a constant temperature of 21 °C during the experiment. Quality control samples included unused samplers for sampler background determination and, in the case of the non-contact sampling experiment, an unused sampler loop placed in the aluminium sampler holder while not worn by an individual for a period of nine hours. Three samplers were placed in the laboratory during sampling in order to monitor the ambient laboratory air during each experiment. Samplers were removed from the

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subject's wrist and analyzed every ninety minutes from the onset of the experiment thus yielding information for a passive sampling duration of up to nine hours for each configuration with one sample per time point. Samples were stored in individual glass vials at -80°C before analysis. All samplers were handled with stainless steel tweezers when transferred from storage vials to thermal desorption tubes.

Sample analysis: Samplers were analyzed by GC×GC-ToFMS once removed from the subject's wrist. All samplers were subject to the instrument and data processing conditions described in Tables 5.1 – 5.3.

Data Processing: The resulting peak table data were then processed and aligned by ChromaToF Statistical Compare software using the parameters described in Table 5.4. All peak table entries reported in all samplers were retained during processing this set of data. The aligned data was exported in .csv format for further processing using Microsoft Excel 2010 software (Microsoft Corporation, USA). For simplicity and to focus on definite skin volatile candidates, peak markers found in the aluminium sampler holder background sample, the laboratory air background and sampler blanks were removed from the data set prior to further evaluation of the data.

5.6 Sampling wrists and ankles experimental procedure

5.6.1 Context

The performance of the new passive sampler was tested in an experiment which involved the simultaneous passive sampling of an individual's wrist and ankle.

The post-analysis evaluation of the performance of the samplers was based on:

1) Evaluation of peak marker unique ion peak area percentage relative standard deviations (%RSDs) of replicate samples post normalization.
2) Principal component analysis of peak table areas, selected using Fisher ratio calculations, across replicates skin samples and between skin sample sets.

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3) Qualitative comparison of tentatively identified analytes found in skin samples to those reported in the literature.

It was hypothesized that the untargeted analysis and comparison of normalized peak area data would reveal relative differences in some analyte abundances between the two sampled regions based on the differing attractiveness of different body regions to malaria vector mosquitoes [5].

5.6.2 Method

Sample Collection: Based on the results of the sampling duration and arrangement experimentation, a sampling procedure was selected which balanced aspects such as sampling duration, number of chromatographic peaks collected and resolution of these peaks in individual chromatograms. The simultaneous sampling of a Caucasian male, aged 26, wrist and ankle was performed in a temperature controlled environment. The ambient temperature throughout sampling was maintained at 21 °C. Passive samplers were worn for a duration of four hours in direct contact with the subject's skin on the non-dominant wrist of the subject and corresponding ankle. Five replicate samplers were worn on each sampled region. Two quality control sampler blanks and three laboratory background samples were also analyzed. In addition, a single sample was collected from the same wrist and ankle of the sampled individual in an alternative environment, namely the subject's home. Samplers from the individual's wrist and ankle were analyzed by GC×GC-ToFMS in alternating order to avoid bias in the results due to the length of storage time. Samplers were sealed in glass vials and frozen at -80 °C for no more than 72 hours before analysis.

Sample analysis: All samplers were subject to the instrument and data processing conditions described in Tables 5.1 – 5.3.

Data Processing: The resulting peak table data were then aligned by ChromaToF Statistical Compare software using the parameters given in Table 5.4. The aligned data was exported in .csv format for further processed using Microsoft Excel 2010

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software (Microsoft Corporation, USA). Processing of the large volume of data generated involved the initial classification of all analyte peak markers into groups. For simplicity, peak markers which corresponded to aligned peak table entries that were not found in at least 80% of skin samples from each respective body area, as well as the corresponding skin sample collected in an alternative environment, were removed as part of the first step of data clean up. This approach identifies compounds which are present in the skin samples without taking human activity into account. Peak markers were further classified according to whether they were present in the laboratory background or sampler blanks.

Normalization: Normalization of unique ion peak areas selected across aligned chromatograms by the ChromaToF Statistical Compare software, as described in Section 4.3.3, involves the use of a normalization constant. The same normalization constant is used as a denominator to scale all the unique ion peak areas in a single sample's peak table data (Eqn. 5.1).

$$N(A)_{ij} = \frac{A_{ij}}{C_j} \ldots \text{Eqn. 5.1}$$

Where $N(A)_{ij}$ is the normalized peak area of the *i*th peak in sample *j*; A_{ij}, is the unique ion peak area prior to normalization and C_j is the corresponding normalization constant for that sample.

Three approaches to the derivation of constants used for the normalization of replicate samples collected from an individual's wrist and ankle were compared. All calculations were performed on tables of data exported to Microsoft Excel. Normalization constants were derived from the mass of PDMS in each individual passive sampler, each sample's mass spectrometry total useful signal (MSTUS) or both applied in succession. The mass spectrometry total useful signal makes use of the sum of all useful peak areas omitting those corresponding to artifacts in the chromatographic data (Eqn 5.2).

$$c_{j\,(MSTUS)} = \sum_{i=1}^{i=n} A_{ij} \ldots \text{Eqn. 5.2}$$

Where $c_{j\,(MSTUS)}$ is the normalization constant derived for sample *j* and *n* is the total number of appropriate peaks in the chromatographic data.

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The effect of using a threshold value for the percentage relative standard deviations (%RSD) of peak markers included in each MSTUS constant was investigated. This involved the exclusion of peak markers which had %RSDs, calculated for replicate skin samples in respective sample sets, larger than a threshold value set for all MSTUS constants. Threshold values used were either 75% or 50%. The peak areas of the remaining peak marker entries which had %RSDs below the threshold value were then summed to give the MSTUS constant for each respective sample. After normalization, the %RSDs of normalized peak marker areas in each respective sample set were compared to examine the effects of each normalization strategy.

Relative peak area comparisons: Analyte unique ion peak areas were normalized using the mass of PDMS corresponding to each respective sampler. Comparison of the average peak areas of replicate skin sample peak table entries to the peak areas of corresponding peak table entries found in the laboratory background and sampler blanks was performed. Peak markers for which the background or sampler blank peak table entry peak area was greater than 10% of the average peak table entry peak area observed in replicate skin samples were omitted from the quantitative comparison. Fisher ratios, as described in Section 4.5.2, were calculated for the normalized peak marker peak areas of the remaining entries corresponding to the two sample sets.

Data exploration: Principal components analysis (PCA) using the covariance matrix of individual sample peak areas selected based on the results of Fisher ratio calculations were used to provide additional validation of the results obtained post normalization of the peak areas. The PCA calculations were performed by importing normalized peak area data for individual analytes found in replicate samples into JMP Pro 12.0.1 (SAS Institute Inc.).

Identification of analytes: Peak markers were assigned tentative identifications based on mass spectral library similarity scores above 750 using ChromaToFs' library search function. In addition, the linear temperature programmed retention index (LTPRI) was calculated using the average first dimension (^1t_R) retention time for each peak marker from replicate samples using Van den Dool's equation [6] and a range of straight chain alkane (C_6-C_{28}) standards. This approach makes use of the peak

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apex as determined when recombining modulated analyte bands by the processing software. Second dimension retention indices were not calculated due to lack of library data for isothermal elution retention indices on the column employed in the second dimension for analytes in the study [7]. The calculated retention indices were compared to literature values reported in the NIST library. LTPRI deviations of up to ten units were included in the results. Due to the limited number of literature LTPRIs available for the column used in the first dimension (Rxi-1 MS) literature results reported using a DB-1 column with similar dimensions were also included in the identification process. Both of these columns have an internal coating of 100% PDMS. The resulting identifications were then compared to compounds reported in the relevant academic literature.

5.7 References

[1] Naude Y, Gorst-Allman P, Rohwer E. A cheap and simple passive sampler using silicone rubber for the analysis of surface water by gas chromatography-time of flight mass spectrometry. Water SA. 2015;41(2, SI):182–188.

[2] Triñanes, S., Pena, M.T., Casais, M.C., Mejuto MC. Development of a new sorptive extraction method based on simultaneous direct and headspace sampling modes for the screening of polycyclic aromatic hydrocarbons in water samples. Talanta. 2015;132:433–442.

[3] Martin HJ, Turner MA, Bandelow S, Edwards L, Riazanskaia S, Thomas CLP. Volatile organic compound markers of psychological stress in skin: a pilot study. J Breath Res. 2016;10:046012.

[4] Mackowiak PA, Wasserman SS, Levine MM. A critical appraisal of 98.6 degrees F, the upper limit of the normal body temperature, and other legacies of Carl Reinhold August Wunderlich. JAMA. 1992 Sep;268(12):1578–1580.

[5] Verhulst NO, Weldegergis BT, Menger D, Takken W. Attractiveness of volatiles from different body parts to the malaria mosquito *Anopheles coluzzii* is affected by deodorant compounds. Sci Rep. 2016;6:27141.

[6] Van den Dool H, Kratz PD. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatogr. 1963;11(3):463–471.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

[7] Mühlen C, Marriott PJ. Retention indices in comprehensive two-dimensional gas chromatography. 2011;2351–60.

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Chapter Six

Sampling method development

Contents

6.1 Introduction

This chapter presents the results of the practical experimentation undertaken in order to develop a new method for the non-targeted sampling and analysis of skin VOCs as described in Chapter 5. The application of the newly developed sampling method for the collection of skin volatiles and comparison of the relative abundances of analytes between samples is explored with respect to the optimal sampling arrangement and reduction of errors during the alignment of chromatographic peaks in individual samples by peak alignment software.

The remainder of this chapter is presented as follows, first the results of sampler positioning and sampling duration experiments as described in Section 5.5 are presented. Subsequently, the results of different approaches to normalization of samples collected from an individual's wrist and ankle, as described in Section 5.6, are presented. Effects of normalization on the data obtained are discussed.

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6.2 Sampling method positioning and duration

The chromatograms obtained during the analysis of samplers used for the comparison of direct skin contact and non-contact sampling arrangements and the effects of sampling duration experimentation are provided in Appendix A1. Individual and aligned peak table data are provided in Appendix A3. This data includes tentative identifications, Chemical abstract service, CAS, registry numbers and similarity scores of chromatographic peaks detected in the collected samples.

6.2.1 Visual analysis

Chromatograms that were obtained from the GC×GC-ToFMS analysis of wrist samplers which had been used in either direct contact or non-contact with the skin surface during sampling for a duration of nine hours are shown in Figure 6.1. Visual investigation and comparison of the two total ion chromatograms (Fig. 6.1a and b) yields some preliminary observations. Firstly, the chromatographic separation conditions are suitable for separating analyte peaks into distinct groups in different regions of the chromatogram.

Region A contains peaks which elute early on in the first dimension (D^1). Peaks in this region have higher intensities in the chromatogram corresponding to the non-contact sampler (Fig. 6.1b) than the contact sampler (Fig. 6.1a). Several high intensity peaks present in Region A of this chromatogram do, however, correspond to artefacts originating from the aluminium bracelet sampler holder.

The sampler worn in direct contact with the skin's surface (Fig. 6.1a) yields much greater peak intensities in Region B after nine hours of sampling when compared to the non-contact arrangement (Fig. 6.1b). The higher peak intensities observed for the direct skin contact arrangement results in greater peak tailing in the second dimension for some polar analytes as well as increased overlap of peaks. Deconvolution algorithms employed by the data processing software can recover

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peak information for overlapping peaks but peak tailing can be problematic for accurate determination of peak areas.

Region C contains many peaks in the contact arrangement (Fig. 6.1a) which are either absent or are present at a much lower intensity in the non-contact set-up (Fig. 6.1b). These peaks elute late in the first dimension and fall outside the temperature program's linear range during the separation. The long retention time on the non-polar column and lower abundance during the non-contact sampling set up implies that these peaks correspond to larger less volatile compounds found on the skin surface such as long chain fatty acids [1].

Further investigation of the effects of sampler arrangement and sampling duration was performed by analyzing the aligned chromatographic peak table data generated by the peak alignment software.

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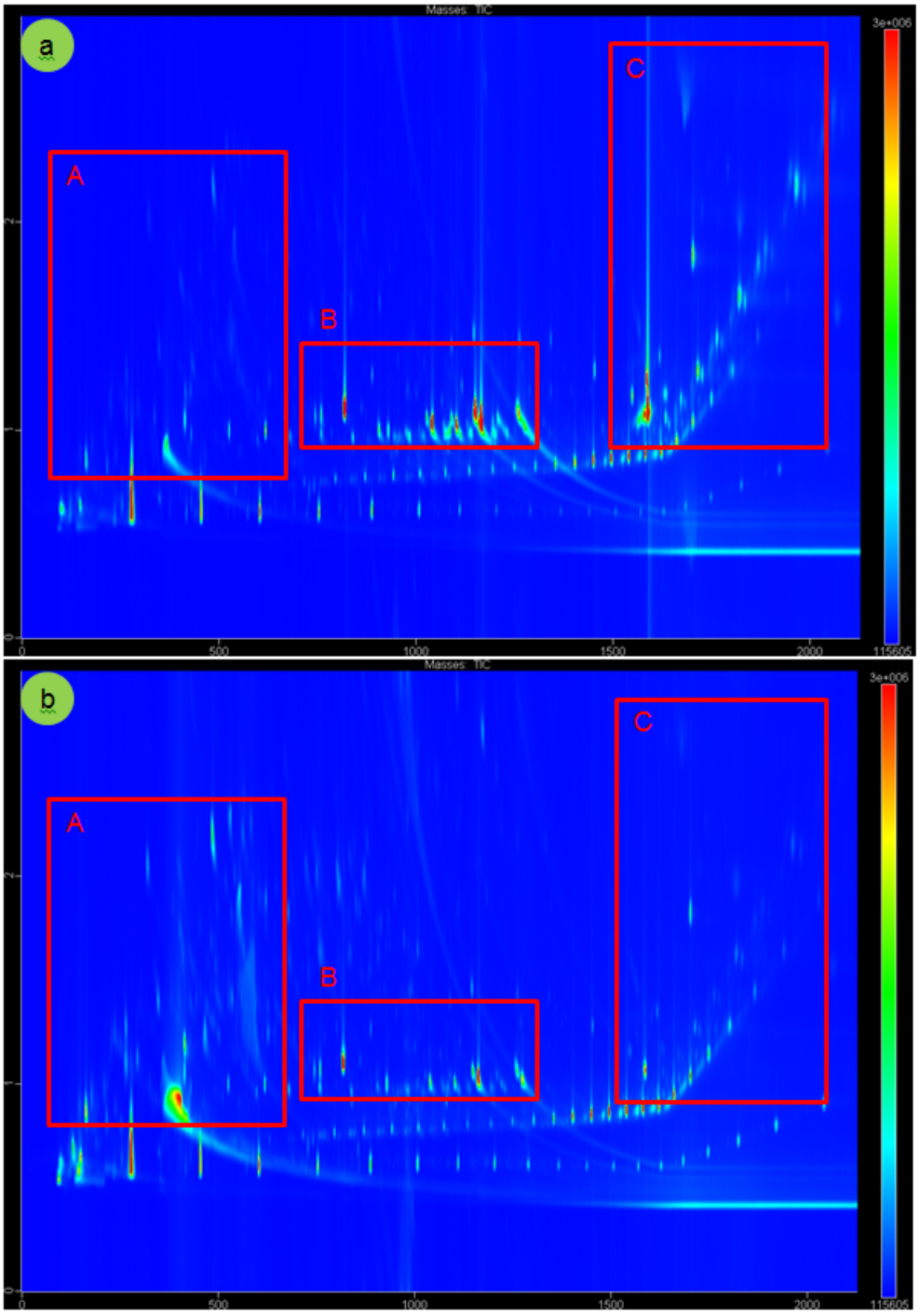

Figure 6.1) Total ion count (TIC) chromatograms for **a)** sampler worn in direct skin contact and **b)** sampler worn in non-contact arrangement after a sampling duration of nine hours. **Region A** contains many peaks which are present at higher intensities in the non-contact sampler arrangement. **Region B** contains peaks corresponding to skin volatiles which are present at a higher intensity in the chromatogram worn in the direct contact arrangement. **Region C** contains many peaks corresponding large organic molecules originating from the skin surface.

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6.2.2 Peak table analysis

Alignment of the twelve skin samples and respective quality control samples resulted in the generation of a chromatographic peak list containing 2624 peak markers. Data processing described in Section 5.5.3 yielded a total of 332 analytes which were found to be aligned exclusively in skin samples from both sampling arrangments (Appendix A3). The effects of the different sampling arrangements on the quality of data obtained were assessed by evaluating the software performance in each case. This was done by quantifying the total number of peaks which were successfully found and aligned in each configuration and for each time interval.

Figure 6.2 shows the total number of peaks successfully found and aligned by the software in each skin sample during the deconvolution and alignment procedure.

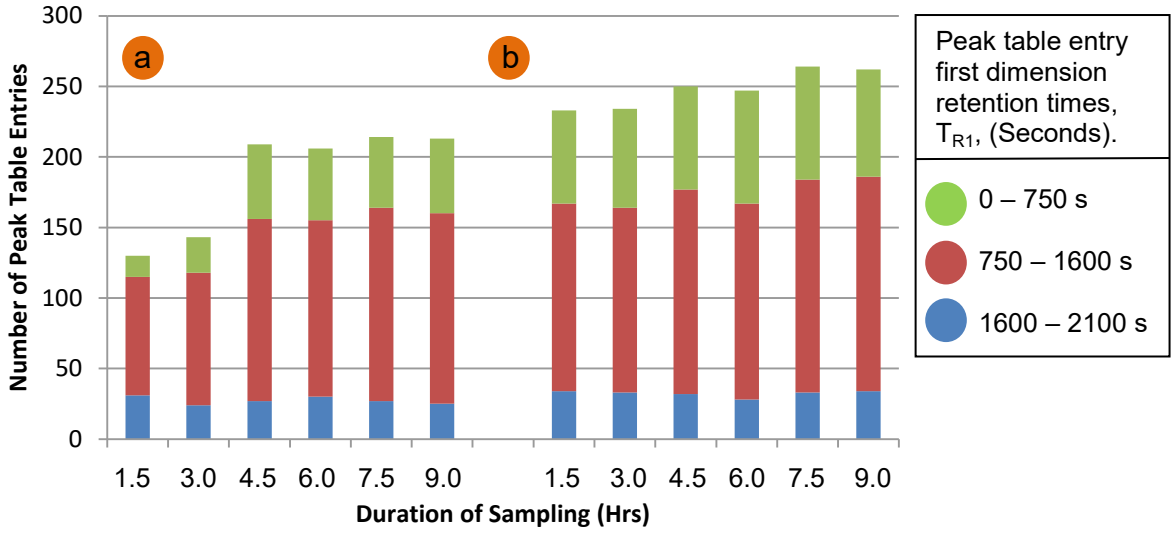

Figure 6.2) The number of peak table entries relative to the duration of sampling for **a)** direct skin contact samplers and **b)** non-contact skin samplers. Each bar is colour coded according to the number of entries which fall between specific retention times in the first dimension (D^1). Peak table entries with retention times between 0 – 750 s (green), 750 – 1600 s (red) and 1600 – 2100 s (blue).

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The total number of peak table entries found in each sample is indicative of the number of chromatographic peaks which have a unique ion signal above the S/N thresholds described in Chapter 5 (Table 5.4). Peaks can be lost during the alignment process if the S/N ratio of the unique ion as determined by the alignment software falls below the lower cut-off limit of 20. Additional false peak markers can be generated for peaks which are saturated or have poor peak shape as described in Chapter 4 (Section 4.3.3). These peak table entries should be manually recombined prior to further data processing as described in Section 5.3.

The total number of peaks detected and aligned in each chromatogram relative to the duration of sampling indicates that more peaks were successfully detected and aligned in the non-contact sampling set-up (Fig. 6.2b) as compared to the contact sampling set-up (Fig. 6.2a) across all durations of sampling. In addition, both sampling methods reach a near maximum number of peaks after 4.5 hours of sampling.

This observation was contrary to the hypothesized delay in analyte uptake by non-contact samplers postulated at the onset of the experiment (Section 5.5.1). Investigation of this observation involved categorizing peak table entries by their primary retention times. An analyte's retention time on the primary column composed of 100% PDMS is proportional to its partition coefficient between the atmosphere and a PDMS passive sampler, with short retention times corresponding to analytes which have small partition coefficients as discussed in Section 2.3.1. Grouping of the peak table entries into three groups, namely, with primary retention times (T_{R1}) between 0 - 750 s, 750 – 1600 s and 1600 – 2100 s, allowed for quantification of the number of peaks found in the three general areas of the chromatograms (Fig. 6.1). The number of peak table entries in each retention time window and their contribution to the total number of peak table entries after each sampling duration are colour coded in Figure 6.2. From these results, it can be observed that the numbers of peak table entries with medium and long retention times, red and blue bars respectively, are similar in when comparing the contact sampling set-up to the non-contact sampling set-up after 4.5 hours of sampling. The major differences between the total number of peak table entries for each set-up and duration of sampling are due to the number of peak table entries present in the low retention

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time window (green bars). This difference is especially prominent for contact sampling with a duration less than 3 hours. This difference between the two sampling arrangements can potentially be explained by the effect of ambient temperature during sampling and its effect on analyte uptake and retention by the PDMS material. The permeability of PDMS was described in Section 2.2.3 (Eqn. 5) and decreases with temperature. The ambient laboratory temperature is maintained at a level approximately 16 °C lower than human body temperature. Thus, samplers placed on an aluminium bracket are most likely operating at a slightly lower ambient temperature than samplers placed in direct contact with the surface of the skin. The higher temperature and resulting lower partitioning of analytes into the samplers in direct contact with the skin surface would result in lower concentrations of poorly retained analytes being collected in comparison to the non-contact samplers.

Missing peak table entries in the aligned peak table are classified as peak table entries which are not detected after detection has been recorded at an earlier sampling duration and reflect mistakes in the peak finding or alignment process. Missing entries were quantified for the second to the sixth sampler in both arrangements. The results of which are summarized in Table 6.1.

Table 6.1) The number of missing peak table entries for contact and non-contact samplers corresponding to their respective sampling durations.

Sampling duration (hours):		3	4.5	6	7.5	9
Contact Samplers	No. of missing entries:	28	24	48	48	58
	Percentage of total number of peaks:	16%	10%	19%	18%	21%
Non-contact Samplers	No of missing entries:	42	44	62	51	57
	Percentage of total number of peaks:	15%	15%	20%	16%	18%

The total number of missing peak table entries in the aligned peak table data increases roughly with the total number of peaks detected, as shown in Figure 6.2, for both sampler set-ups. More missing peak table entries are found in the data for

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non-contact samplers which have a greater total number of peaks detected across all time intervals. There is, however, very little difference in the percentage of missing peak table entries if the two different set-ups are compared to each other. This implies that the benefit to the quality of alignment obtained by wearing the skin samplers in a non-contact arrangement is minimal.

6.3 Skin wrist and ankle VOC analysis

Chromatograms obtained after the collection and analysis of samplers worn on a human subject's wrist and ankle, as described in Section 5.6.2, are provided in Appendix A2. Individual and aligned peak tables are provided in Appendix A3. Initial alignment of the peak tables generated by GC×GC-ToFMS analysis of the skin samples, background samples and quality control samples yielded a total of 3136 peak markers. Peak markers not found in the appropriate number of skin samples, as described in data processing methodology (Section 5.6.2), were removed leaving a total of 1196 peak markers. The remaining peak markers were further categorised according to whether they were detected in the sampler blanks or the laboratory background. Different approaches to global normalization of the unique mass signal areas for each sample were investigated. Semi-quantitative evaluation and exploration of the differences in signal areas of analytes between the ankle and the wrist was performed by application of Fisher ratios (FR) and principal components analyses (PCA) using the peak areas corresponding to analyte specific unique masses selected during alignment.

6.3.1 Normalization of signals areas

The proposed sampling method making use of PDMS loops as passive samplers does not facilitate the inclusion of internal standards during the collection and analysis stages of the developed methodology. In addition, endogenous metabolites which are released from the skin surface at a constant and reliable rate are not

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reported to the best of our knowledge in current academic literature. Normalization of analyte peak areas is therefore limited to the application of global normalization constants post data acquisition [2]. Normalization of peak areas is required primarily to compensate for the difference in sampler volumes and surface areas between the bracelet and anklet samplers which will enable the comparison of analyte signals between the two sampler types. Global normalization involves the selection of a scaling factor which is applied uniformly to all peak areas in an individual sample as described in Section 5.6.2, (Eqn. 5.1). The choice of an appropriate normalization constant is an important aspect of metabolomics data processing and has an effect on the quality of subsequent feature detection and biomarker discovery processes [2]. Several normalization constants were derived and compared based on their ability to reduce variation in the skin sample sets obtained from the wrist and ankle of a sampled individual. Normalization constants were derived from either the mass of PDMS material present in each sampler, the total useful mass spectrometry signal (MSTUS), Section 5.6.2 (Eqn. 5.2), of each sample or a combination of the two strategies applied in succession.

