# METABOLOMIC INVESTIGATIONS INTO HUMAN APOCRINE SWEAT SECRETIONS

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## Abstract

Human axillary odour is formed by the action of *Corynebacteria* or *Stephyloccui* bacteria on odourless axilla sections. Several groups have identified axillary odorants, including 3-methyl-2-hexanoic acid (3M2H) and 3-hydroxy-3-methyl-hexenoic acid (HMHA), and how they are pre-formed and bound to amino acid conjugates. However, there is currently a lack of LC-MS methodologies and no reported NMR methods, that are required to further identify the non-volatile constituents, which would provide further information to allow understanding of the underlying physiological biochemistry of malodour.

This work has incorporated a three-pronged approach. Firstly, a global strategy, through the use of NMR and LC-MS, provided a complementary unbiased overview of the metabolite composition. Metabolites were identified based on acquired standards, accurate mass and through the use of in-house or online databases. Furthermore, spectra of biological samples are inherently complex, thus, requiring a multivariate data analysis (MVDA) approach to extract the latent chemical information in the data. Secondly, semi-targeted LC-MS/MS methodologies has been used to identify metabolites with a common structural core (i.e. odour precursors) and provide structural information for the reliable identification of known and unknown metabolites. Finally, a targeted LC-MS/MS method provided an increase in specificity and sensitivity to accurately quantify known metabolites of interest (odour precursors).

Initially, all methodologies were developed through the use of either an artificial sweat matrix (global strategy) or through the use of synthetic standards (semi-targeted or targeted strategy). The sample complexity was then increased by applying the methodologies to an ASG5 apocrine cell line, in order to provide further knowledge into apocrine cell metabolism and to identify whether there could be any potential male or female differences due to differences in circulating hormones. Changes in the cell metabolism were identified, and both the NMR and LC-MS data could differentiate between control, tamoxifen- and β-estradiol-treated. However, it is

difficult to attribute these changes to specific pathways, as these hormones or the vehicle used (ethanol) are likely to produce a ripple effect across the cell's metabolism. Nonetheless, NMR spectroscopy quantified 25 metabolites with lactate being the most abundant at 19.1 mM, while HILIC-MS could detect a range of lipids, nucleotides, amino acids, fatty acids and vitamins.

The methodologies were then applied to human apocrine sweat collected from six volunteers across five days. NMR spectroscopy was able to identify 25 and quantify 19 metabolites, with lactate being the most abundant at 13.2 mM. LC-MS/MS readily identified 12 amino acid conjugates with HMHA being the most abundant. Furthermore, a possible 20 unidentified conjugates were detected (LC-MS/MS semi-targeted methodologies) as well as putatively identifying 473 metabolites (LC-MS global methodologies). MVDA techniques such as principal component analysis (PCA) illustrated that intra-individual variation was greater than inter-individual variation, as well as secretions from both the left and right arm being consistent with one another. Moreover, MVDA illustrated the complementary nature of both NMR and MS, as the data acquired with the two types of instrumentation showed the same trends, even though these trends were based on different subsets of metabolites.

The work presented herein, has successfully used a number of analytical technologies to investigate metabolite content of human apocrine sweat. It has been shown that a number of complementary techniques and multivariate analysis can provide a valuable insight into the underlying physiology of malodour.

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*"It is very obvious that we have many different kinds of smells, all the way from the odour of violets and roses up to asafoetida. But until you can measure their likeness and differences you can have no science of odour."*