The initial data processing involved the alignment of all replicate skin samples, blank samples and laboratory background chromatograms. During the alignment process, unique quantitative ions were selected for each peak table entry across all samples. All further data processing was based on the peak areas of these unique mass signals.

Further processing involved the selection of peak markers that were present above the corresponding background laboratory signal, as described in the “Relative peak area comparisons” section of Section 5.6.2. This process involved the removal of peak markers for which the corresponding laboratory background samples or sampler blanks contained corresponding signals with a peak area, normalized to the mass of the PDMS in each sampler, greater than 10% of the average signal area observed in the respective skin samples. This threshold percentage was selected as a conservative estimate in order to focus the data processing on the volatiles most likely to be of skin origin. This process yielded 869 peak markers which could be compared between the two sampled skin regions. None of the peak table entries corresponding to the peak markers which were selected during this process had

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been flagged as having saturated masses by the ChromaToF software during the initial data processing. Normalization by mass spectral total useful signal (MSTUS) makes use of the sum total of peak areas corresponding to useful signals in each chromatogram as a scaling factor. The inclusion of only useful MS signals aims to exclude contributions from artefacts in the chromatograms [2] but can be extended to exclude peak markers which correspond to poor quality chromatographic peaks. Selection of peak markers which were suitable for inclusion as part of the mass spectral total useful signal (MSTUS) calculation was therefore based on the %RSDs of peak marker areas between replicate skin samples in respective sample sets. This was done with the aim to find an appropriate way to derive a MSTUS normalization constant that could be applied to normalize all peak areas in a sample. Selection of peaks based on their reproducible signal area results in peaks with poor shape being excluded. In addition, poorly retained peaks for which the peak area decreases during storage and highly retained peaks whose areas may increase during storage and handling of the stored samples are also omitted from the MSTUS calculation. The effect of each normalization constant on the skin sample sets was evaluated based on the distribution of %RSDs of all peak markers in each sample set post normalization.

The results were visualized as box-and-whisker plots. Box-and-whisker plots represent the distribution of values in a data set by separating them into four quartiles. The median of the range of values in the data set becomes the boundary between the second and third quartiles and is represented by a horizontal black line in the plot. The second and third quartiles of the data are represented by the boxes above and below the median line. The upper or lower limits of these boxes correspond to limits of half of all the data points. The whiskers extending above and below the boxes indicate the minimum and maximum values of the range of values in the data set. The lower whisker corresponds to the first quartile while the upper whisker corresponds to the fourth quartile in the data set.

In addition to the box-and-whisker plots, the resulting increase or decrease in the percentage of peak markers which can be considered as usable for potential biomarker candidates, with %RSDs below 30% post normalization [3], was quantified in each case. The distributions of %RSD values post normalization are summarized

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in Figures 6.3 and 6.4 for the bracelet and anklet sample sets respectively. The percentage of useful biomarker candidates in each case are summarized in Table 6.2.

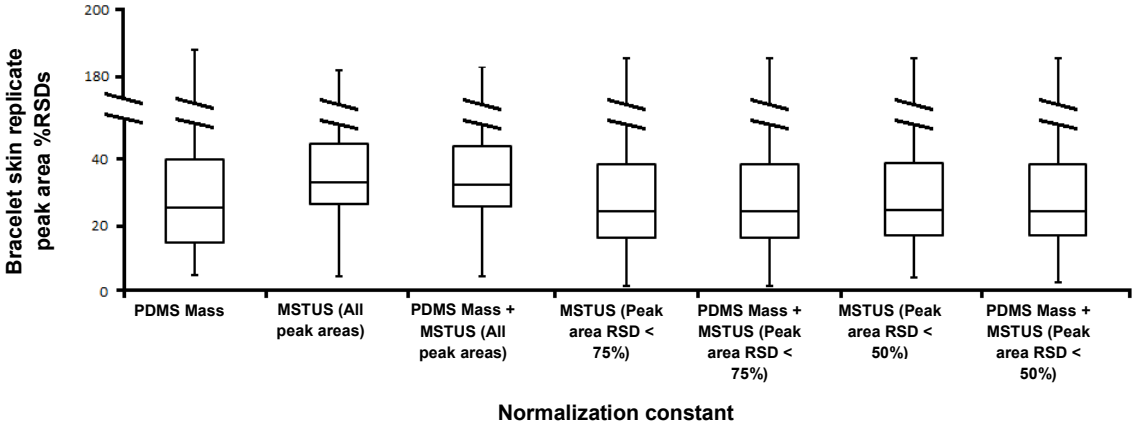

Figure 6.3) Box-and-whisker plots representing the distribution of %RSD values of the replicate bracelet unique ion signal areas after normalization of each sample. Normalization constants were derived based on the mass of PDMS sorbent in the sampler, the mass spectral total useful signal (MSTUS) or a combination of both applied in succession.

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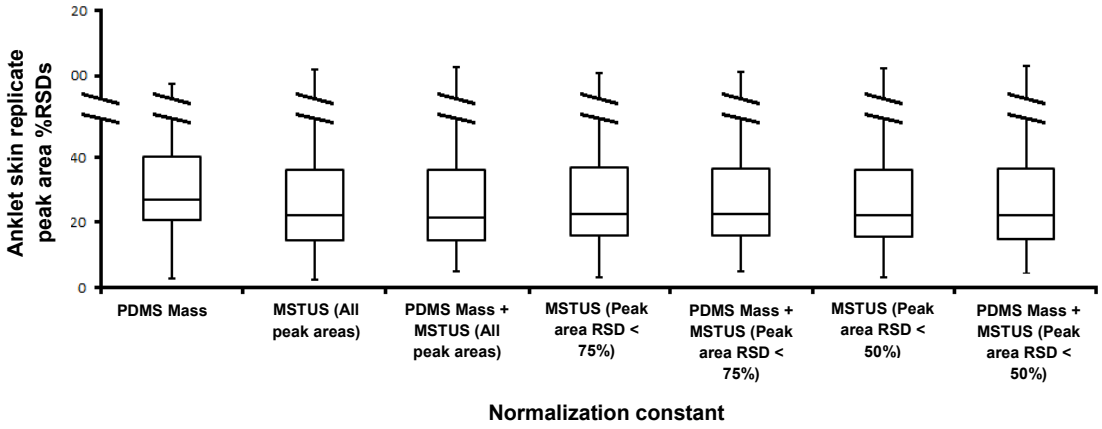

Figure 6.4) Box-and-whisker plots representing the distribution of %RSD values of the replicate anklet unique ion signal areas after normalization of each sample. Normalization constants were derived based on the mass of PDMS sorbent in the sampler, the mass spectral total useful signal (MSTUS) or a combination of both applied in succession.

Table 6.2) The effects of normalization constants derived from the mass of PDMS material, the mass spectral total useful signal (MSTUS) or a combination of the two approaches applied in succession on the percentage of peak markers which can be used as potential biomarker candidates for bracelet and anklet sample sets.

Normalization constant:	PDMS Mass	MSTUS (All peak areas)	PDMS Mass + MSTUS (All peak areas)	MSTUS (RSD < 75%)	PDMS Mass + MSTUS (RSD < 75%)	MSTUS (RSD < 50%)	PDMS Mass + MSTUS (RSD < 50%)
Percentage of bracelet peaks with RSD<30%	60.6	38.9	42.2	63.8	63.8	62.3	63.3
Percentage of anklet peaks with RSD<30%	59.1	66.4	66.9	66.3	67.3	67.2	67.9

Figures 6.3 and 6.4 demonstrate that the choice of normalization constant has an effect on the quality of results obtained. Comparison of the effect of normalization based on the mass of PDMS material in each individual sampler versus normalization to the MSTUS using all peak areas in each sample shows different trends for the bracelet and anklet sample sets. Normalization based on the PDMS mass yields three-quarters of the total number of peaks with peak area variabilities below 40% (Figures 6.3 and 6.4) for both sampler types with approximately 60% of

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peaks being usable for biomarker discovery (Table 6.2). This is not the case when comparing the results of normalization using the MSTUS if all peak areas are included. This approach introduces significantly greater variability to the bracelet data set while reducing variability in the anklet data set. Excluding peaks with large variability from the MSTUS normalization constant has a beneficial effect on the results of MSTUS normalization of bracelet data. This trend is not observed in the corresponding anklet results. However, the effect of decreasing the %RSD threshold of inclusion for the MSTUS calculation from 75% to 50% has a very small effect on the quality of normalized data from both sampler types. Normalization based on the combined effects of the mass of PDMS and MSTUS applied in succession for each sample does not significantly change the quality of the results obtained when compared to just using the MSTUS normalization in either case.

6.3.2 Semi-quantitative sample set comparison

Comparison of analyte peak areas between the bracelet and anklet sample sets was performed for all analytes which had a corresponding sampler blank and laboratory background peak area less than 10% of the average replicate skin sample equivalent. Of the original 1196 peak markers identified as potential skin volatiles, 869 were classified as relevant for comparison between sampled skin regions. Fisher ratios were calculated for each peak marker in the aligned wrist and ankle data using the formula described in Section 4.5.2 (Eqn. 5.1). Repeatability of measurement is important for accurate feature detection using Fisher ratios. Poor quality data with large peak area variability results in relevant features which differ between samples not being identified. Fisher ratios were calculated using data normalized to the PDMS sampler masses or the MSTUS of each sample using a sample replicate %RSD threshold of 75% for inclusion of a peak marker in the MSTUS normalization constant. The full list of analytes and calculated Fisher ratios are provided in Appendix A3.

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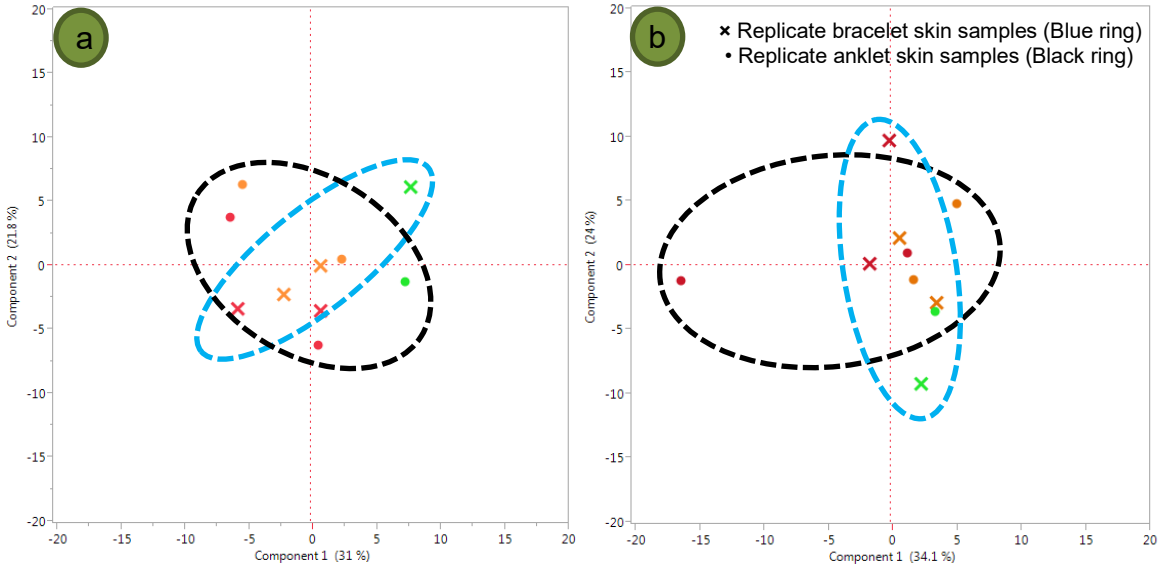

Figure 6.5) PCA of log peak areas of replicate bracelet **(x)** and anklet **(•)** samples using peak markers with a Fisher ratio below one and normalized using **a)** PDMS mass and **b)** MSTUS with a %RSD threshold of 75% for inclusion. Marker colours indicate the duration of storage time prior to analysis with less than 10 hours after sampling **(Red)**, 16-24 hours **(Orange)** and 36-48 hours **(Green)**.

Principal components analyses (PCA) were performed using log transformed normalized peak area data generated using the two different strategies of normalization. This approach was used to validate the results of the normalization process by comparing the samples based on peak markers which had a very small Fisher ratio. In addition, a threshold Fisher ratio was sought which could be used to identify peak markers which contributed to differences between the two sampled regions. Principal components analysis of peak markers with a Fisher ratio of less than one unit was performed for data sets which had been normalized using the two different approaches (Figure 6.5a and b). The peak markers used for the PCA had %RSD values below 45% within each sample set.

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Principal components analysis of peak markers which are present at similar levels in both bracelet and anklet samples should exhibit clustering and overlap of the two groups in the principal components scatterplot. This is true for the data sets generated (blue and black rings) using both the PDMS mass (Fig. 6.5a) and MSTUS (Fig. 6.5b) normalization approach with the first and second principal components describing similar amounts of variation in each case. In addition, samples are colour coded according to the period of time which passed between sample collection and analysis. During this time samples were frozen at -80°C. Clustering of data points by the storage time of analysis may result from small variations in peak areas due to changes in analyte concentrations in the passive samplers over time during storage.

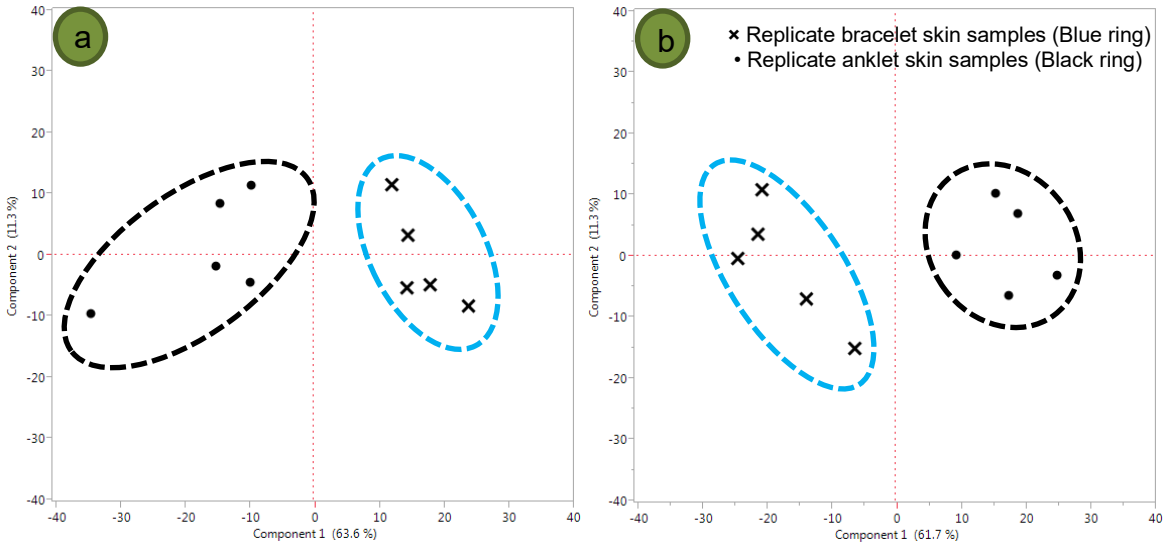

Figure 6.6) PCA of log peak areas of the replicate bracelet **(x)** and anklet **(•)** samples using peak table entries with a Fisher ratio above two and normalized using **a)** PDMS mass and **b)** MSTUS with a %RSD threshold of 75% for inclusion.

Principal components analysis of entries with Fisher ratios larger than two shows clustering of replicate samples and clear separation between the bracelet and anklet sample sets with separation between the two groups accounting for most of the variation in the data (Fig. 6.6). The inversion of bracelet and anklet replicate data is observed as a result of the difference in the normalization constants derived in each case. The normalization constants derived using the MSTUS of anklet samples are smaller relative to those derived from the bracelet samples when compared to normalization constants derived from the masses of PDMS. This highlights the effect

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that post acquisition data treatment can have on the results of non-targeted metabolomics investigations.

6.4 References

[1] Tobin DJ. Biochemistry of human skin—our brain on the outside. Chem Soc Rev. 2006;35(1):52–67.

[2] Wu Y, Li L. normalization methods in quantitative metabolomics. J Chromatography A. 2016;1430:80–95.

[3] Naz S, Vallejo M, García A, Barbas C. Method validation strategies involved in non-targeted metabolomics. J Chromatography A. 2014;1353:99–105.

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Chapter Seven

Application of skin sampling

Contents

7.1 Introduction

This chapter presents the qualitative and semi-quantitative analysis of the volatiles collected using the newly developed sampling method on an individual's wrist and ankle.

The range of analytes collected and tentatively identified as potential skin volatiles using PDMS loop samplers were compared to those previously reported in the literature which had been collected using other techniques. The results are presented in the context of potential applications of the sampling method to the study of the skin microbiome and to studying the ecology of human blood-host seeking mosquitoes. This was focused on chemical signals triggering electrophysiological or behavioural responses in the major malaria vector in Africa, *Anopheles gambiae* sensu stricto *(*from here on referred to as *An. gambiae*) [1]. As well as, *Aedes aegypti,* a vector of dengue and yellow fever viruses in Africa, Asia and the Americas [2].

The skin volatile samples were collected as described in Section 5.6.2 from the wrist and ankle of a Caucasian male subject, aged 26. The two-dimensional chromatograms are available in Appendix A2 while the full peak tables of individual samples and aligned peak markers is available in Appendix A3. This data includes

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the unique mass selected for each analyte peak in aligned chromatograms, similarity scores and CAS numbers for tentatively identified analytes as well as signal to noise ratios for each peak.

7.2 Qualitative evaluation of skin VOCs

The tentative identification of the potential skin VOCs present in the aligned bracelet and anklet chromatograms was based on mass spectral library similarity ≥ 75% and supplemented by linear retention index library comparisons when available. Of the approximately 1200 peak table entries in the aligned data approximately 700 were assigned tentative identifications and 88 are discussed in more detail with regard to their biological context. The library search yielded tentative compound identifications from a broad range of chemical classes. Notable chemical classes included saturated, unsaturated and aromatic carboxylic acids, aldehydes, ketones and alcohols. Many of these have been previously reported as components of skin secretions in academic literature. In addition, several lactones, as well as sulphur and nitrogen containing compounds, were tentatively identified. Some of these have been identified as natural products of skin borne microbiota and semiochemicals for blood-host seeking mosquitoes. It must be noted that the total number of peaks collected in this study using two-dimensional gas chromatography is larger than reported in many investigations making use of a single separation dimension, however many compounds which are reported as originating from the skin in prominent review articles were not tentatively identified in this current work [26, 89]. This can be attributed to the selectivity of the sampling technique, as well as, a lack of library mass spectra match and retention indices for many compounds. The Fisher ratios reported were calculated using peak areas normalized to the mass of each passive sampler.

Many of the tentatively identified skin volatiles were carboxylic acids (Table 7.1) and ketones (Table 7.2). Saturated straight chained carboxylic acids ranged from C_3 - C_{18} excluding C_5 and C_{11}. The two missing carboxylic acids have been reported as

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components of skin emissions [26] however they were not identified in any skin samples collected. Far fewer branched, unsaturated and aromatic carboxylic acids were identified. The majority of the carboxylic acids tentatively identified have been previously reported by researchers collecting human skin chemicals on glass beads in conjunction with GC-MS analysis [12]. Odd chain length carboxylic acids and ketones reported are likely not of biological origin and rather dietary intake [90]. Straight chained carboxylic acids have been widely studied for their potential role in the host seeking behaviour of anthropophilic blood feeding mosquitoes [27-31] and have been employed in odour traps to attract the malaria vector *An. gambiae* in the wild [32]. Bacteria are known to produce a diverse range of volatile carboxylic acids [33]. With some species commonly found on human skin being able to produce short chained fatty acids from substrates found in skin secretions [34]. The branched 3-methylbutanoic acid is present in the headspace of a wide range of skin borne *Staphylococcaceae* [35] and may play a role in the attraction of *An. gambiae* mosquitoes [31].

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Table 7.1) Compounds containing a carboxylic acid moiety which were tentatively identified after GC×GC-ToF analysis of PDMS bracelets and anklets. Corresponding calculated and literature LTPRI values, as well as Fisher ratios for PDMS normalized analyte specific unique mass signal areas are provided. Comparison of the compounds detected using the new sampler to literature reports using alternative passive sampling methodologies are shown.

Carboxylic acids tentatively identified in skin samples				Skin sampling techniques reported in literature		
Identification	**LTPRI (Calc.)**	**LTPRI (Lit.) [3-11]**	**Fisher Ratio (PDMS)**	**Glass beads [12, 13]**	**SPME - (CAR/DVB/ PDMS) [14-22]**	**PDMS [23-25]**
Saturated straight chained aliphatic carboxylic acids						
Acetic acid	645	646	0.2	X		
Propanoic acid	704	715	0.005	X	X	
Butanoic acid	792	795	0.9		X	X
Hexanoic acid	974	973	0.1	X		X
Heptanoic acid	1066	1063	0.7	X		
Octanoic acid	1158	1158	0.2	X	X	X
Nonanoic acid	1255	1268	2.2	X		
Decanoic acid	1351	1347	8.5	X		
Dodecanoic acid	1546	1546	15.7	X		
Tridecanoic acid	1645	NA	9.6	X		
Tetradecanoic acid	1745	1748	12.9	X	X	X
Pentadecanoic acid	1842	1839	27.1	X		
Hexadecanoic acid	1945	1942	30.3	X		
Heptadecanoic acid	2045	2039	8.7	X		
Octadecanoic acid	2144	2142	12.5	X		
Saturated branched chained aliphatic carboxylic acids						
Butanoic acid, 3-methyl-	842	851	0.9	X		
Hexanoic acid, 2-ethyl-	1110	NA	NQ	X		
Unsaturated aliphatic carboxylic acids						
9,12-Octadecadienoic acid	2111	2106	2.8	X		
Aromatic carboxylic acids						
Benzoic acid	1154	1160	2.8	X		

***NA – Not Available, *NQ – Not Quantifiable**

Ketones which were identified as potential skin volatiles included a range of methyl-ketones from C_7 – C_{13} and C_{15}. The absence of 2–tetradecanone is consistent with skin volatile literature [26]. Straight chained and branched/unsaturated ketones have been previously identified as skin volatiles using a variety of sampling techniques. The odd numbered straight chained methyl ketones 2-nonanone, 2-undecanone, 2-tridecanone and 2-pentadecanone have been identified as volatiles released by bacteria found on the skin surface [35]. Among the branched, unsaturated methyl ketones 6-methyl-5-hepten-2-one has attracted interest as a potential olfactory cue for *An. gambiae* [30, 50-52] and *Ae. aegypti* [53] mosquitoes but has also been shown to exhibit a repellent effect [54]. Geranyl acetone and 6-methyl-3,5-heptadiene-2-one have been detected in incubated sweat samples which elicit a response in electroantennography (EAG) measurements for *An. gambiae* [50]. A

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group of various five member ring ketones were tentatively identified which have not been reported in skin volatile literature to the best of the authors' knowledge. These compounds, as well as 1-hydroxy-2-butanone, have previously been identified as bacterial natural products [33].

Table 7.2) Compounds containing a ketone moiety which were tentatively identified after GC×GC-ToF analysis of PDMS bracelets and anklets. Corresponding calculated and literature LTPRI values, as well as Fisher ratios for PDMS normalized analyte specific unique mass signal areas are provided. Comparison of the compounds detected using the new sampler to literature reports using alternative passive sampling methodologies are shown.

Ketones tentatively identified in skin samples				**Skin sampling techniques reported in literature**		
Identification	**LTPRI (Calc.)**	**LTPRI (Lit.) [5,9,36-48]**	**Fisher Ratio (PDMS)**	**Glass beads [12,13]**	**SPME - (CAR/DVB/ PDMS) [14-22]**	**PDMS [23-25]**
Straight chained aliphatic ketones						
2-Heptanone	869	863	2.5		X	
2-Octanone	969	966	NQ			X
2-Nonanone	1071	1070	NQ			
2-Decanone	1168	NA	7.0	X	X	
2-Undecanone	1270	1274	7.5			
2-Dodecanone	1374	1373	2.4			
2-Tridecanone	1473	NA	7.2			
2-Pentadecanone	1677	1682	21.8			
2-Hexadecanone	1777	1778	8.7			
Unsaturated straight chained aliphatic ketones						
5-Hepten-2-one, 6-methyl-	963	963	1.1	X	X	X
6-Methyl-3,5-heptadiene-2-one	1077	1076	25.2	X		
2-Undecanone, 6,10-dimethyl-	1385	NA	0.9			
Geranyl acetone	1429	1426	18.5		X	
5-member ring ketones						
Cyclopentanone	755	749	NQ			
2-Cyclopenten-1-one	806	799	1.1			
2-Cyclopenten-1-one, 3-methyl-	686	NA	NQ			
Cyclopent-4-ene-1,3-dione	850	846	10.6			
2-Cyclopenten-1-one, 2-methyl-	875	881	0.7			
2-Cyclopenten-1-one, 3-methyl-	931	923	0.1			
3-Methylcyclopentane-1,2-dione	997	NA	3.3			
1,2-Cyclopentanedione	895	NA	2.9			
Aromatic ketones						
Acetophenone	1032	1033	0.9		X	
Benzophenone	1584	1577	4.3			X
Other ketones						
1-Hydroxy-2-butanone	747	748	0.3			

***NA – Not Available, *NQ – Not Quantifiable**

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A range of short chain length saturated and unsaturated aldehydes were tentatively identified in the skin volatile samples (Table 7.3). Many of these had been previously identified by the collection of skin volatiles on SPME fibers making use of modified PDMS absorbant or PDMS patches in combination with GC-MS analysis [14-25]. Saturated straight chain aldehydes, with chain lengths C_7 – C_{10}, and benzaldehyde have been found to elicit a response in EAG experiments involving *Ae. aegypti* [53]. The abundance of these saturated aldehydes in the laboratory environment resulted in comparisons between the wrist and ankle not being achievable. This is consistent with the high emission rates for these compounds from human skin [88].

Table 7.3) Compounds containing an aldehyde moiety which were tentatively identified after GC×GC-ToF analysis of PDMS bracelets and anklets. Corresponding calculated and literature LTPRI values, as well as, Fisher ratios for PDMS normalized analyte specific unique mass signal areas are provided. Comparison of the compounds detected using the new sampler to literature reports using alternative passive sampling methodologies are shown.

Aldehydes tentatively identified in skin samples				**Skin sampling techniques reported in literature**		
Identification	**LTPRI (Calc.)**	**LTPRI (Lit.)** [9,36,37,40,45,55-62]	**FR (PDMS)**	**Glass beads** [12,13]	**SPME - (CAR/DVB/ PDMS)** [14-22]	**PDMS** [23-25]
Straight chained saturated aliphatic aldehydes						
Hexanal	773	780	NQ		X	X
Heptanal	876	874	NQ	X	X	X
Octanal	978	981	NQ	X	X	X
Nonanal	1081	1081	NQ	X	X	X
Decanal	1181	1184	NQ	X	X	X
Unsaturated straight chained aliphatic aldehydes						
2-Butenal	631	624	2.5		X	
2-Octenal	1032	1032	2.1		X	
2-Nonenal	1132	1137	0.1		X	
2-Decenal	1233	1229	12.4			
2-Undecenal	1345	1346	4.6			X
Other aldehydes						
Furfural	808	804	10.2		X	X
2-Furancarboxaldehyde, 5-methyl-	931	926	0.4			
Benzaldehyde	928	925	0.5		X	
Benzene-acetaldehyde	1007	1011	8.1		X	
***NA – Not Available, *NQ – Not Quantifiable**						

A range of aliphatic and aromatic ring containing alcohols were tentatively identified from the skin samples collected (Table 7.4). The majority of these have been identified in human skin emanations using a variety of sampling techniques. Benzyl

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alcohol and 1-butanol have been detected in the headspace of cultured *S. epidermis* [49]. The branched aliphatic alcohols 2-ethyl-1-hexanol and 2,6-dimethyl-7-Octen-2-ol have been shown to generate a response in EAG studies involving *Ae. aegypti* [53]. Compounds containing a phenol moiety have been found to generate a response in electrophysiological studies for *An. gambiae* [30].

Table 7.4) Compounds containing an alcohol moiety which were tentatively identified after GC×GC-ToF analysis of PDMS bracelets and anklets. Corresponding calculated and literature LTPRI values, as well as, Fisher ratios for PDMS normalized analyte specific unique mass signal areas are provided. Comparison of the compounds detected using the new sampler to literature reports using alternative passive sampling methodologies are shown.

Alcohols tentatively identified in skin samples				**Skin sampling techniques reported in literature**		
Identification	**LTPRI (Calc.)**	**LTPRI (Lit.) [8,37,48,63-68]**	**FR (PDMS)**	**Glass beads [12,13]**	**SPME - (CAR/DVB/ PDMS) [14-22]**	**PDMS [23-25]**
Straight chained and branched aliphatic alcohols						
1-Butanol	658	657	NQ		**X**	
1-Octanol	1055	1061	1.8		**X**	**X**
1-Nonanol	1152	1151	2.2			
1-Hexanol, 2-ethyl-	1013	1013	NQ	**X**	**X**	**X**
7-Octen-2-ol, 2,6-dimethyl-	1058	1058	2.8			
Aromatic ring containing alcohols						
Phenol	965	959	0.008	**X**	**X**	
Phenol, 2-methyl-	1034	1033	0.7			
Benzyl alcohol	1006	1007	1.6	**X**	**X**	
p-Cresol	1055	1051	0.3			
Ethanol, 2-phenoxy-	1184	1194	12.9		**X**	
Benzenemethanol, α,α-dimethyl-	1058	1057	NQ			

***NA – Not Available, *NQ – Not Quantifiable**

A variety of branched butenolides (Table 7.5) which have not been reported in skin emanations to the best of the authors' knowledge were tentatively identified in the skin samples. These compounds are found in the headspace of several *actinomyces* and *streptomyces* species of bacteria and are believed to play a role as signaling compounds by members of these genera [33].

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Table 7.5) Compounds containing a lactone moiety which were tentatively identified after GC×GC-ToF analysis of PDMS bracelets and anklets. Corresponding calculated and literature LTPRI values, as well as, Fisher ratios for PDMS normalized analyte specific unique mass signal areas are provided. Comparison of the compounds detected using the new sampler to literature reports using alternative passive sampling methodologies are shown.

Lactones tentatively identified in skin samples				Skin sampling techniques reported in literature		
Identification	**LTPRI (Calc.)**	**LTPRI (Lit.)** [37,55,58,63,69,70]	**FR (PDMS)**	**Glass beads** [12,13]	**SPME - (CAR/DVB/ PDMS)** [14-22]	**PDMS** [23-25]
Butanolides						
Butyrolactone	866	864	3.7		**X**	
Butenolides						
2(5H)-Furanone	868	871	11.0			
2(3H)-Furanone, 5-methyl-	838	836	0.002			
2(3H)-Furanone, 5-hexyldihydro-	1420	1414	8.1			
2(3H)-Furanone, dihydro-5-pentyl-	1315	1320	1.3			
2(3H)-Furanone, 5-heptyldihydro-	1529	1521	0.3			
2(3H)-Furanone, 5-butyldihydro-	1207	1208	3.4			
10-Methylundec-2-en-4-olide	2055	NA				

***NA – Not Available, *NQ – Not Quantifiable**

Terpenes have been reported in many skin volatile studies (Table 7.6). Members of this class are, however, abundant in fragrances and food stuff and thus most likely of exogenous origin [26]. α-Pinene has been detected in the headspace of human skin [21] and blood [72]. Studies have linked limonene to individuals who are poorly attractive to *An. gambiae* mosquitoes [13]. Linalool has been shown to be EAG active when tested on *Ae. aegypti* [53] and can be repellent for *An. gambiae* [54].

Table 7.6) Terpenes which were tentatively identified after GC×GC-ToF analysis of PDMS bracelets and anklets. Corresponding calculated and literature LTPRI values, as well as Fisher ratios for PDMS normalized analyte specific unique mass signal areas are provided. Comparison of the compounds detected using the new sampler to literature reports using alternative passive sampling methodologies are shown.

Terpenes tentatively identified in skin samples				Skin sampling techniques reported in literature		
Identification	**LTPRI (Calc.)**	**LTPRI (Lit.)** [71]	**FR (PDMS)**	**Glass beads** [12,13]	**SPME - (CAR/DVB/ PDMS)** [14-22]	**PDMS** [23-25]
Linalool	1084	1081	0.3		**X**	
α-Pinene	925	931	NQ		**X**	
Limonene	1019	1020	NQ	**X**	**X**	

***NA – Not Available, *NQ – Not Quantifiable**

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Compounds containing nitrogen are not commonly reported in skin volatile literature (Table 7.7). Benzonitrile and pyrrole have been reported in the headspace of medically important pathogenic bacteria species *Pseudomonas aeruginosa* [75]. Indole and 3-methylindole have been shown to elicit a response in EAG studies involving *An. gambiae* mosquitoes [30, 50] and indole has been found to have a concentration dependent role in the reduction of the attractiveness of an attractive mixture of skin derived compounds in behavioural experiments [51]. Olfactory neurons in *Ae. aegypti* and *An. gambiae* are sensitive to pyridine [76]. The tentatively identified 5,10-diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-α:1',2'-δ]pyrazine has not previously been reported in human skin secretions to the best of the authors' knowledge. It has, however, been reported as a bacterial natural product with antifungal activity [77].

Table 7.7) Compounds containing a nitrogen moiety which were tentatively identified after GC×GC-ToF analysis of PDMS bracelets and anklets. Corresponding calculated and literature LTPRI values, as well as Fisher ratios for PDMS normalized analyte specific unique mass signal areas are provided. Comparison of the compounds detected using the new sampler to literature reports using alternative passive sampling methodologies are shown.

Nitrogen containing compounds tentatively identified in skin samples				Skin sampling techniques reported in literature		
Identification	**LTPRI (Calc.)**	**LTPRI (Lit.)[44,58,74,77]**	**FR (PDMS)**	**Glass beads [12-13]**	**SPME - (CAR/DVB/ PDMS) [14-22]**	**PDMS [23-25]**
Benzonitrile	947	940	42.1			
Pyrrole	729	727	2.0			
Pyridine	726	726	2.9	X	X	
Indole	1252	1257	0.0	X		
Indole, 3-methyl-	1304	NA	1.5			
5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-α:1',2'-δ]pyrazine	1850	NA	0.3			

***NA – Not Available, *NQ – Not Quantifiable**

The organosulphide compound dimethyl sulfone (Table 7.8) has been found in melanocyte skin cells [79]. Benzothiazole has been detected in human skin volatiles [80] and together with dimethyl trisulphide in exhaled breath [81]. Benzothiazole elicits an EAG response for *An. gambiae* [82]. Bacterial action may convert methionine to dimethyl trisulphide [83] where an elevated level of this compound has been suggested as a marker for pathogenic *Pseudomonas aeruginosa* in sepsis [84].

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The tentative identifications of many compounds presented here are of analytes which have been previously identified in skin volatile literature as well as in studies investigating the volatiles emitted by skin borne bacteria species. However, the number of compounds tentatively identified in the skin samples reflects only a minority of peaks identified as potential skin volatiles using the untargeted data processing methodology. The absence of compounds which have previously been reported in the context of skin or resident skin bacteria studies is not indicative of the absence of the analyte from the samples as many peaks had MS matches below 75% and therefore were not included here. The full peak tables of potential skin volatiles including Fisher ratios are included in Appendix A3. Many unidentified peaks have Fisher ratios indicative of differences in abundances between the two sampled regions. In addition, bacterial volatiles collected from *in vitro* cultures are influenced by experimental parameters such as the culture medium used [85] and may not correspond to the volatile profile of *in vivo* colonies.

Table 7.8) Compounds containing a sulphur moiety which were tentatively identified after GC×GC-ToF analysis of PDMS bracelets and anklets. Corresponding calculated and literature LTPRI values, as well as Fisher ratios for PDMS normalized analyte specific unique mass signal areas are provided. Comparison of the compounds detected using the new sampler to literature reports using alternative passive sampling methodologies are shown.

Sulphur containing compounds tentatively identified				**Skin sampling techniques reported in literature**		
Identification	**LTPRI (Calc.)**	**LTPRI (Lit.)[78]**	**FR (PDMS)**	**Glass beads [12,13]**	**SPME - (CAR/DVB/ PDMS) [14-22]**	**PDMS [23-25]**
Dimethyl sulfone	872	NA	56.4			
Dimethyl trisulphide	940	943	0.6			
Benzothiazole	1187	1187	10.7		X	

***NA – Not Available, *NQ – Not Quantifiable**

7.3 Application study: Mosquito olfactory cues

Comparison of the relative abundances of chemical compounds which have been identified as invoking either an electrophysiological or behavioural response from anthropophilic blood-host seeking mosquitoes is summarized in Figure 7.1.

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Differences in the relative abundances of compounds between different skin regions have the potential to affect the attractiveness of different skin regions to blood-host seeking mosquitoes [86]. The comparison of log normalized average analyte peak areas reveals clear differences and similarities in abundances of the selected compounds between the wrist and ankles of the sampled individual. Aliphatic carboxylic acids (Green block) with short chain lengths, C_2 – C_9, were found to have very similar abundances in both sampled regions while longer chain lengths, C_{10} – C_{16}, were more abundant in the wrist sample. Interestingly, tetradecanoic acid (C_{14}) has been found to play an important role in the formulation of synthetic blends which are attractive to the major malaria vector *An. gambiae* [87]. Among the ketones and alcohols (purple and orange blocks respectively) geranyl acetone, 6-methyl-3,5-heptadiene-2-one and 2-phenoxy-ethanol were more abundant in the wrist sample than the ankle. All of the compounds compared have %RSDs below 40% for both wrist and ankle samples, however, greater numbers of samples should be analyzed in order to calculate the statistical significance of differences in compound abundances between the two sampled regions.

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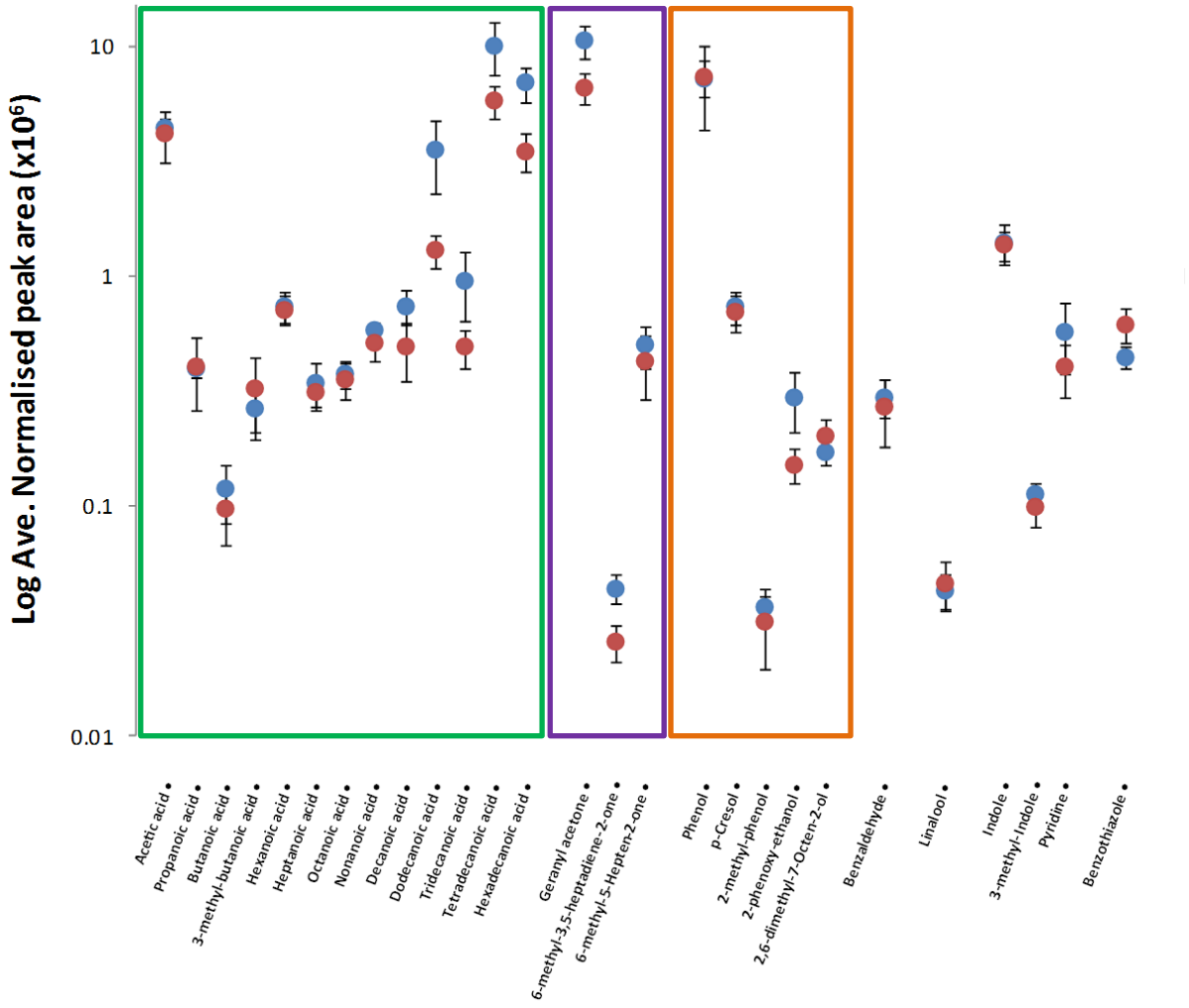

Figure 7.1) Graph comparing the log average normalized peak areas between wrist and ankle samples of compounds potentially associated with behavioural or electrophysiological responses by anthropophilic mosquito species. Carboxylic acids, ketones and alcohols are labelled using green, purple and orange blocks respectively.

7.4 References

[1] Lanzaro GC, Lee Y. Speciation in *Anopheles gambiae* — The Distribution of Genetic Polymorphism and Patterns of Reproductive Isolation Among Natural Populations. Anopheles Mosquitoes - New insights into malaria vectors. 2013. p. 173–196.

[2] Powell JR, Tabachnick WJ. History of domestication and spread of *Aedes aegypti*--a review. Mem Inst Oswaldo Cruz. 2013;108(August):11–17.

[3] Ciccioli P, Cecinato A, Brancaleoni E, Frattoni M, Liberti A. Use of carbon adsorption traps combined with high resolution gas chromatography - mass spectrometry for the analysis of polar and non-polar C4-C14 hydrocarbons involved in photochemical smog formation. J Hi Res Chromatogr. 1992;15(2):78–84.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

[4] Buttery RG, Ling LC, Stern DJ. Studies on popcorn aroma and flavor volatiles. J Agric Food Chem. 1997;45(3):837–843.

[5] Habu T, Flath RA, Mon TR, Morton JF. Volatile components of Rooibos tea (Aspalathus linearis). J Agric Food Chem. 1985;33(2):249–254.

[6] Osorio C, Alarcon M, Moreno C, Bonilla A, Barrios J, Garzon C, et al. Characterization of Odor-Active Volatiles in Champa (Campomanesia lineatifolia R. P.). J Agric Food Chem. 2006;54(2):509–516.

[7] Iranshahi M, Amin G, Sourmaghi MS, Shafiee A, Hadjiakhoondi A. Sulphur-containing compounds in the essential oil of the root of Ferula persica Wild. var. persica. Flavour Fragr J. 2006;21(2):260–261.

[8] Dib ME, Djabou N, Desjobert L-M, Allali, H.; Tabti B, Muselli A, Costa J. Characterization of volatile compounds of Daucus crinitus Desf. headspace solid phase microextraction as alternative technique to hydrodistillation. Chem, Centr J. 2010;4(16):1–15.

[9] Paolini J, Muselli A, Bernardini A-F, Bighelli A, Casanova J, Costa J. Thymol derivatives from essential oil of Doronicum corsicum L. Flavour Fragr J. 2007;22(6):479–487.

[10] Johnson CI, Urso A, Geleta L. Broad spectrum analysis of municipal and industrial effluents discharged into the Peace, Athabasca and Slave river basins: characterization of effluent samples, 1994 - Volume 1 of 2, Northern River Basins Study Project Report No. 121. Norther River Basins Study. 1997.

[11] Richmond R, Pombo-Villar E. Use of persistent trace gas chromatography artifacts for the calculation of pseudo-Sadtler retention indices. J Chromatogr A. 1998;811(1-2):241–245.

[12] Bernier UR, Kline DL, Barnard D, Schreck C, Yost RA, Barnard DR, et al. Analysis of Human Skin Emanations by Gas Identification of Volatile Compounds That Are Candidate Attractants for the Yellow Fever Mosquito (*Aedes aegypti*). Anal Chem. 2000;72(4):747–756.

[13] Verhulst NO, Beijleveld H, Qiu YT, Maliepaard C, Verduyn W, Haasnoot GW, et al. Relation between HLA genes, human skin volatiles and attractiveness of humans to malaria mosquitoes. Infect Genet Evol. 2013;18:87–93.

[14] Dormont L, Bessiere J-M, McKey D, Cohuet A. New methods for field collection of human skin volatiles and perspectives for their application in the chemical ecology of human-pathogen-vector interactions. J Exp Biol. 2013;216(15):2783–2788.

[15] Zhang ZM, Cai JJ, Ruan GH, Li GK. The study of fingerprint characteristics of the emanations from human arm skin using the original sampling system by SPME-GC/MS. J Chromatogr B Anal Technol Biomed Life Sci. 2005;822(1-2):244–252.

[16] Curran AM, Ramirez CF, Schoon AA, Furton KG. The frequency of occurrence and discriminatory power of compounds found in human scent across a population determined by SPME-GC/MS. J Chromatogr B Anal Technol Biomed Life Sci. 2007;846(1-2):86–97.

[17] Mochalski P, King J, Unterkofler K, Hinterhuber H, Amann A. Emission rates of selected volatile organic compounds from skin of healthy volunteers. J Chromatogr B Anal Technol Biomed Life Sci. 2014;959:62–70.

[18] Acevedo CA, Sanchez EY, Reyes JG, Young ME. Volatile profiles of human skin cell cultures in different degrees of senescence. J Chromatogr B Anal Technol Biomed Life Sci. 2010;878(3-4):449–455.

[19] Prada PA, Curran AM, Furton KG. The evaluation of human hand odor volatiles on various textiles: A comparison between contact and noncontact sampling methods. J Forensic Sci. 2011;56(4):866–881.

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

[20] de la Mata AP, McQueen RH, Nam SL, Harynuk JJ. Comprehensive two-dimensional gas chromatographic profiling and chemometric interpretation of the volatile profiles of sweat in knit fabrics. Anal Bioanal Chem. Analytical and Bioanalytical Chemistry. 2016;1905–1913.

[21] Curran AM, Rabin SI, Prada P a., Furton KG. Comparison of the volatile organic compounds present in human odor using SPME-GC/MS. J Chem Ecol. 2005;31(7):1607–1619.

[22] Curran AM, Prada PA, Furton KG. The differentiation of the volatile organic signatures of individuals through SPME-GC/MS of characteristic human scent compounds. J Forensic Sci. 2010;55(1):50–57.

[23] Penn DJ, Oberzaucher E, Grammer K, Fischer G, Soini HA, Wiesler D, et al. Individual and gender fingerprints in human body odour. J R Soc Interface. 2007;4(13):331–340.

[24] Jiang R, Cudjoe E, Bojko B, Abaffy T, Pawliszyn J. A non-invasive method for in vivo skin volatile compounds sampling. Anal Chim Acta. 2013;804(August):111–119.

[25] Riazanskaia S, Blackburn G, Harker M, Taylor D, Thomas CLP. The analytical utility of thermally desorbed polydimethylsilicone membranes for in-vivo sampling of volatile organic compounds in and on human skin. Analyst. 2008;133(8):1020-1027.

[26] de Lacy Costello B, Amann A, Al-Kateb H, Flynn C, Filipiak W, Khalid T, et al. A review of the volatiles from the healthy human body. J Breath Res. 2014;8(1):014001.

[27] Knols BGJ, van Loon JJA, Cork A, Robinson RD, Adam W, Meijerink J, et al. Behavioural and electrophysiological responses of the female malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) to Limburger cheese volatiles. Bull Entomol Res. 1997;87(02):151-159.

[28] Van Den Broek IVF, Den Otter CJ. Olfactory sensitivities of mosquitoes with different host preferences (*Anopheles gambiae* s.s., An. arabiensis, An. quadriannulatus, An. m. atroparvus) to synthetic host odours. J Insect Physiol. 1999;45(11):1001–1010.

[29] Smallegange RC, Qiu YT, van Loon JA, Takken W. Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae* sensu stricto (Diptera: Culicidae). Chem Senses. 2005;30(2):145–152.

[30] Qiu YT, van Loon JJA, Takken W, Meijerink J, Smid HM. Olfactory coding in antennal neurons of the malaria mosquito, *Anopheles gambiae*. Chem Senses. 2006;31(9):845–863.

[31] Smallegange RC, Qiu YT, Bukovinszkiné-Kiss G, Van Loon JJA, Takken W. The effect of aliphatic carboxylic acids on olfaction-based host-seeking of the malaria mosquito *Anopheles gambiae* sensu stricto. J Chem Ecol. 2009;35(8):933–43.

[32] Okumu FO, Killeen GF, Ogoma S, Biswaro L, Smallegange RC, Mbeyela E, et al. Development and field evaluation of a synthetic mosquito lure that is more attractive than humans. PLoS One. 2010;5(1):1-7

[33] Citron CA, Rabe P, Dickschat JS. The scent of bacteria: Headspace analysis for the discovery of natural products. J Nat Prod. 2012;75(10):1765–1776.

[34] James AG, Casey J, Hyliands D, Mycock G. Fatty acid metabolism by cutaneous bacteria and its role in axillary malodour. World J Microbiol Biotechnol. 2004;20(8):787–793.

[35] Lemfack MC, Ravella SR, Lorenz N, Kai M, Jung K, Schulz S, et al. Novel volatiles of skin-borne bacteria inhibit the growth of Gram-positive bacteria and affect quorum-sensing controlled phenotypes of Gram-negative bacteria. Syst Appl Microbiol. 2016;39(8):503–515.

[36] DeMilo AB, Lee C-J, Moreno DS, Martinez AJ. Identification of volatiles derived from Citrobacter freundii fermentation of a trypticase soy broth. J Agric Food Chem. 1996;44(2):607–612.

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

[37] Sun G, Stremple P. Retention Index Characterization of Flavor, Fragrance and Many Other Compounds on DB-1 and DB-XLB. J&W Scientific. 2003.

[38] Paolini J, Desjobert J.M, Costa J, Bernardini A.F, Castellini C.B, Cioni P.L, Flamini G, Morelli I. Composition of essential oils of Helichrysum italicum (Roth) G. Don fil subsp. italicum from Tuscan archipelago islands. Flavour Fragr J. 2006;21(5):805–808.

[39] Stashenko EE, Villa HS, Combariza MY. Comparative study of Colombian rue oils by high resolution gas chromatography using different detection systems. J Microcolumn Sep. 1995;7(2):117–122.

[40] Paolini J, Costa J, Bernardini A. Analysis of the essential oil from aerial parts of Eupatorium cannabinum subsp. corsicum (L.) by gas chromatography with electron impact and chemical ionization mass spectrometry. J Chromatogr A. 2005;1076(1-2):170–178.

[41] de Souza PP, Cardeal ZD, Augusti R, Morrison P, Marriott PJ. Determination of volatile compounds in Brazilian distilled cachaca by using comprehensive two-dimensional gas chromatography and effects of production pathways. J Chromatogr A. 2009;1216(14):2881-2890.

[42] Cozzani S, Muselli A, Desjobert J-M, Bernardini A-F, Tomi F, Casanova J. Chemical composition of essential oil of Teucrium polium subsp. capitatum (L.) from Corsica. Flavour Fragr J. 2005;20(4):436–441.

[43] Muselli A, Rossi P-G, Desjobert J-M, Bernardini A-F, Berti L, Costa J. Chemical composition and antibacterial activity of Otanthus maritimus (L.) Hoffmanns. Link essential oils from Corsica. Flavour Fragr J. 2007;22(3):217–223.

[44] Helmig D, Pollock W, Greenberg J, Zimmerman P. Gas chromatography mass spectrometry analysis of volatile organic trace gases at Mauna Loa Observatory, Hawaii. J Geophys Res. 1996;101(D9):14697–14710.

[45] Park B-S, Lee K-G, Takeoka GR. Comparison of three sample preparation methods on the recovery of volatiles from taheebo (Tabebuia impetiginosa Martius ex DC). Flavour Fragr J. 2004;19(4):287–292.

[46] Tai C-Y, Ho C-T. Influence of glutathione oxidation and pH on thermal formation of Maillard-type volatile compounds. J Agric Food Chem. 1998;46(6):2260–2265.

[47] Lu G, Yu T-H, Ho C-T. Generation of flavor compounds by the reaction of 2-deoxyglucose with selected amino acids. J Agric Food Chem. 1997;45(1):233–236.

[48] Lee K-G, Lee S-E, Takeoka GR, Kim J-H, Park B-S. Antioxidant activity and characterization of volatile constituents of beechwood creosote. J Sci Food Agric. 2005;85(9):1580–1586.

[49] Verhulst NO, Beijleveld H, Knols BG, Takken W, Schraa G, Bouwmeester HJ, et al. Cultured skin microbiota attracts malaria mosquitoes. Malar J. 2009;8:302.

[50] Dekker T, Posthumus M a, Beek T a V a N. Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. J Chem Ecol. 2000;26(6):1367–1382.

[51] Qiu YT, Smallegange RC, Van Loon JJA, Takken W. Behavioural responses of *Anopheles gambiae* sensu stricto to components of human breath, sweat and urine depend on mixture composition and concentration. Med Vet Entomol. 2011;25(3):247–255.

[52] Hiscox A, Otieno B, Kibet A, Mweresa CK, Omusula P, Geier M, et al. Development and optimization of the Suna trap as a tool for mosquito monitoring and control. Malar J. 2014;13(1):257.

UNIVERSITEIT VAN PRETORIA
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[53] Logan JG, Birkett MA, Clark SJ, Powers S, Seal NJ, Wadhams LJ, et al. Identification of human-derived volatile chemicals that interfere with attraction of *Aedes aegypti* mosquitoes. J Chem Ecol. 2008;34(3):308–322.

[54] Menger DJ, Van Loon JJA, Takken W. Assessing the efficacy of candidate mosquito repellents against the background of an attractive source that mimics a human host. Med Vet Entomol. 2014;28(4):407–413.

[55] Bendiabdellah A, El Amine Dib M, Djabou N, Allali H, Tabti B, Costa J, et al. Biological activities and volatile cinstituents of Daucus muricatus L. from Algeria. Chem Centr J. 2012;6(48):1–22.

[56] Peng CT. Prediction of retention indices. V. Influence of electronic effects and column polarity on retention index. J Chromatogr A. 2000;903(1-2):117–143.

[57] Rapior S, Breheret S, Talou T, Bessiére J-M. Volatile flavor constituents of fresh Marasmius alliaceus (garlic Marasmius). J Agric Food Chem. 1997;45(3):820–825.

[58] Kim JS. Einfluss der Temperatur beim Rösten von Sesam auf Aroma und antioxidative Eigenschaften des Öls. Technischen Universität Berlin zur Erlangung des akademischen Grades; 2001.

[59] Lin J, Rouseff RL. Characterization of aroma-impact compounds in cold-pressed grapefruit oil using time-intensity GC-olfactometry and GC-MS. Flavour Fragr J. 2001;16(6):457–463.

[60] Wu C-M, Wang Z, Wu QH. Volatile compounds produced from monosodium glutamate in common food cooking. J Agric Food Chem. 2000;48(6):2438–2442.

[61] Zhang Y, Ho C-T. Formation of meatlike aroma compounds from thermal reaction of inosine 5'-monophosphate with cysteine and glutathione. J Agric Food Chem. 1991;39(6):1145–1148.

[62] de Feo V, Bruno M, Tahiri B, Napolitano F, Senatore F. Chemical composition and antibacterial activity of essential oils from Thymus spinulosus Ten. (Lamiaceae). J Agric Food Chem. 2003;51(13):3849–3853.

[63] Takeoka G, Perrino, C. J, Buttery R. Volatile constituents of used frying oils. J Agric Food Chem. 1996;44(3):654–660.

[64] Brat P, Rega B, Alter P, Reynes M, Brillouet J-M. Distribution of volatile compounds in the pulp, cloud, and serum of freshly squeezed orange juice. J Agric Food Chem. 2003;51(11):3442–3447.

[65] Mattinen M-L, Tuominen J, Saarela K. Analysis of TVOC and certain selected compounds from indoor air using GC/FID-RIM technique. Indoor Air. 1995;5(1):56–61.

[66] Chang LP, Sheng LS, Yang MZ, An DK. Retention index of essential oil in temperature-programmed capillary column gas chromatography. Acta Pharm Sin. 1989;24(11):847–852.

[67] Coen M, Engel R, Nahrstedt A. Chavicol β-D-glucoside, a phenylpropanoid heteroside, benzyl-β-D-glucoside and glycosidically bound volatiles from subspecies of Cedronella canariensis. Phytochemistry. 1995;40(1):149–155.

[68] Ciccioli P, Cecinato A, Brancaleoni E, Frattoni M, Liberti A. Use of carbon adsorption traps combined with high resolution gas chromatography - mass spectrometry for the analysis of polar and non-polar C4-C14 hydrocarbons involved in photochemical smog formation. J Hi Res Chromatogr. 1992;15(2):75–84.

[69] Lee C-J, DeMilo AB, Moreno DS, Mangan RL. Identification of the volatile components of E802 Mazoferm steepwater, a condensed fermented corn extractive highly attractive to the Mexican fruit fly (Diptera: Tephritidae). J Agric Food Chem. 1997;45(6):2327–2331.

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UNIVERSITY OF PRETORIA
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[70] Chen J, Ho C-T. Volatile compounds generated in serine-monosaccharide model systems. J Agric Food Chem. 1998;46(4):1518–1522.

[71] Bendahou M, Muselli A, Grignon-Dubois M, Benyoucef M, Desjobert J-M, Bernardini A-F, et al. Antimicrobial activity and chemical composition of Origanum glandulosum Desf. essential oil and extract obtained by microwave extraction: Comparison with hydrodistillation. Food Chem. 2008;106(1):132–139.

[72] Deng C, Zhang X, Li N. Investigation of volatile biomarkers in lung cancer blood using solid-phase microextraction and capillary gas chromatography-mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2004;808(2):269–277.

[73] Bartelt RJ. Calibration of a commercial solid-phase microextraction device for measuring headspace concentrations of organic volatiles. Anal Chem. 1997;69(3):364–372.

[74] Zhang Y, Chien M, Ho.C.-T. Comparison of the volatile compounds obtained from thermal degradation of cysteine and glutathione in water. J Agric Food Chem. 1988;36(5):992–996.

[75] Bean HD, Dimandja JD, Hill JE. Bacterial volatile discovery using solid phase microextraction and comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry. J Chromatogr B. 2012;901:41–46.

[76] Tauxe GM, Macwilliam D, Boyle SM, Guda T, Ray A. Targeting a dual detector of skin and CO2 to modify mosquito host seeking. Cell. 2013;155(6):1365–79.

[77] Li H, Liu L, Zhang S, Cui W, Lv J. Identification of antifungal compounds produced by Lactobacillus casei AST18. Curr Microbiol. 2012;65(2):156–161.

[78] Rochat S, Laumer JY de Saint, Chaintreau A. Analysis of sulfur compounds from the in-oven roast beef aroma by comprehensive two-dimensional gas chromatography. J Chromatogr A. 2007;1147(1):85–94.

[79] Kwak J, Gallagher M, Ozdener MH, Wysocki CJ, Goldsmith BR, Isamah A, et al. Volatile biomarkers from human melanoma cells. J Chromatogr B Anal Technol Biomed Life Sci. 2013;931:90–96.

[80] Dormont L, Bessière J, McKey D, Cohuet A. New methods for field collection of human skin volatiles and perspectives for their application in the chemical ecology of human-pathogen-vector interactions. J Exp Biol. 2013;216(Pt 15):2783–2788.

[81] Caldeira M, Perestrelo R, Barros AS, Bilelo MJ, Morête A, Câmara JS, et al. Allergic asthma exhaled breath metabolome : A challenge for comprehensive two-dimensional gas chromatography. J Chromatogr A. 2012;1254:87–97.

[82] Qiu YT, Smallegange RC, Smid H, Loon JJ a Van, Galimard A, Posthumus M a, et al. GC-EAG analysis of human odours that attract the malaria mosquito *Anopheles gambiae* sensu stricto. Proc Netherlands Entomol Meet. 2004;15:59–64.

[83] Schulz S, Dickschat JS. Bacterial volatiles: the smell of small organisms. Nat Prod Rep. 2007;24(4):814.

[84] Bos LDJ, Sterk PJ, Schultz MJ. Volatile Metabolites of Pathogens: A Systematic Review. PLoS Pathog. 2013;9(5):1–8.

[85] Tait E, Perry JD, Stanforth SP, Dean JR. Identification of Volatile Organic Compounds Produced by Bacteria Using HS-SPME-GC – MS. 2014;363–373.

[86] Verhulst NO, Weldegergis BT, Menger D, Takken W. Attractiveness of volatiles from different body parts to the malaria mosquito Anopheles coluzzii is affected by deodorant compounds. Sci Rep. 2016;6(January):27141.

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[87] van Loon JJA, Smallegange RC, Bukovinszkiné-Kiss G, Jacobs F, De Rijk M, Mukabana WR, et al. Mosquito Attraction: Crucial Role of Carbon Dioxide in Formulation of a Five-Component Blend of Human-Derived Volatiles. J Chem Ecol. 2015;41(6):567–573.

[88] Mochalski P, King J, Unterkofler K, Hinterhuber H, Amann A. Emission rates of selected volatile organic compounds from skin of healthy volunteers. J Chromatogr B Anal Technol Biomed Life Sci. 2014;959:62–70.

[89] Filipiak W, Mochalski P, Troppmair J, Unterkofler K, Agapiou A, Davis C, et al. A compendium of volatile organic compounds (VOCs) released by human cell lines. Curr Med Chem. 2016;23(999):1–1

[90] Vlaeminck, B., Fievez, V., Cabrita, A. R. J., Fonseca, A. J. M. & Dewhurst, R. J. Factors affecting odd- and branched-chain fatty acids in milk: A review. *Animal Feed Science and Technology* 131, 389–417 (2006).

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Chapter Eight

Conclusions, recommendations and future work

Contents

7.1 Conclusions and recommendations

The initial evaluation of the effects of wearing passive samplers in direct contact or non-contact arrangements with respect to the skin surface for a range of sampling durations, presented in Chapter 6, was undertaken with the objective of exploring parameters affecting the sampling procedure. The aim of which was to ensure that the widest range of analytes were collected while preserving the resolution of chromatographic peaks and simplicity of mass spectra that would facilitate aspects of the GC×GC-MS data processing such as peak finding and alignment. This work indicated that the separation conditions employed were adequate for the analysis of skin volatiles collected in the passive samplers. Sampling times of up to nine hours could be used without difficulties arising due to saturation of the MS detector by major analyte peaks given the large sorptive volumes of the samplers employed. Evaluation of the total number of peak entries collected after different durations of sampling in both sampler arrangements revealed that four hours of sampling is sufficient to collect a large number of volatiles. Further investigation of the correlation between the total number of analytes collected and the partitioning characteristics of the analytes into PDMS indicated the important role that the temperature may play during sampling, with slightly lower temperatures apparently allowing for greater retention of volatile analytes which did not readily partition into PDMS. This effect

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can potentially be used to reduce sampling durations required in future investigations. It also emphasises the importance of the contribution of temperature during sampling and for samples being compared.

An important aspect of the second project aim was the correct alignment of chromatographic peaks in the data processing of the method. The performance of the software was assessed based on the percentage of missing peak entries relative to the total number of peak table entries after different durations of sampling. The number of missing peak table entries was found to have a correlation with the total number of chromatographic peaks and therefore the duration of sampling. Visual inspection of chromatograms obtained, as well as assesment of the total number (Section 6.2.2) and retention times (Appendix A3) of compounds collected at inceasing time increments, lead to the conclusion that little improvement was gained by placing samplers in a non-contact arrangement compared to those placed in direct skin contact. The simpler method of sampling, involving the samplers being placed in direct contact with the skin surface was thus preferred. It is, however, crucial to examine and assess the quality of the chromatographic data presented to the software prior to peak alignment to ensure good results are obtained.

The second part of the experimentation involved the collection of skin volatiles from an individual's wrist and ankle and was undertaken using the conditions recommended by the first set of experiments.

The application of global normalization constants to compensate for the differences in sorptive volumes and surface areas of the bracelet and anklet samplers was an important aspect of the sampling methodology. Normalization of each sampler's peak area signals based on the mass of PDMS in each sampler provides an easy way to normalize corresponding peak areas from different samples. This approach has the disadvantage of not taking the effect of sample storage or fluctuations in instrument performance into account during normalization. The alternative global normalization method which is popular among metabolomics studies is derived from the mass spectral total useful signal (MSTUS) of each sample. By using threshold limits on the variability of peak areas included in this calculation one can omit peak areas corresponding to chromatographic peaks with poor reproducibility and has the

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potential to yield more accurate normalization results. Investigation of the distribution of variances revealed that a threshold value for the RSD of 75% for the inclusion of peaks in the MSTUS calculation provides similar or improved results to normalization using the mass of each PDMS sampler.

The use of principal components analyses guided by Fisher ratio calculations, making use of the most unique quantitative ion signal area for analytes found in wrist and ankle samples, allowed for the visualization of the data post normalization. This revealed that both strategies for normalization show potential for facilitating the comparison of samples. The two different approaches to normalization provide different relationships between the bracelet and anklet normalization constants in each case. This highlights the effect normalization can have of post-acquisition data analysis and the quality of results obtained. The selection of an appropriate method for deriving normalization constants will benefit from a more detailed validation making use of reference standards of known concentration.

The passive sampling technique performs favourably in a real life sampling situation with 60% of peaks having peak area %RSDs below 30% when normalized to the mass of PDMS of each sampler and this strategy for normalization was used during the investigation of potential applications of the new passive sampling methodology (Chapter 7). Feature detection based on the combination of analysis of variances between samples, by application of Fisher ratios, and principal component analysis (PCA), for the reduction in dimensionality of the results, provides an effective way to use the newly developed sampling method as a metabolomics investigative tool.

The number of chromatographic peaks found across skin samples and qualitative investigation of many of these peaks, presented in Chapter 7, highlights the broad range of analytes that are found in skin emanations and detectable using GC×GC-ToFMS. Several compounds which were detected have not been previously found in skin emanations to the best of the authors' knowledge. Many compounds which were identified have links to active fields of research which require semi-quantitative analyses to be performed.

This was emphasised by application of the sampling method to study and comparison of the relative abundances of potential human semiochemicals which

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may be used by female members of anthropophilic mosquito species during blood-host selection. The data presented highlights the ability of the sampling method to identify chemical compounds which differ in abundance between the wrist and ankle of a sampled individual. The good precision of the results (RSDs<45%) obtained for analyte abundance is an improvement over current skin volatile analyses found in the academic literature.

The application of this technique also provides a potential tool for the *in vivo* study of the complex volatile profiles emitted by skin borne bacteria which may help researchers understand the effects of disease and medical treatments such as antibiotic usage on the human microbiome.

These results in combination with the cost effectiveness of the sampler design highlight the utility of the new method aimed at large scale metabolomics studies.

7.2 Future work

The simplicity of the investigated sampling procedure along with the increased total number of chromatographic peaks separated and detected, when compared to current sampling and analysis approaches, shows promise for this new method's implementation in studies of human skin emanations. The method will benefit from improvements to the sampling methodology which reduces the number of chromatographic peaks corresponding to compounds present in the external sampling environment. This will allow for easier identification of skin volatiles and improved quantitative results for some analytes. Assessment of the uptake rates of skin volatiles by PDMS can be investigated in more detail by collecting replicate samples at increasing time intervals. Improved data interpretation can be implemented by addressing aspects such as peak area normalization and the possible inclusion of internal standards in the passive sampler material. The inclusion of high-resolution mass spectrometry for the identification of analytes will aid the qualitative application of the technique by facilitating volatile identification and discovery of compounds which lack mass spectra library data.

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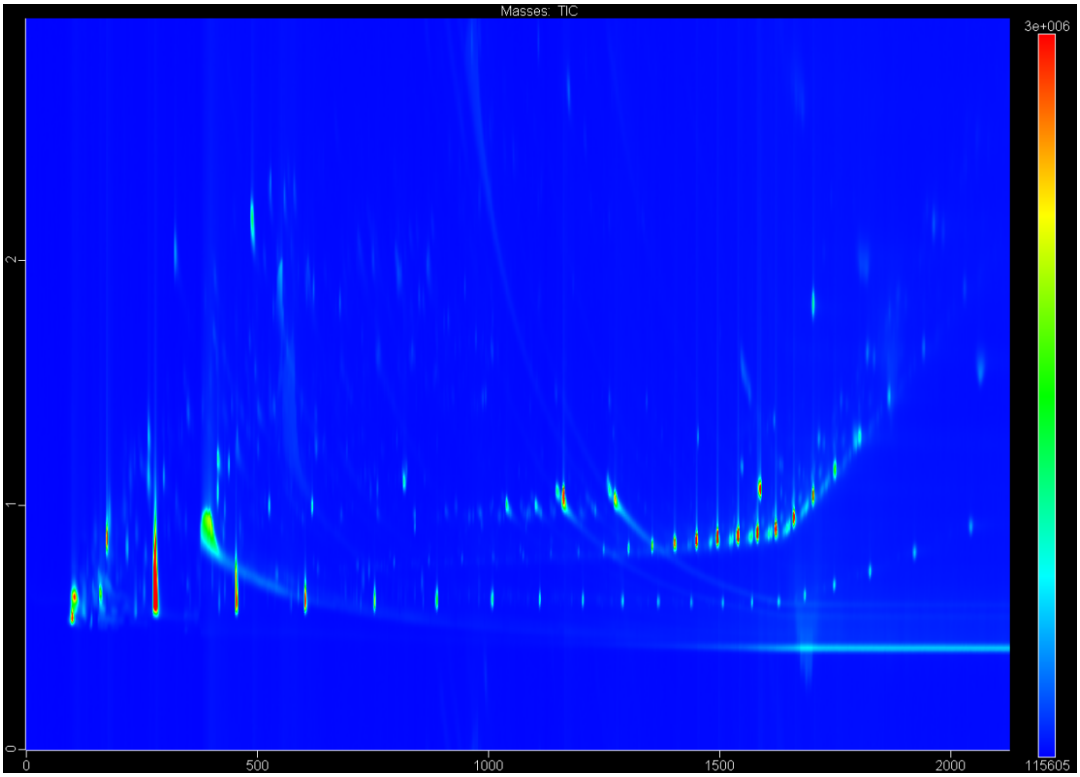
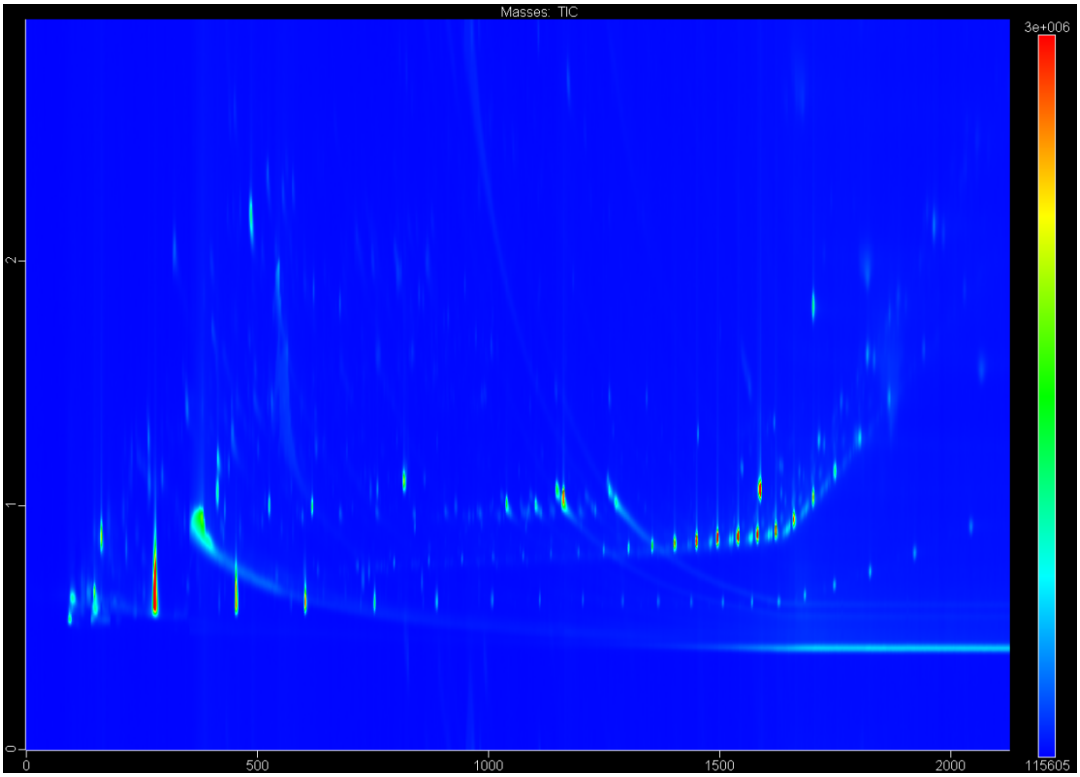
APPENDIX A1: Contour plots of total ion chromatograms (GC×GC-ToFMS) collected for the comparison of contact and non-contact samplers over a range of sampling durations.



Chromatogram A1.1 Direct skin contact sampler 1, (1.5 hours).



Chromatogram A1.2 Direct skin contact sampler 2, (3.0 hours).

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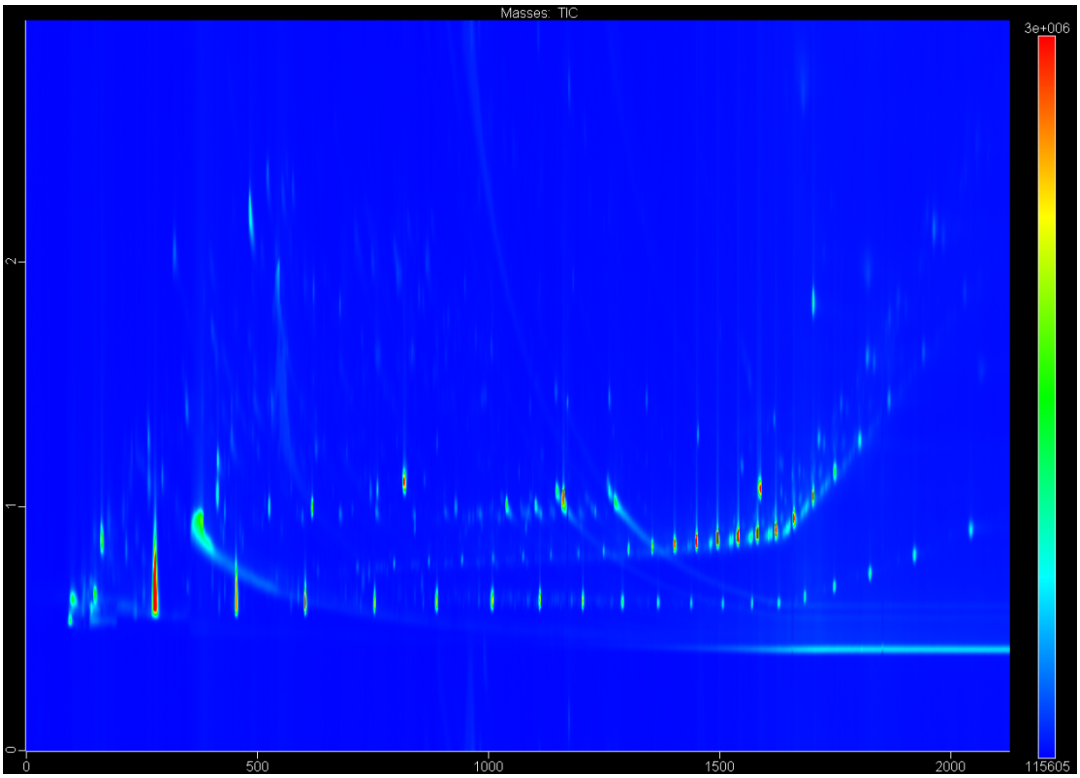
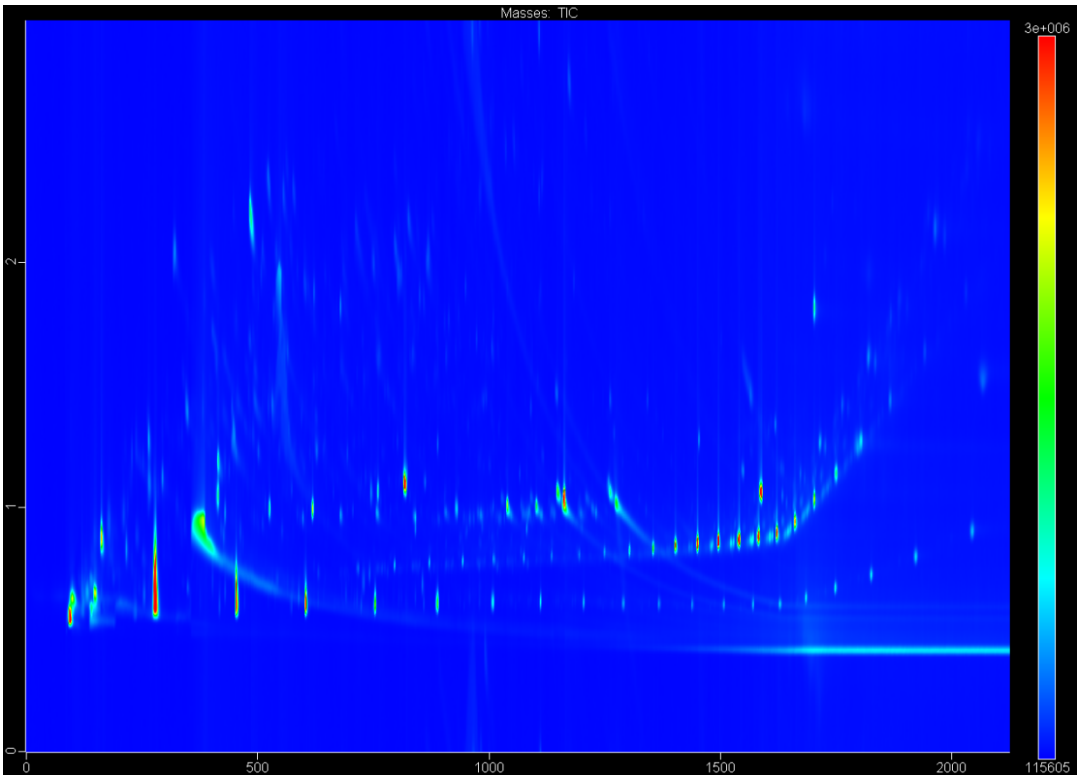


Chromatogram A1.3 Direct skin contact sampler 3, (4.5 hours).



Chromatogram A1.4 Direct skin contact sampler 4, (6.0 hours).

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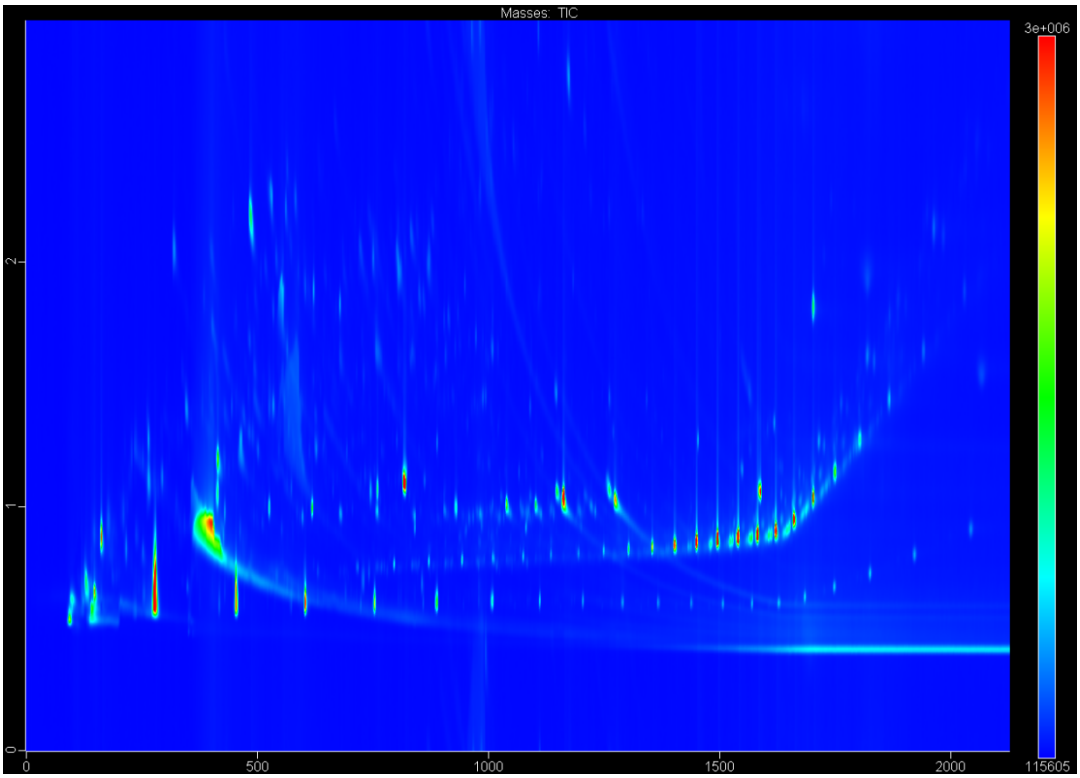
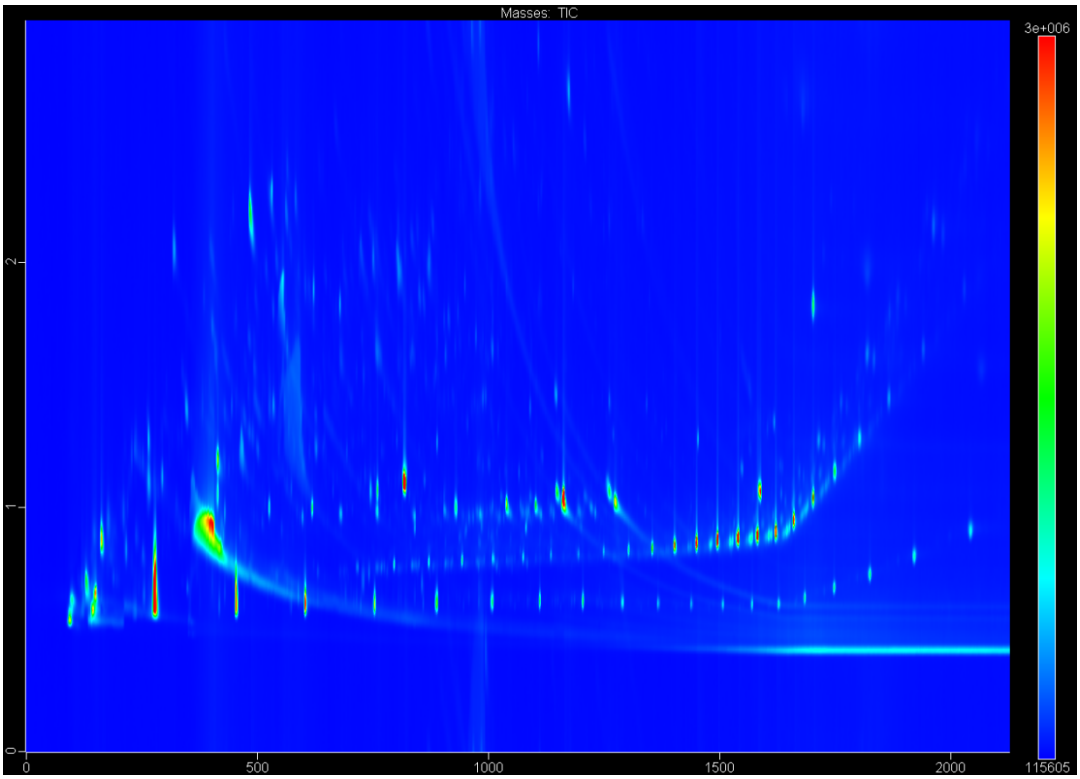


Chromatogram A1.5 Direct skin contact sampler 5, (7.5 hours).



Chromatogram A1.6 Direct skin contact sampler 6, (9.0 hours).

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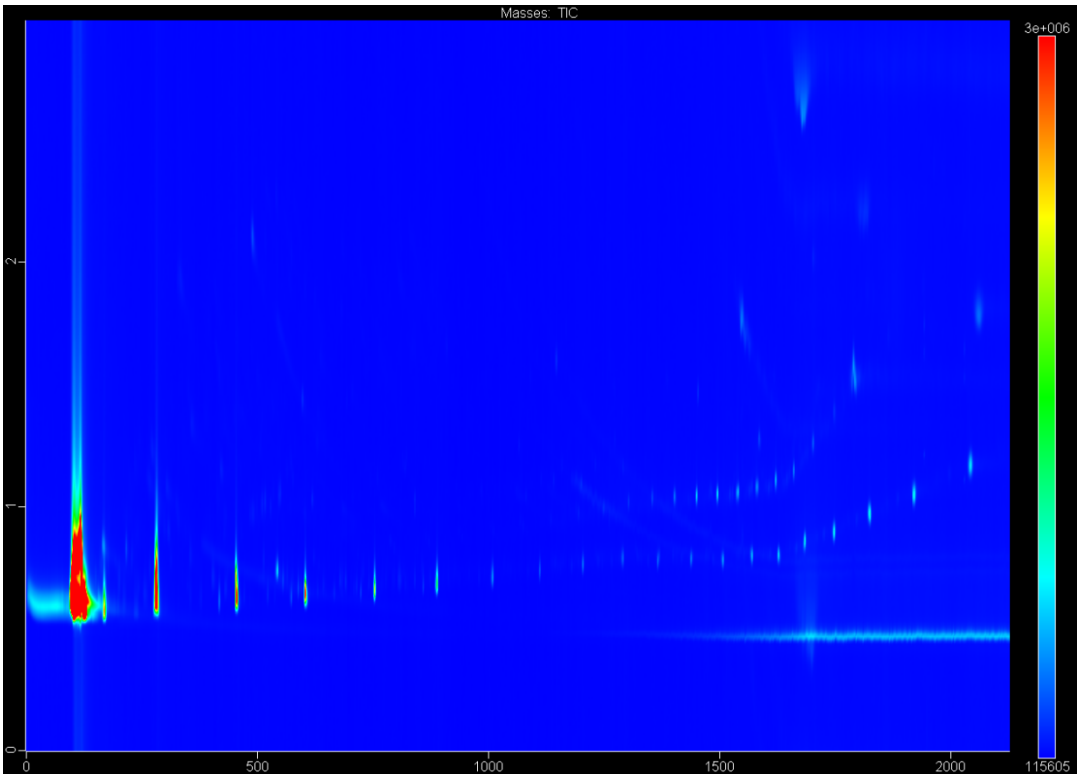
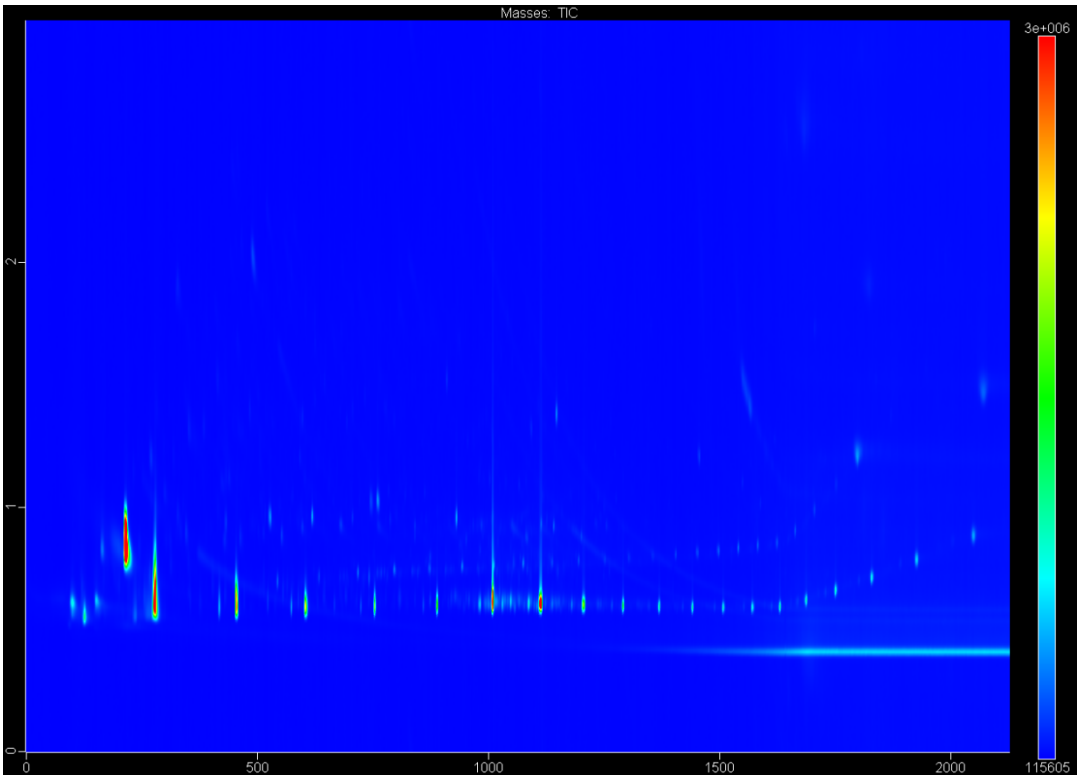


Chromatogram A1.7 Direct skin contact sampler blank.



Chromatogram A1.8 Laboratory blank 1.

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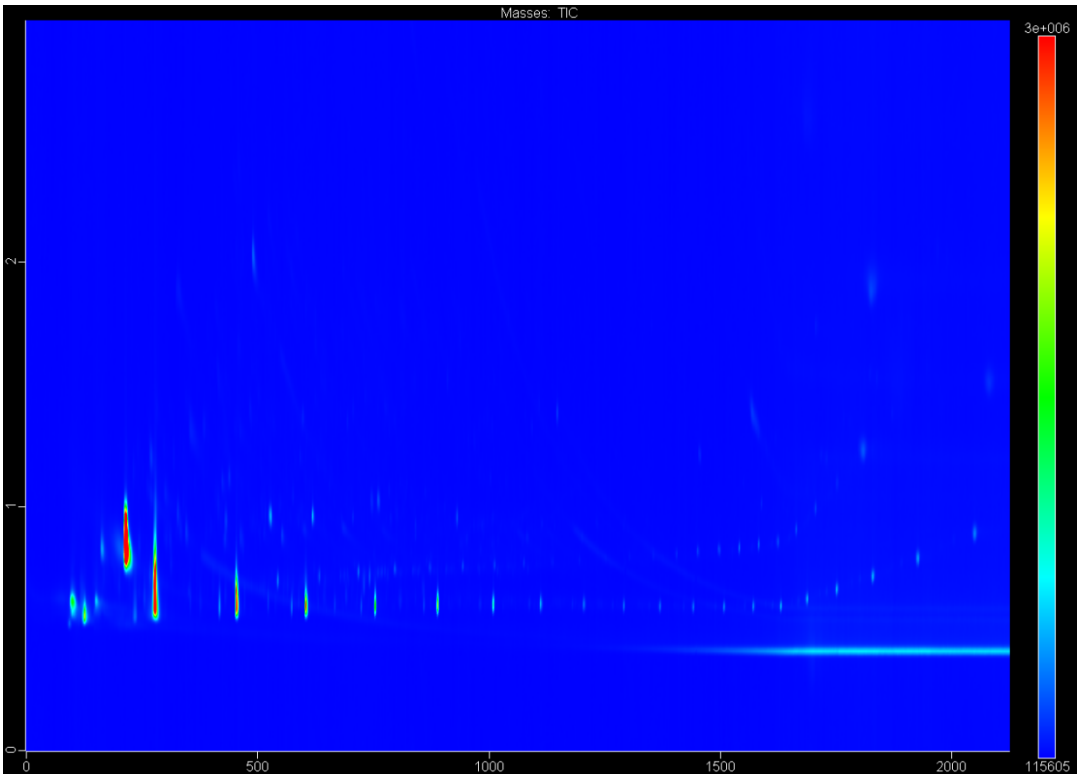
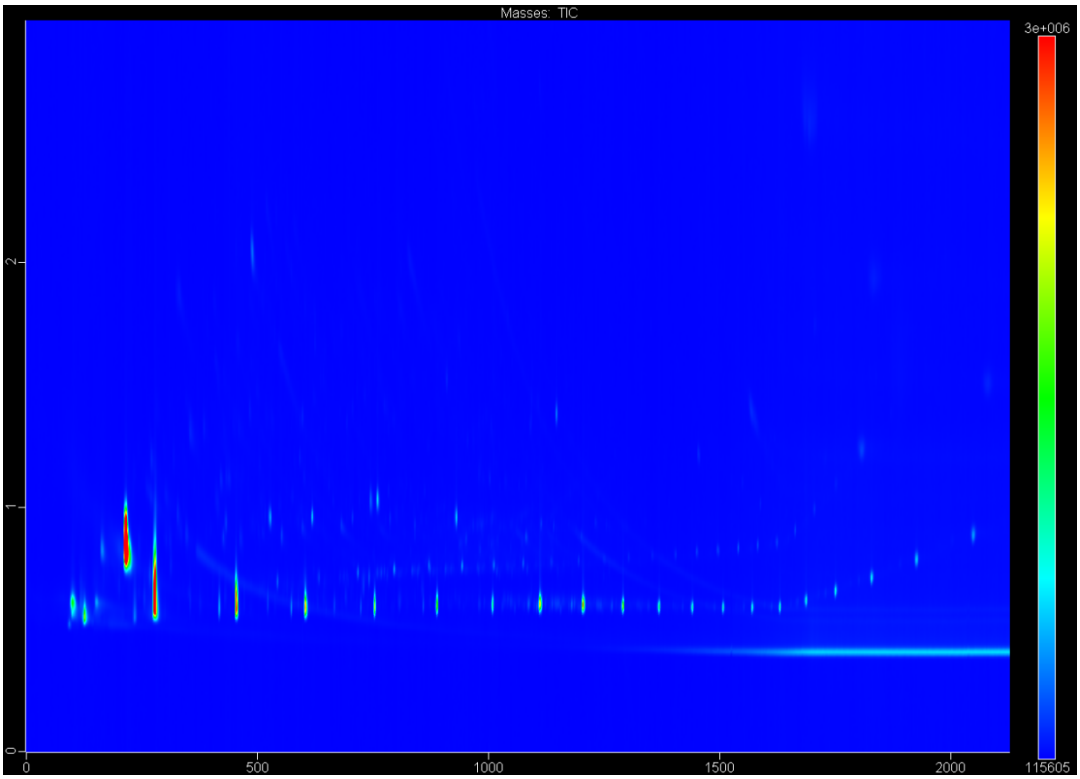


Chromatogram A1.9 Laboratory blank 2.



Chromatogram A1.10 Laboratory blank 3.

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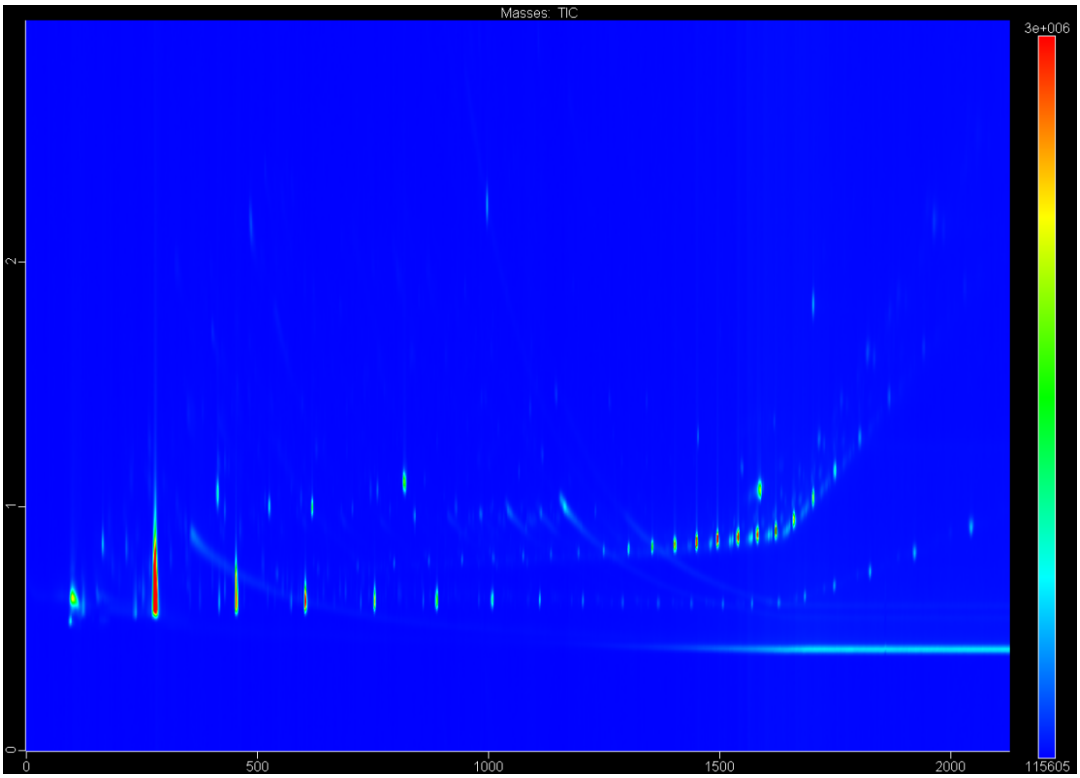
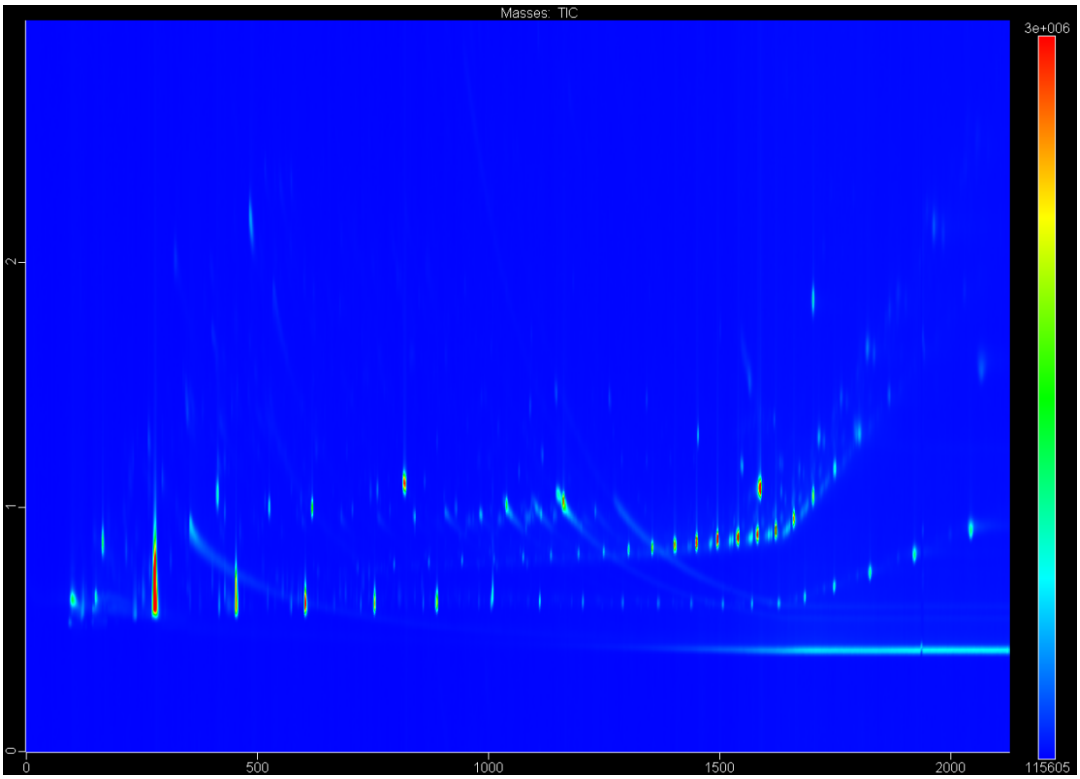


Chromatogram A1.11 Non-contact skin sampler 1 (1.5 hours).



Chromatogram A1.12 Non-contact skin sampler 2 (3.0 hours).

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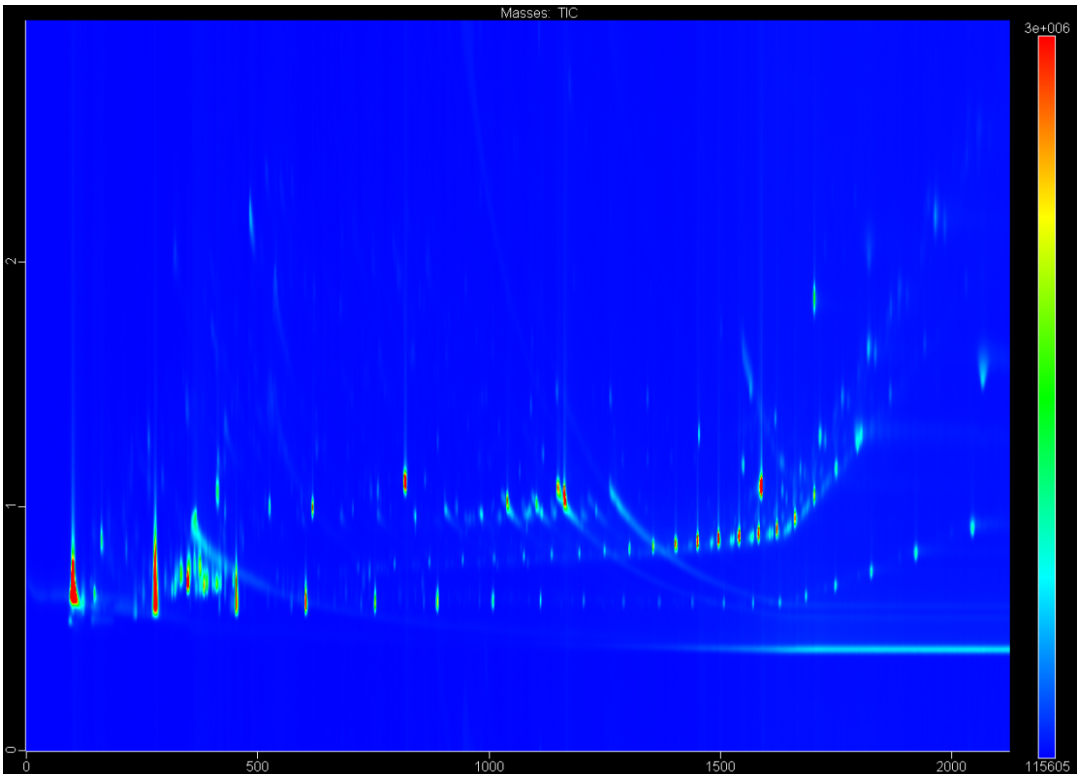
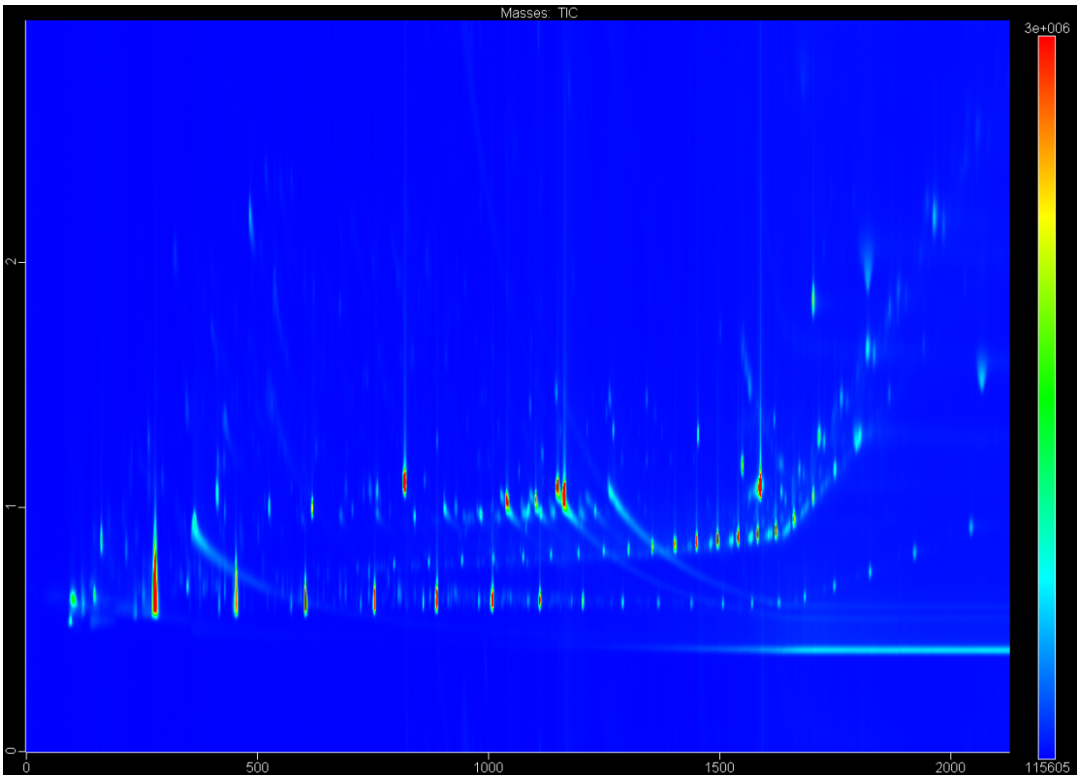


Chromatogram A1.13 Non-contact skin sampler 3 (4.5 hours).



Chromatogram A1.14 Non-contact skin sampler 4 (6.0 hours).

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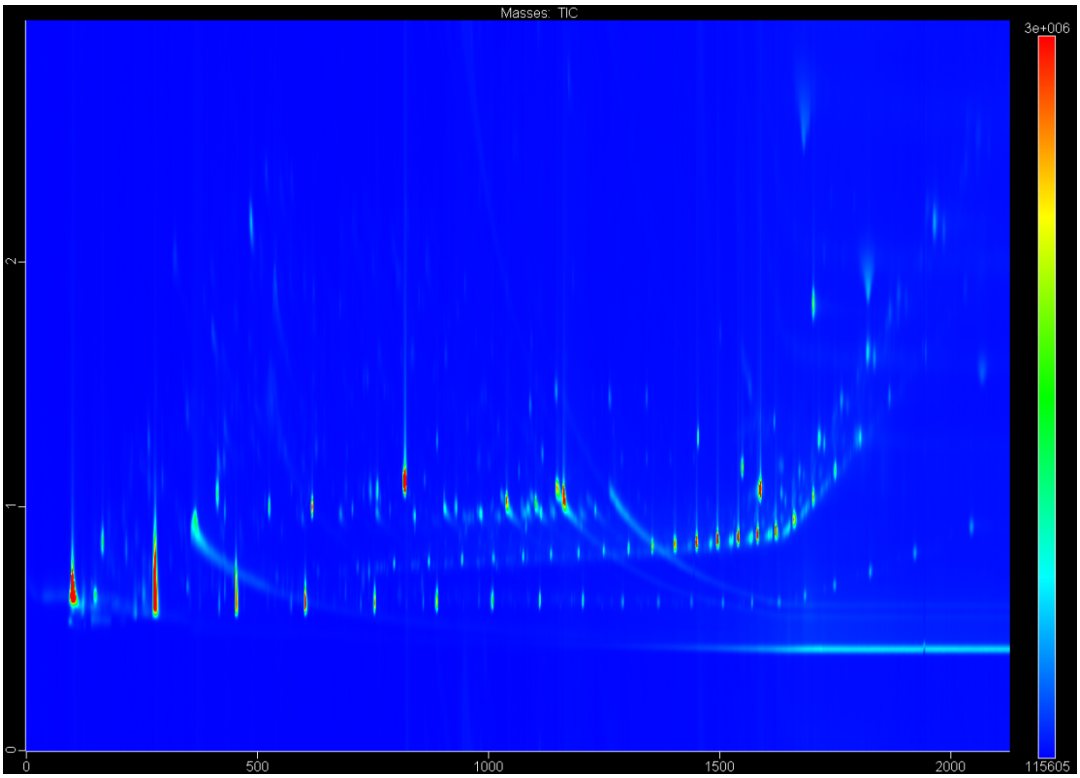
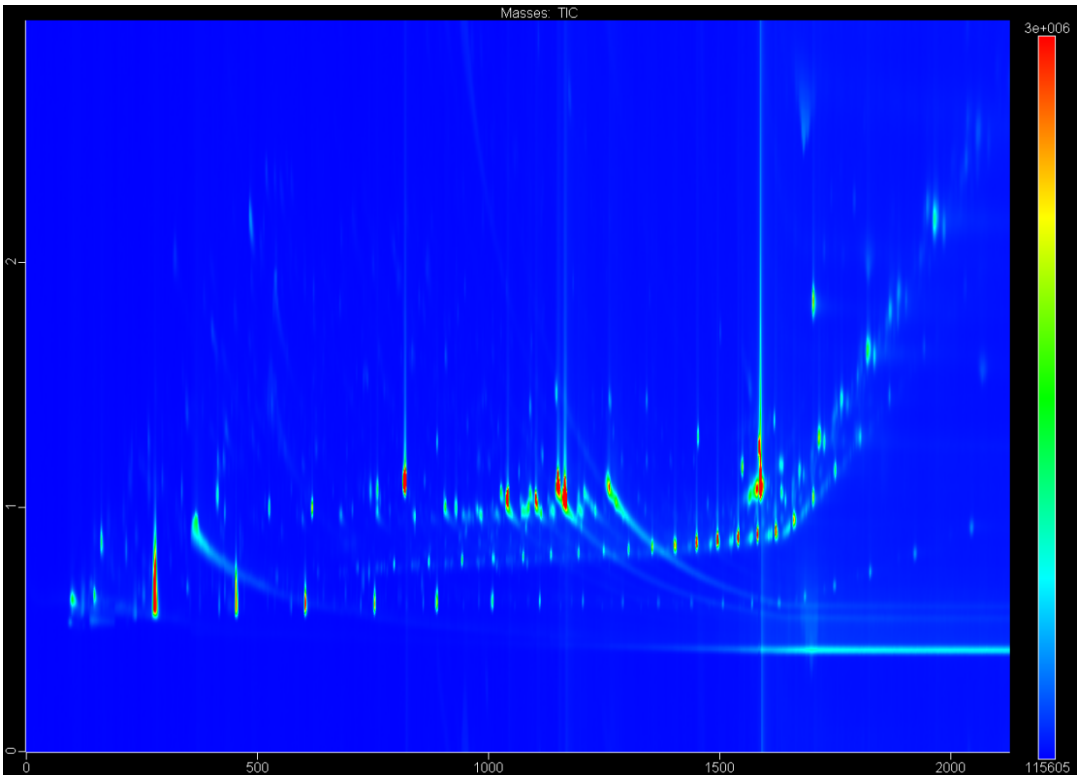


Chromatogram A1.15 Non-contact skin sampler 5 (7.5 hours).



Chromatogram A1.16 Non-contact skin sampler 6 (9.0 hours).

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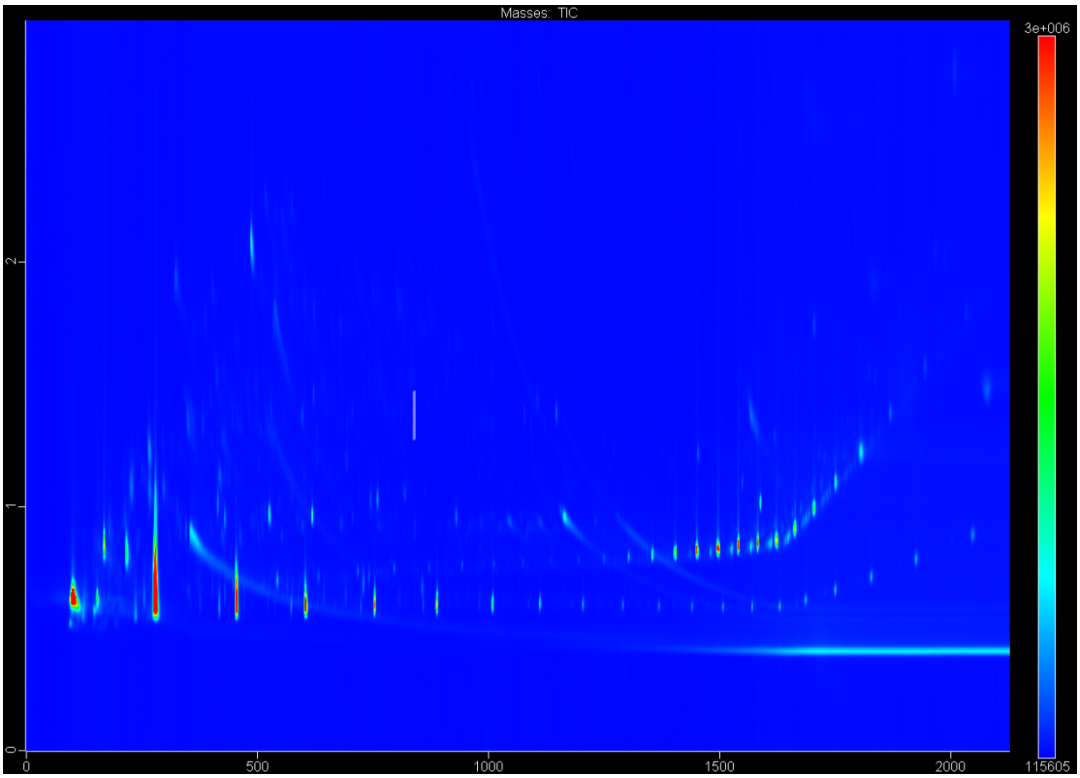
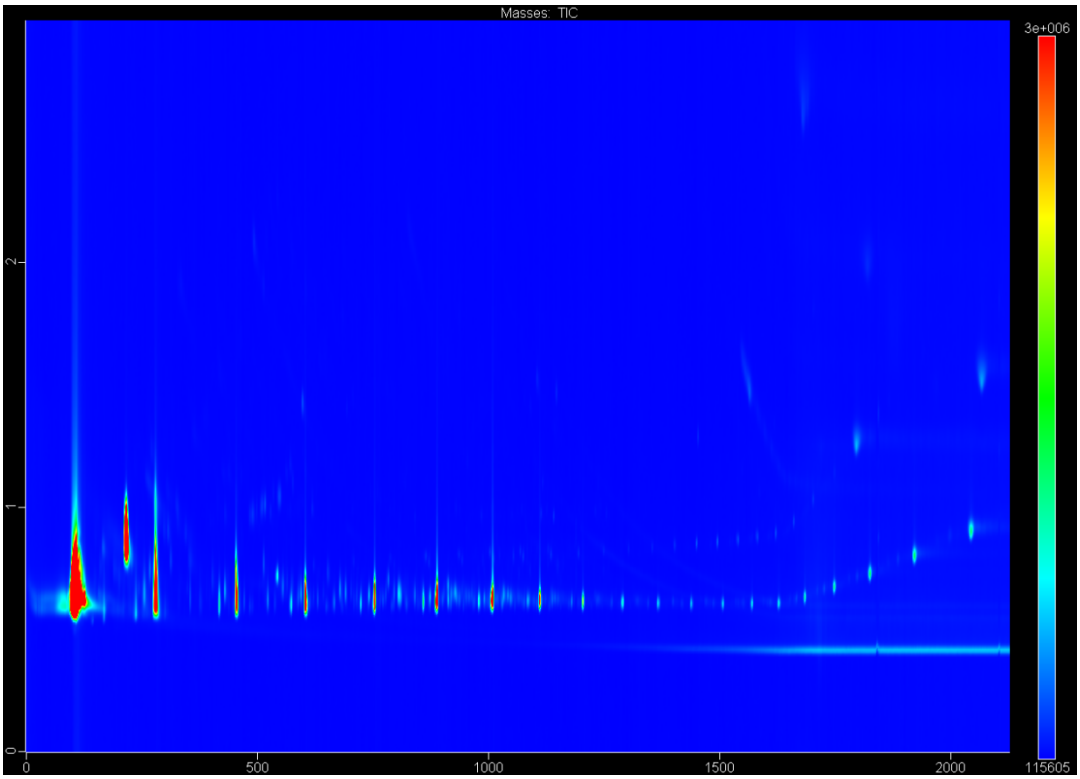


Chromatogram A1.17 Non-contact skin sampler aluminium bracelet background.



Chromatogram A1.18 Non-contact skin sampler bracelet background.

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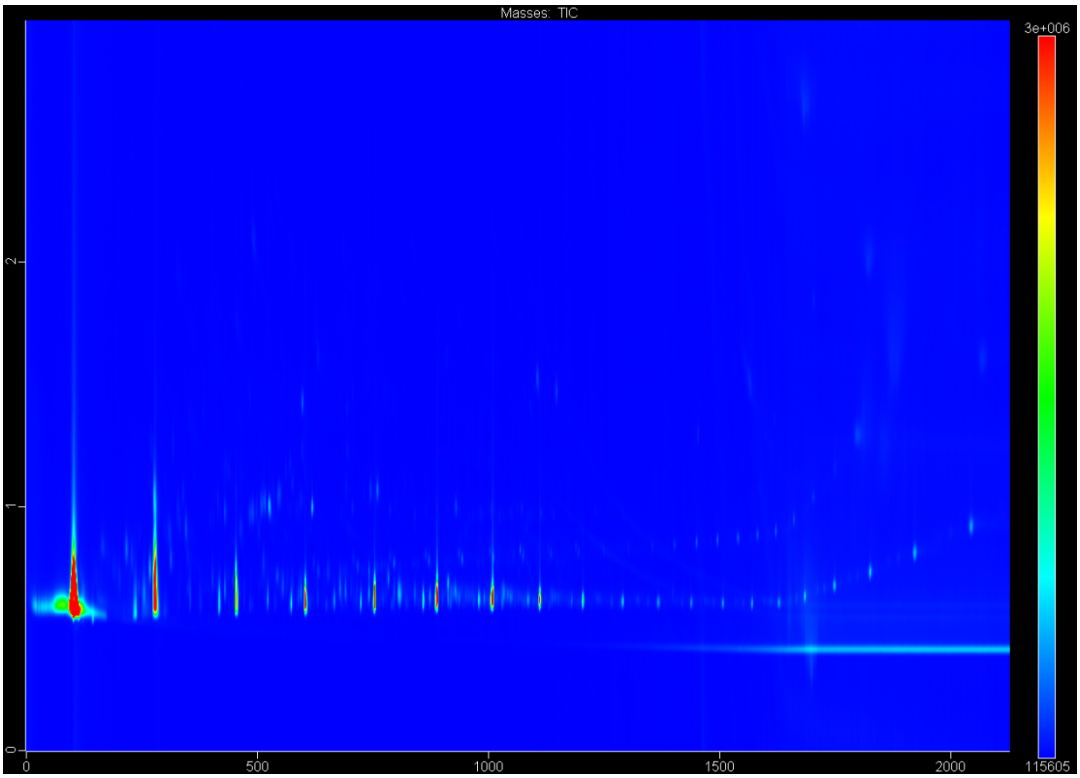
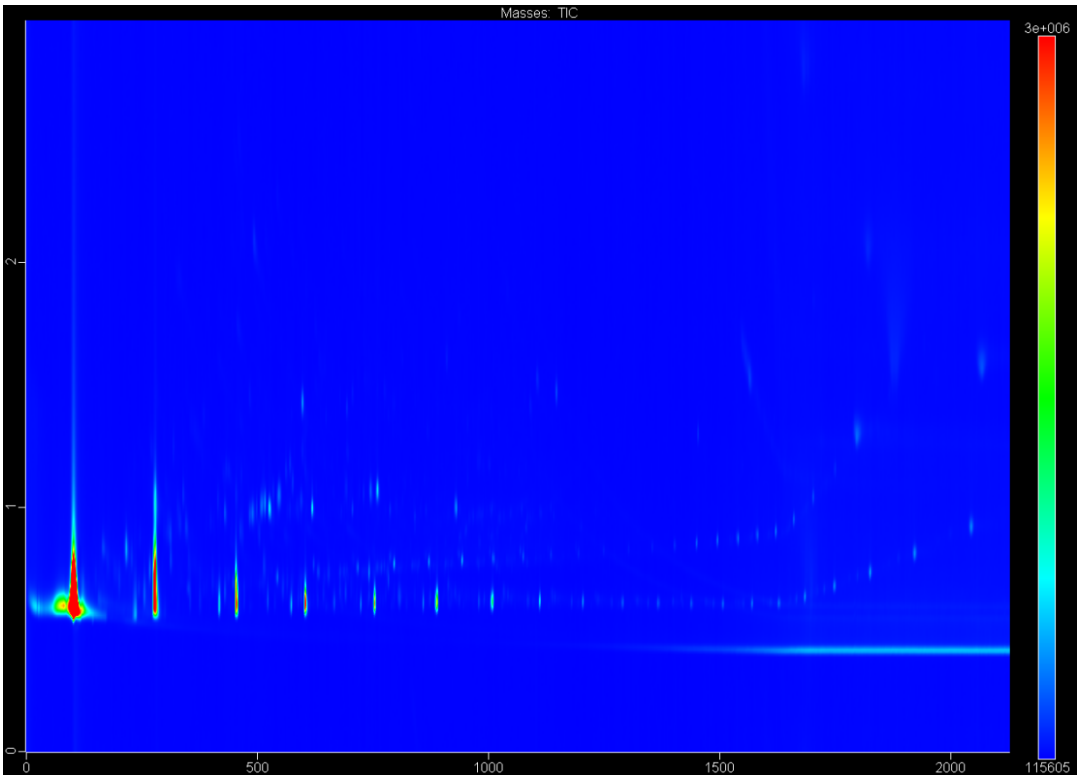


Chromatogram A1.19 Non-contact skin sampler laboratory background 1.



Chromatogram A1.20 Non-contact skin sampler laboratory background 2.

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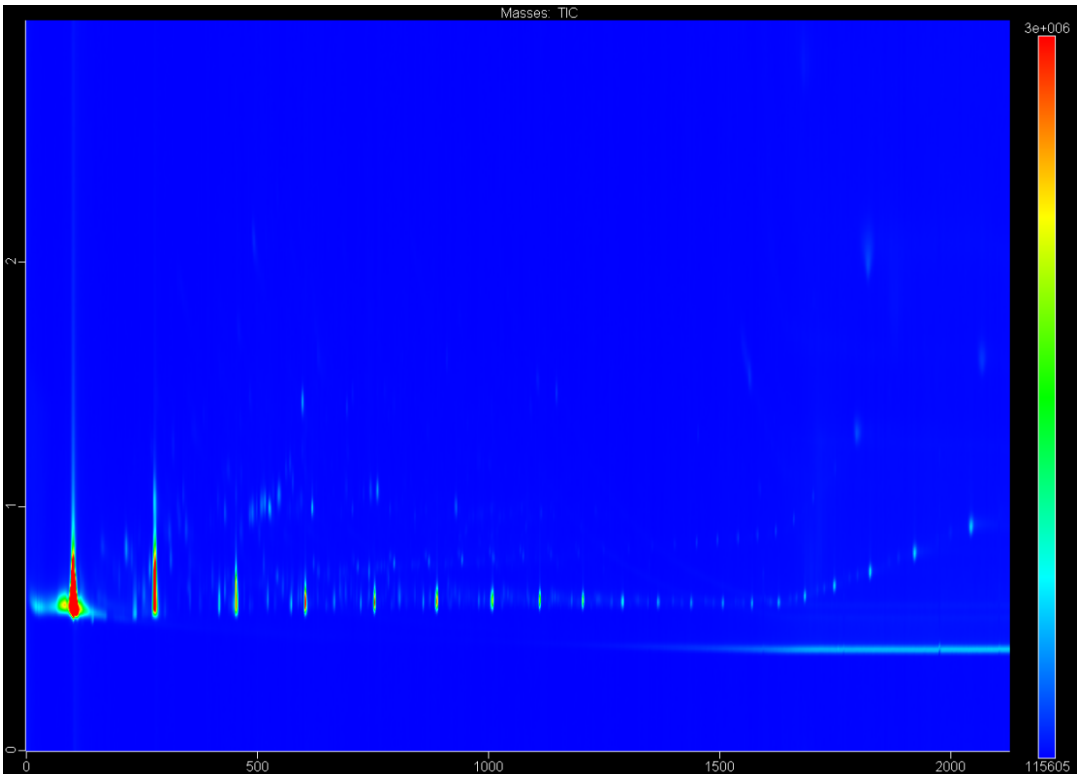

Chromatogram A1.21 Non-contact skin sampler laboratory background 3.

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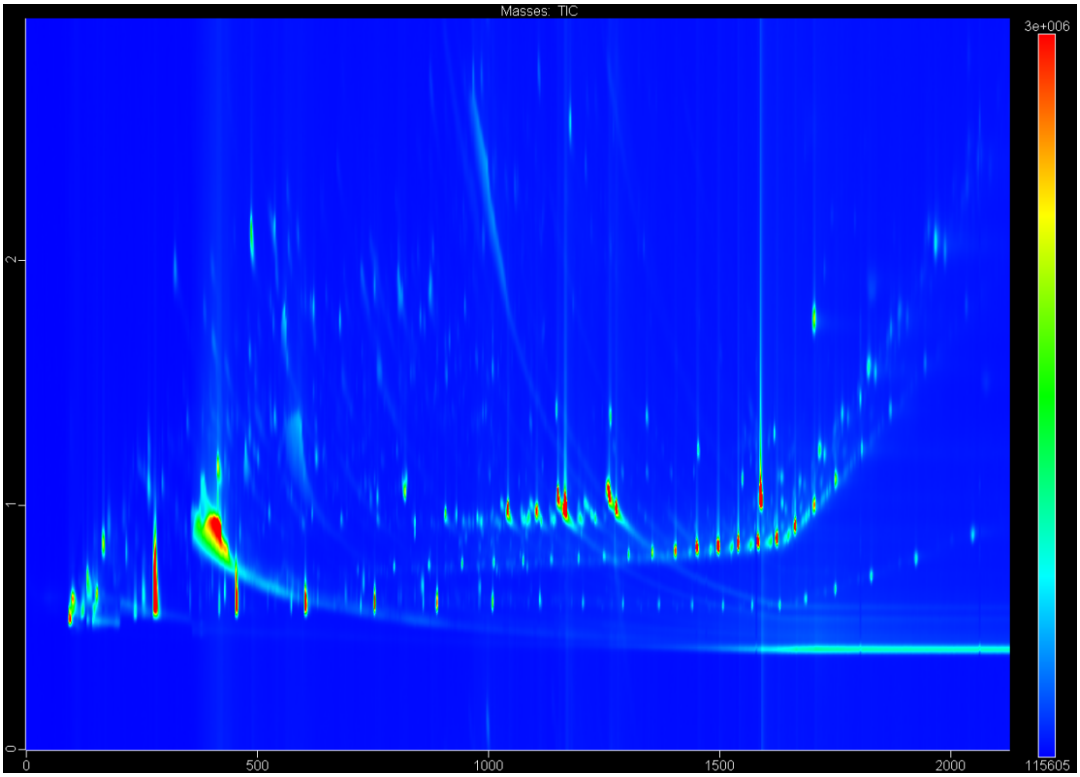
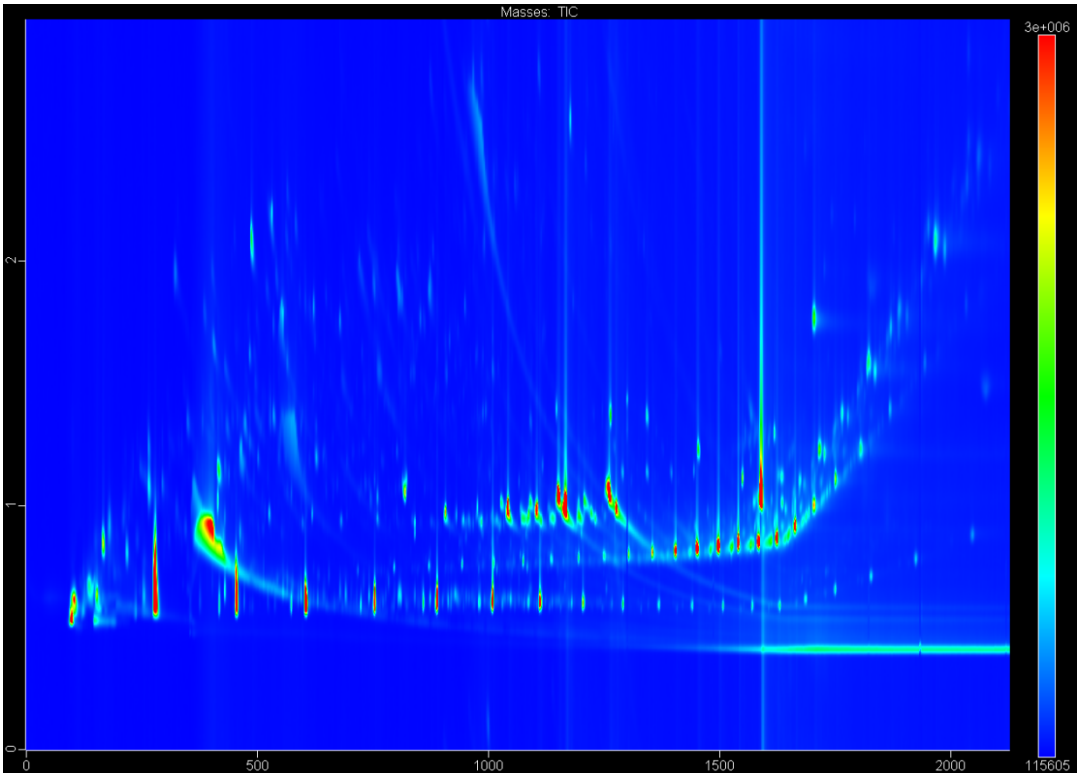
Appendix A2: Contour plots of total ion chromatograms (GC×GC-ToFMS) obtained from the analysis of samplers used in the comparison of wrist and ankle experimentation.



Chromatogram A2.1 Bracelet skin sample 1 of 5.



Chromatogram A2.2 Bracelet skin sample 2 of 5.

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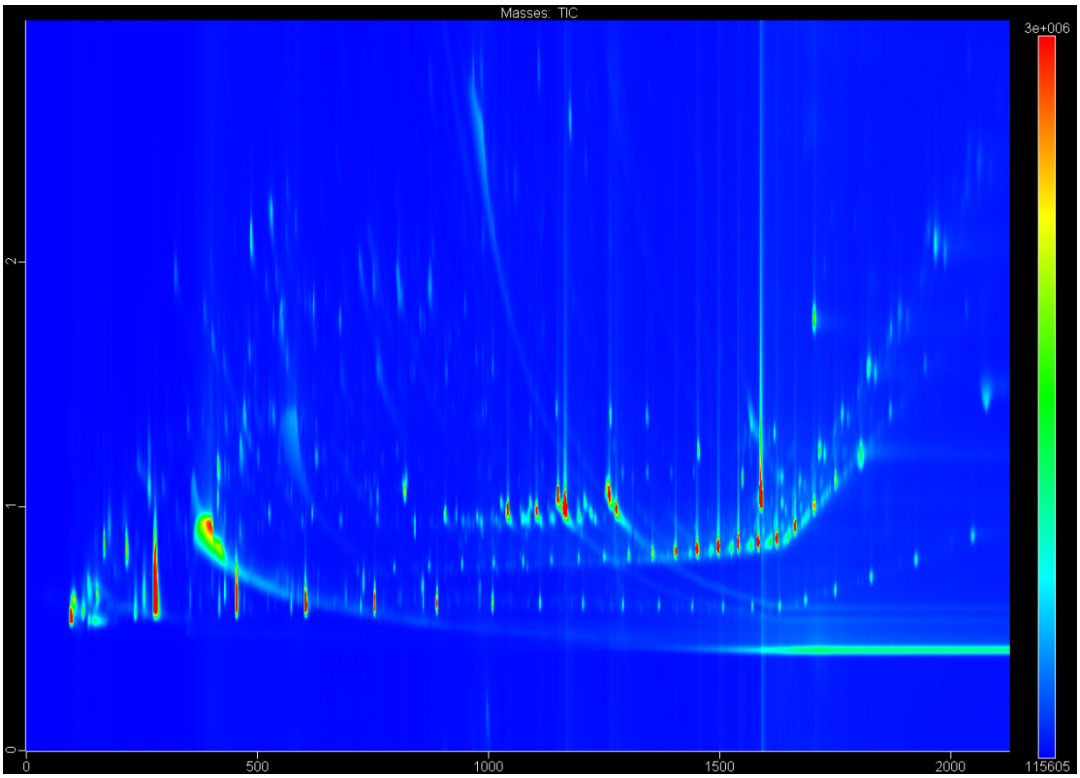
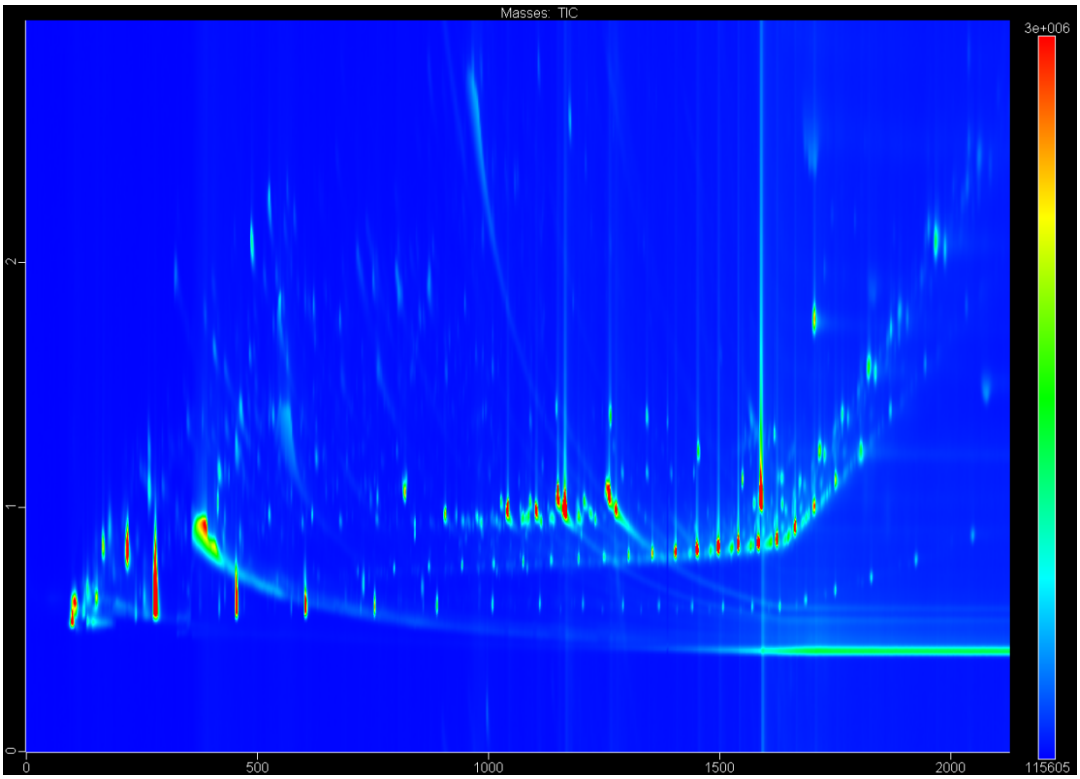


Chromatogram A2.3 Bracelet skin sample 3 of 5.



Chromatogram A2.4 Bracelet skin sample 4 of 5.

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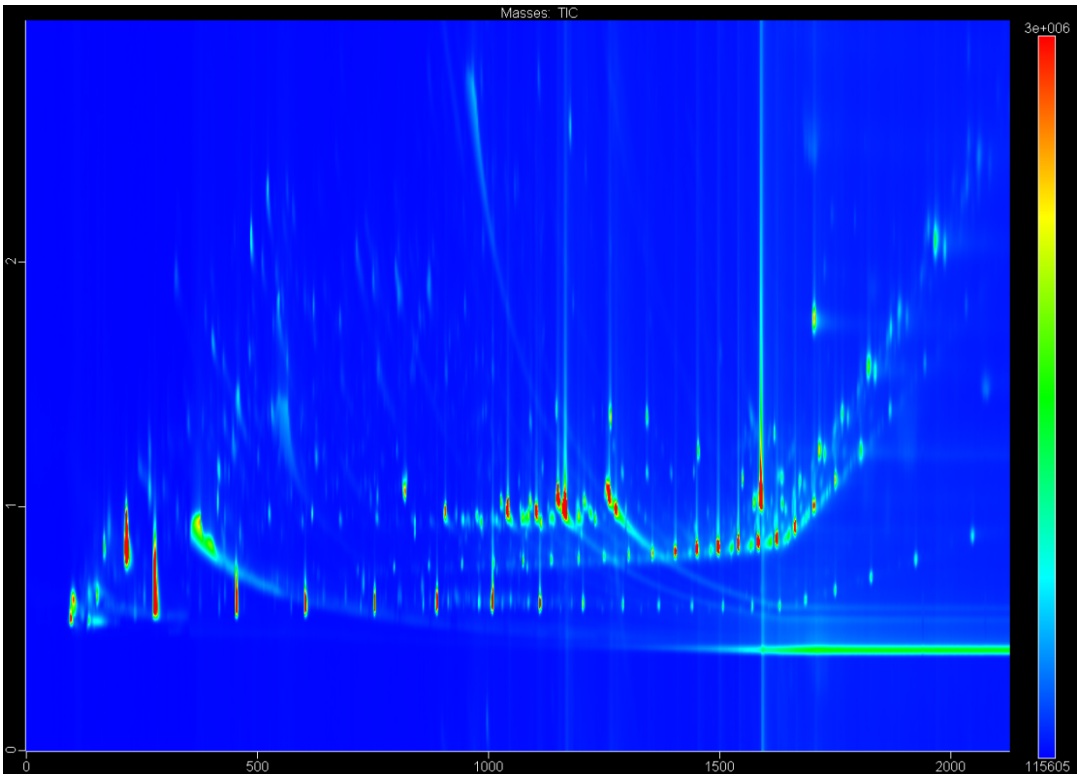
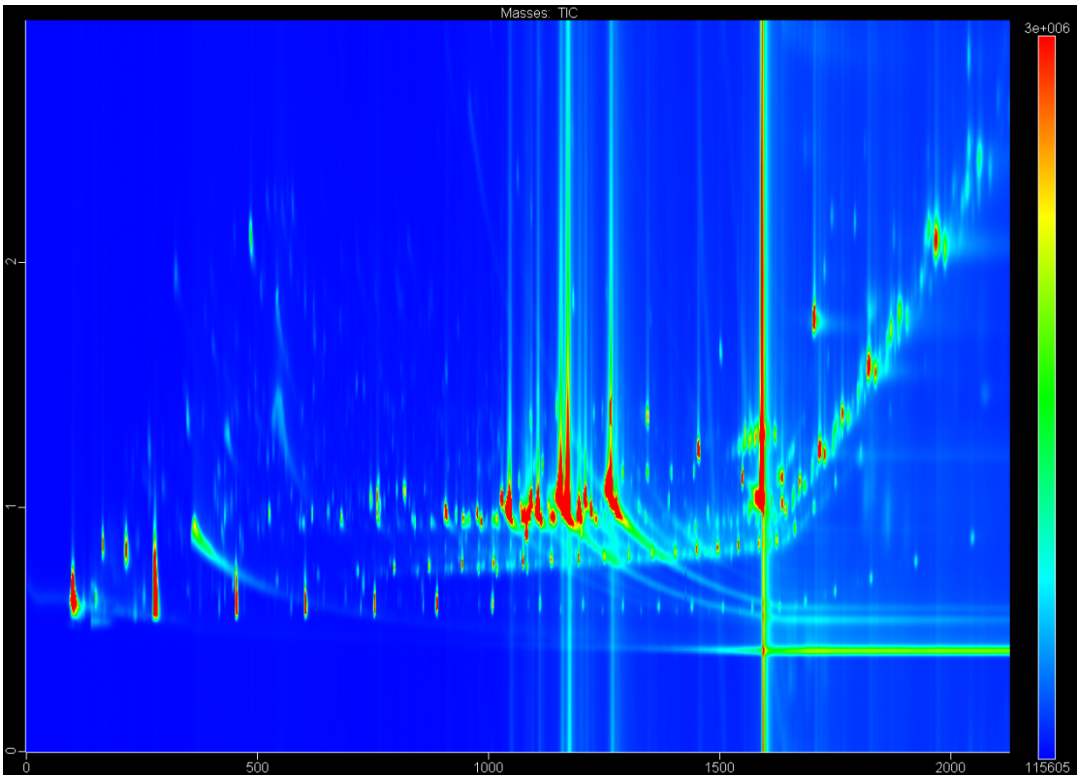


Chromatogram A2.5 Bracelet skin sample 5 of 5.



Chromatogram A2.6 Bracelet skin sample alternative environment.

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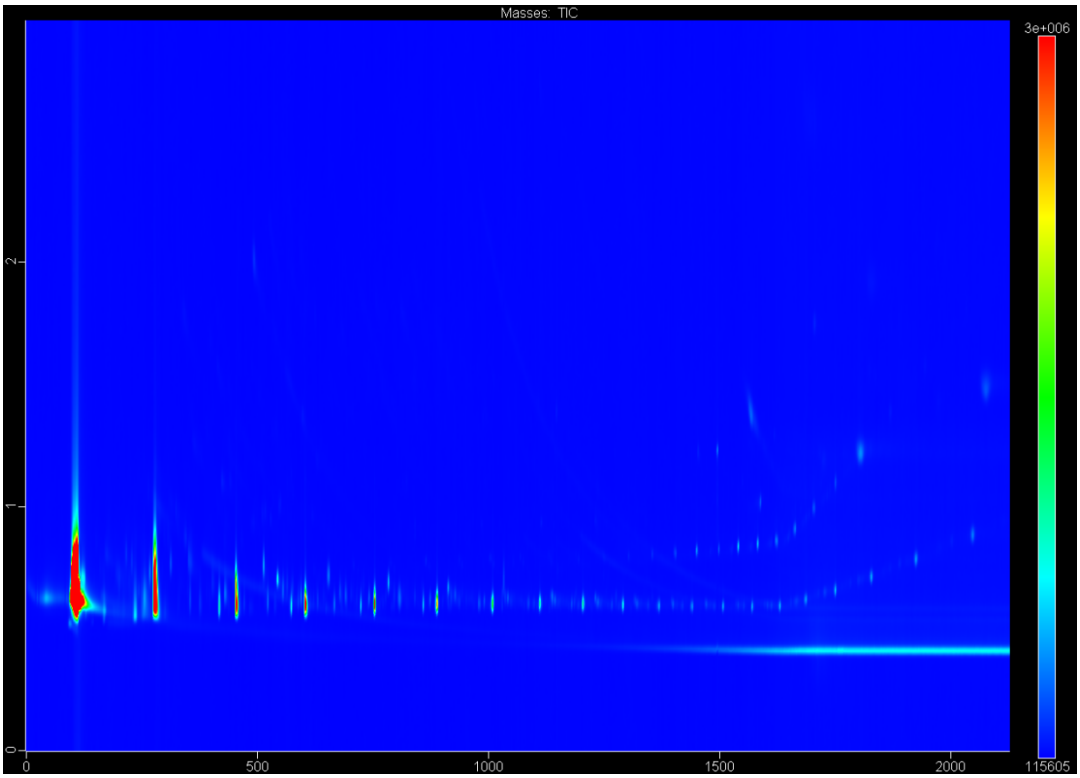
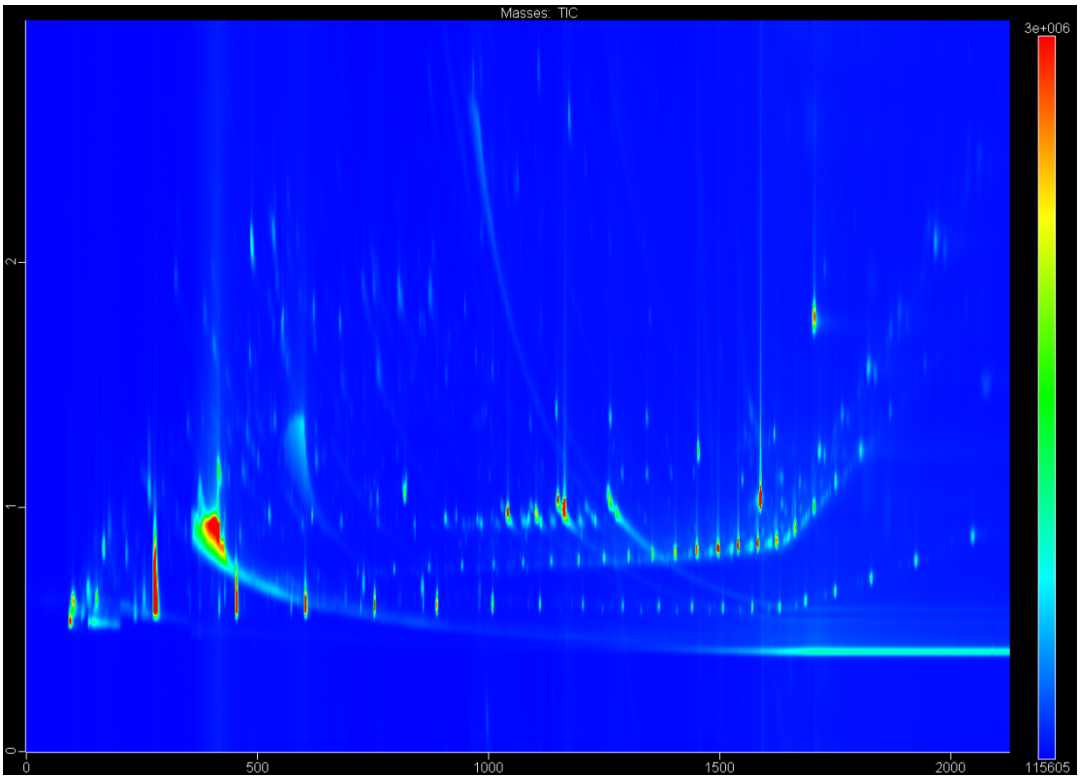


Chromatogram A2.7 Bracelet quality control blank.



Chromatogram A2.8 Anklet skin sample 1 of 5.

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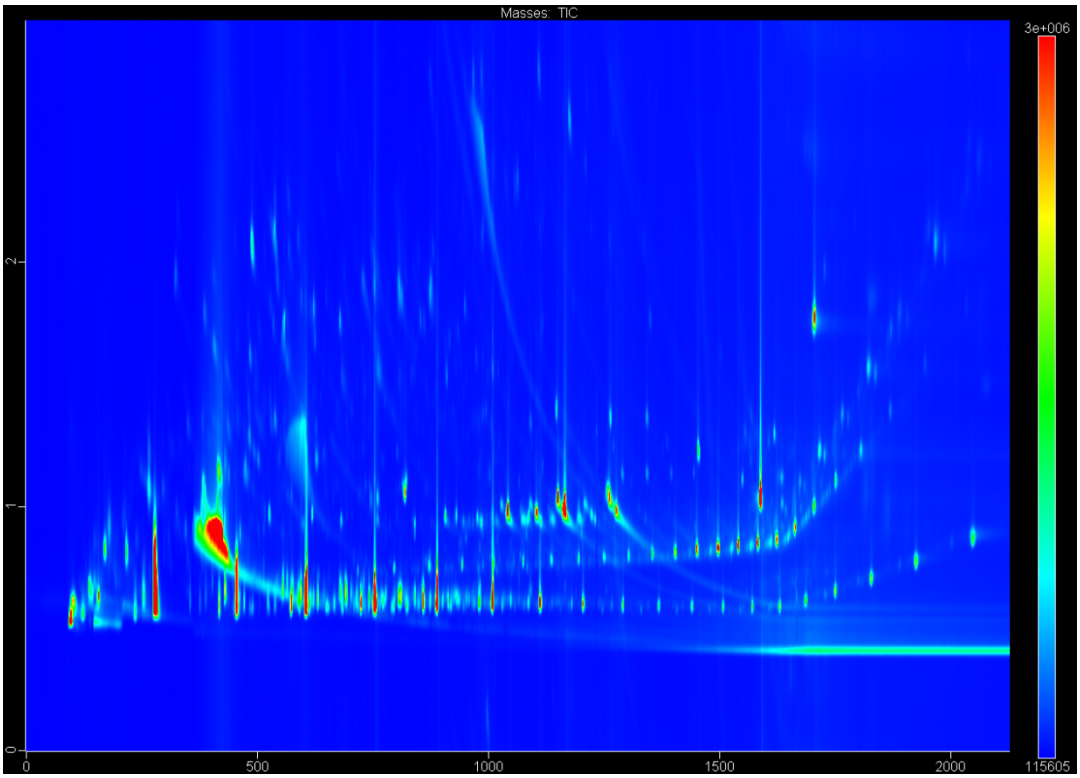
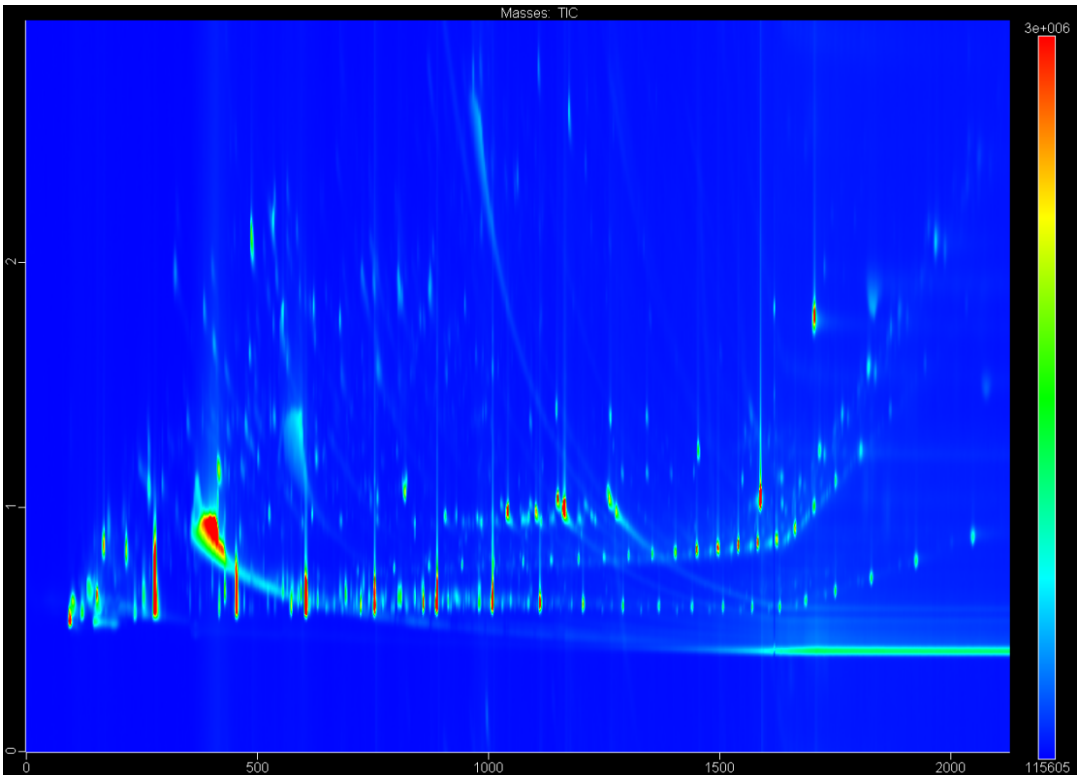


Chromatogram A2.9 Anklet skin sample 2 of 5.



Chromatogram A2.10 Anklet skin sample 3 of 5.

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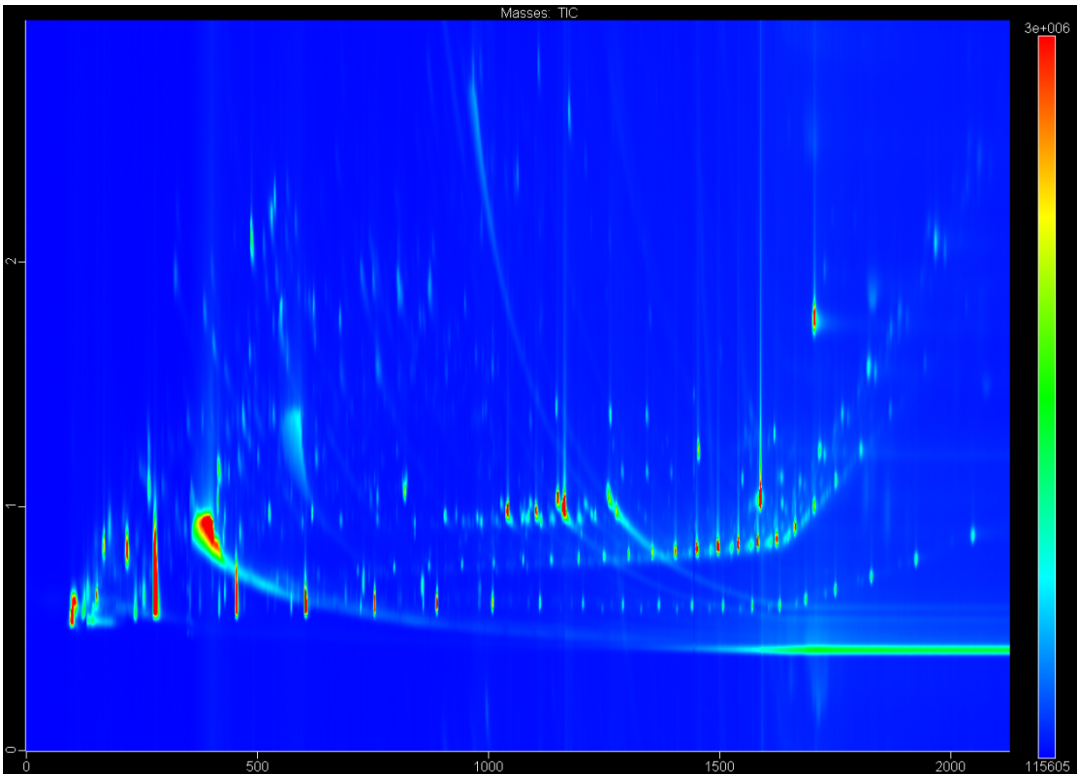
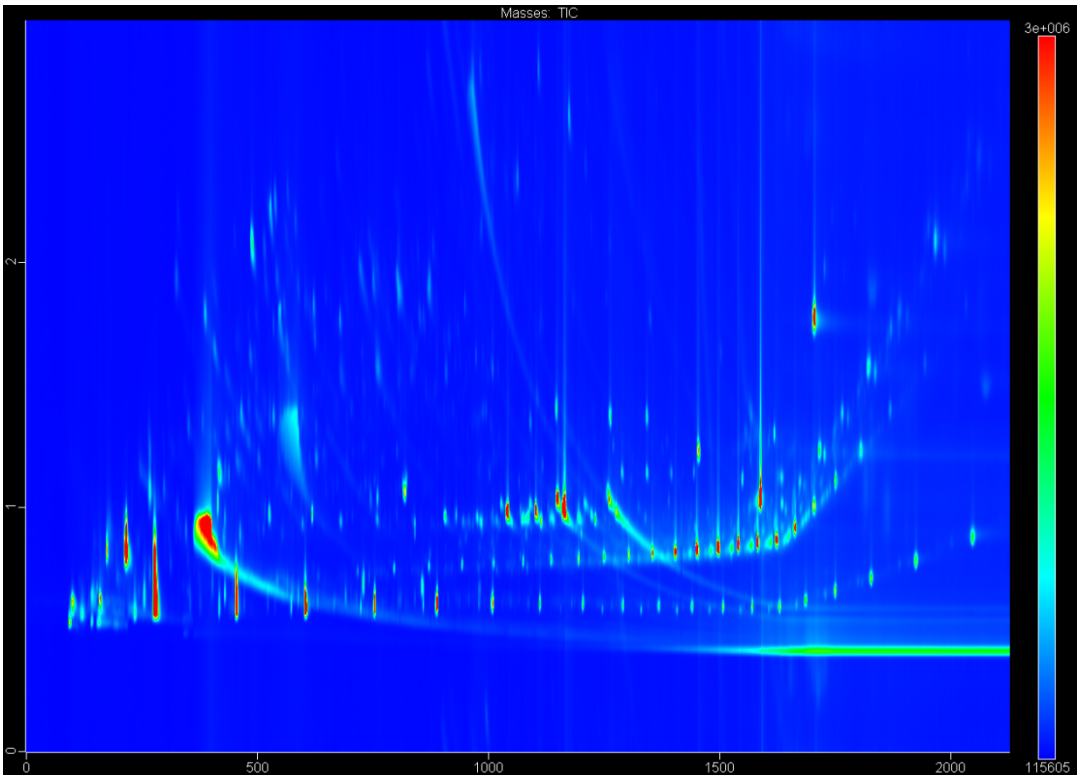


Chromatogram A2.11 Anklet skin sample 4 of 5.



Chromatogram A2.12 Anklet skin sample 5 of 5.

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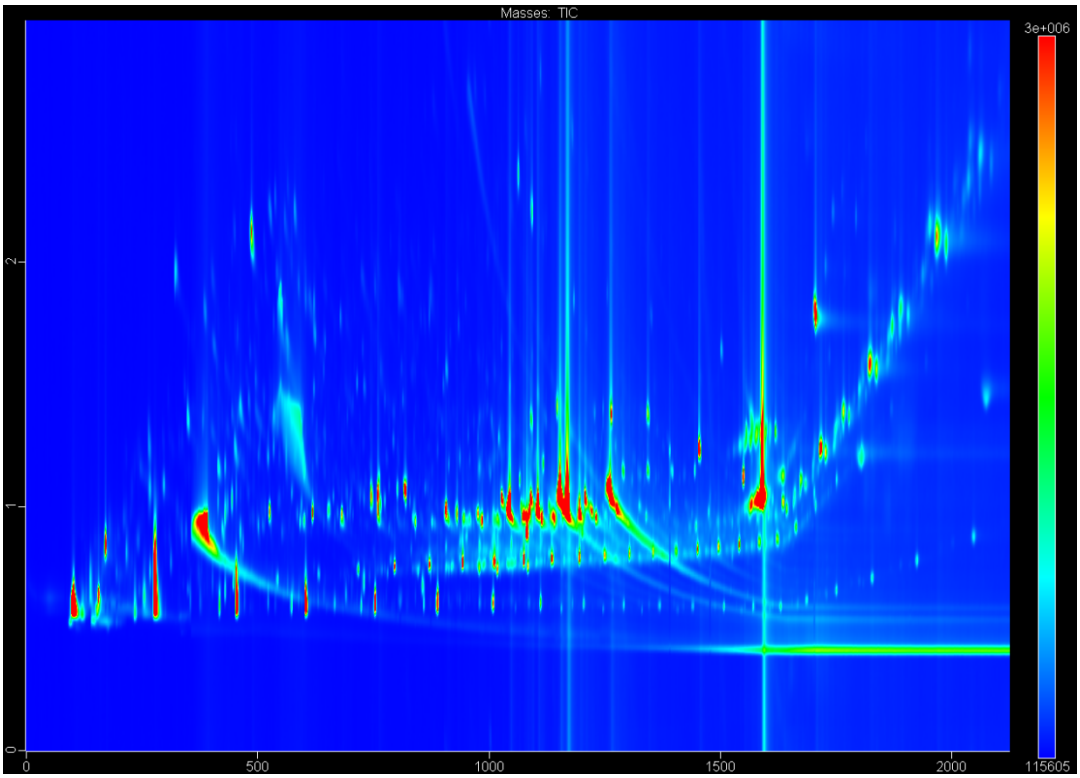
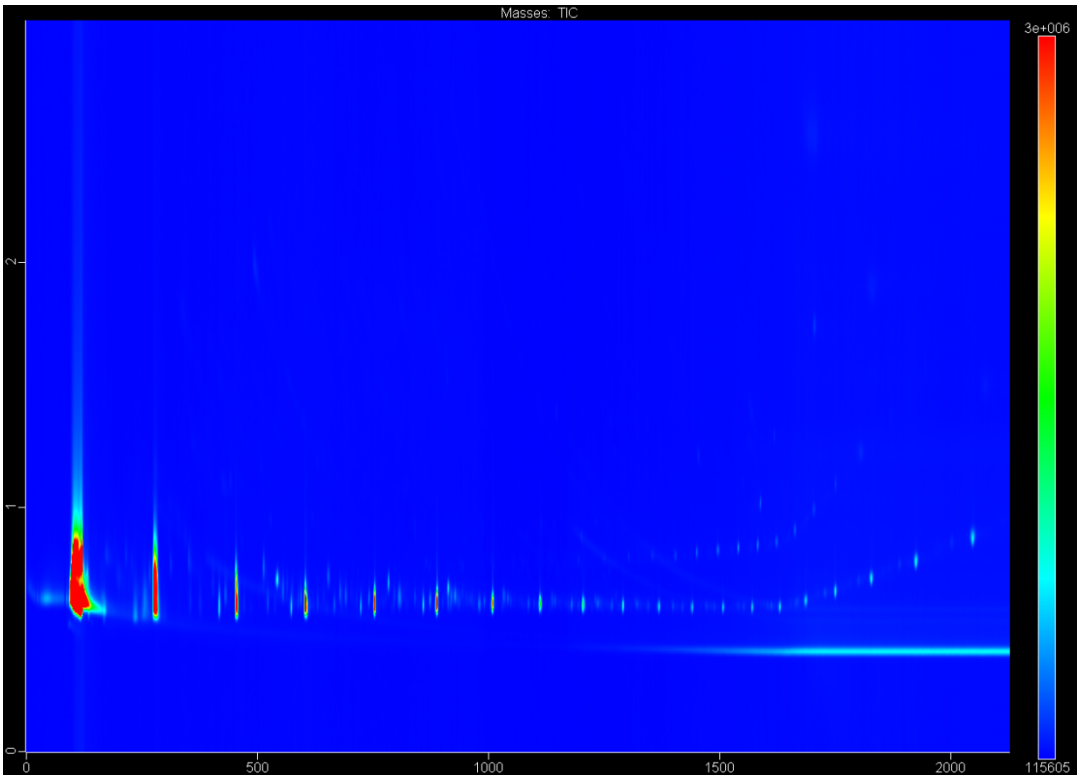


Chromatogram A2.13 Anklet skin sample alternative environment.



Chromatogram A2.14 Anklet quality control blank.

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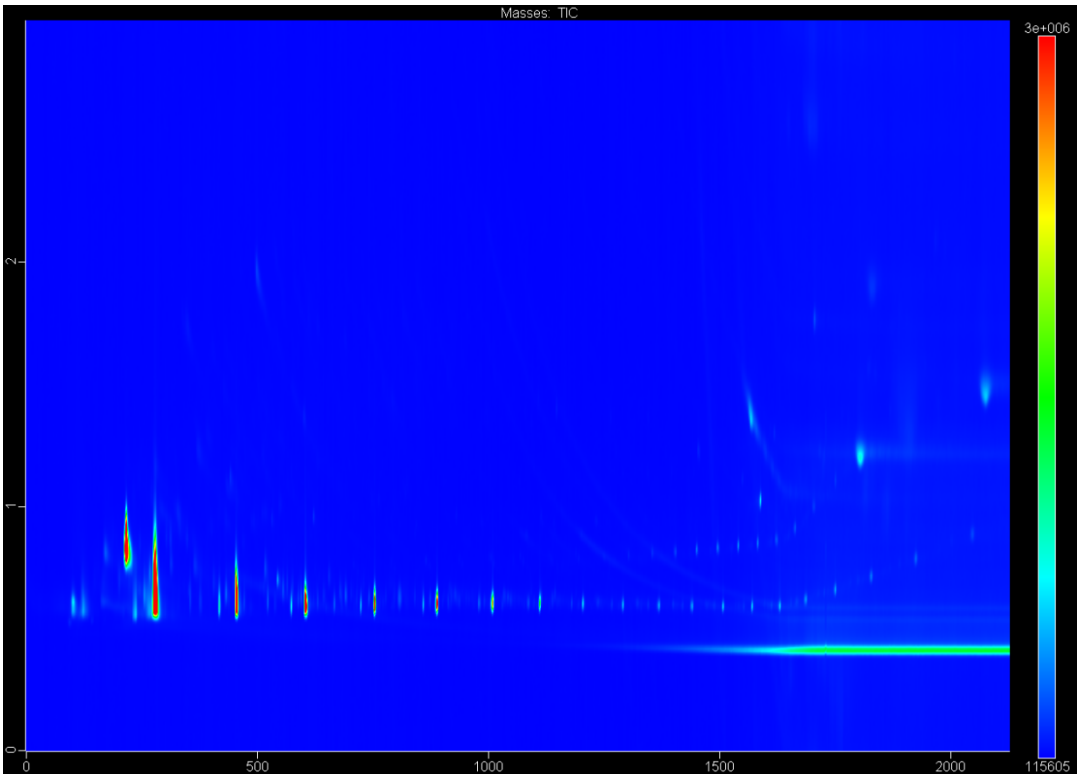
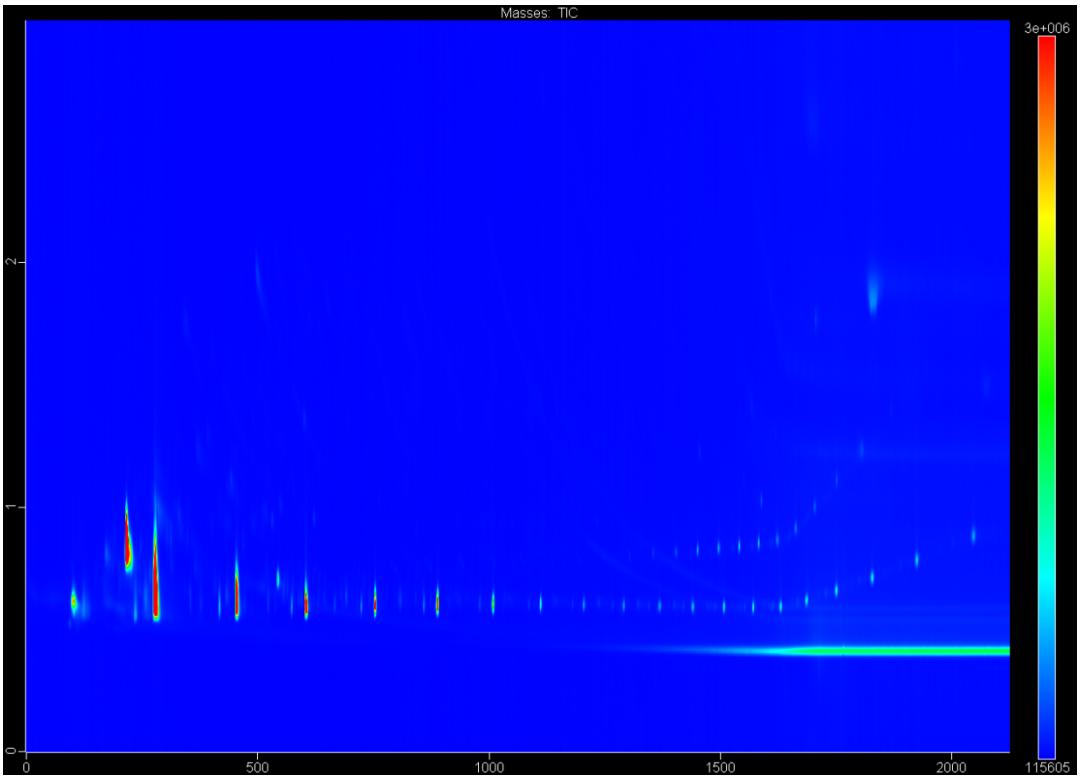


Chromatogram A2.15 Laboratory background 1.



Chromatogram A2.16 Laboratory background 2.

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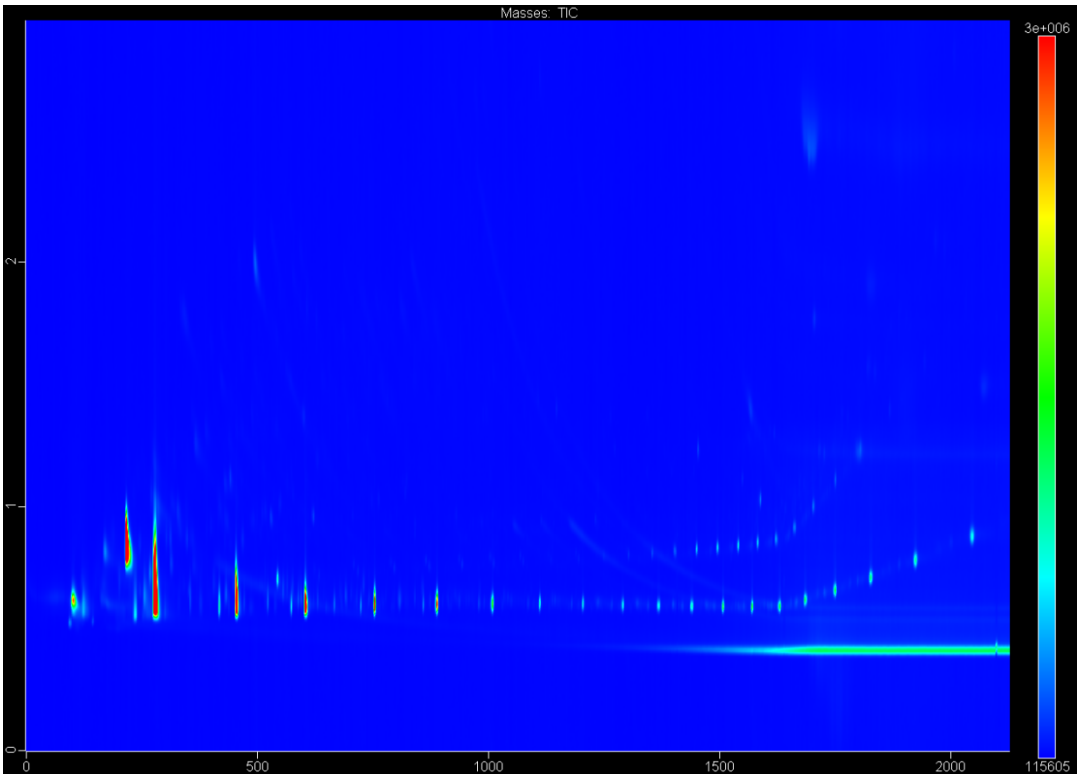

Chromatogram A2.17 Laboratory background 3.