— ALEXANDER GRAHAM BELL, 1914

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# Abbreviations

| | |
|---|---|
| 2M3H-Cys | S-[1-(2-hydroxy-1-methylethyl)-ethyl]-L-cysteine |
| 3M2H-Gln | N-α-3-methylhex-2-enoyl-L-glutamine |
| 3M2H-Cys-Gly | S-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cysteinylglycine |
| A. A | Amino acid |
| apoD | Apolipoprotein D |
| $B_0$ | Magnetic field |
| CE | Collision energy |
| CID | Collision induced dissociation |
| COSY | Correlation spectroscopy |
| Ctr | Mean Centered |
| d | Doublet |
| dd | Doublet of doublets |
| DIMS | Direct injection mass spectrometry |
| DP | Declustering potential |
| E2 | β-Estradiol |
| EIC | Extracted ion chromatogram |
| EPI | Enhanced product ion |
| ESI | Electrospray ionization |
| Eth | Ethanol |
| FA | Fatty acid |
| FFA | Free fatty acid |
| FT | Fourier transform |
| FWHM | Full width at half maximum |
| $\gamma$ | Magnetogyric ratio |
| GC-MS | Gas chromatography mass spectrometry |
| $h$ | Planck's constant ($6.62 \times 10^{-34}$ $m^2$ kg/s) |
| HILIC | Hydrophilic interaction liquid chromatography |
| HMBC | Hetronuclear multiple bond coherence |
| HMDB | Human metabolite database |
| HMHA-Gln | N-α-3-hydroxy-3-methylhexanoyl- L-glutamine |
| HMQC | Hetronuclear multiple quantum coherence |
| HPLC | High performance liquid chromatography |
| $I$ | Nuclear spin quantum number |
| Im | Immonium ion $[R\text{-}CH{=}NH_2]^+$ |
| IDA | Information dependent acquisition |
| J-res | J-resolved spectroscopy |
| $k$ | Boltzman constant ($1.38 \times 10^{-23}$ J $K^{-1}$) |

| | |
|---|---|
| LC | Liquid chromatography |
| LCFA | Long chain fatty acid |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| LV | Latent variable |
| m | Multiplet |
| $\mu$ | Nuclear magnetic moment |
| MeCN | Acetonitrile |
| MHC | Major histocompatibility complex |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MVA | Multivariate analysis |
| NMR | Nuclear magnetic resonance |
| ns | Number of transients |
| OD | Optical density |
| PC | Principle component |
| PCA | Principal component analysis |
| PC-DA | Principal component discriminant analysis |
| PI | Precursor ion |
| PLS-DA | Partial least squares discriminant analysis |
| PPM | Parts per million |
| PR | Pattern recognition |
| QC | Quality control |
| QNP | Quattro nucleus probe |
| QqQLit | Hybrid triple quadrupole linear ion trap |
| QTOF | Quadrupole time of flight |
| QTRAP | See QqQLit |
| R | Correlation coefficient |
| RD | Relaxation delay |
| RF | Radio frequency |
| RP | Reversed phase |
| s | Singlet |
| S/N | Signal to noise |
| SRM | Single reaction monitoring |
| t | Triplet |
| TAM | Tamoxifen |
| TIC | Total ion chromatogram |
| TOCSY | Total correlation spectroscopy |
| TSP | Trimethylsilyl [2,2,3,3-$^{2}H_4$] propionate |
| TXI | Triple resonance inverse probe |

| | |
|---|---|
| Unt | Untreated |
| UPLC | Ultra performance liquid chromatography |
| UV | Unit variance |
| VFA | Volatile fatty acid |
| VIP | Variable importance in projection |
| $\nu_0$ | Larmor frequency |

# Chapter 1

## 1 Introduction

### 1.1 Metabolomics

Metabolomics is the global study of all the naturally occurring low molecular weight molecules, called metabolites, in biological samples such as cells, biofluids (e.g. urine and blood) or tissues. These low molecular weight molecules, which make up the 'metabolome', are derived from the interaction of the genome with its environment and are not merely the end products of gene expression, but also form part of the metabolic status of a biological system.

When 'metabonomics' was first introduced it was defined as "*the quantitative measurement of the time related multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification*" (Nicholson *et al.*, 1999). Whereas metabolomics has been described as "*a comprehensive and quantitative analysis of all metabolites*" (Fiehn, 2001). These terms are often used interchangeably, although groups adhering to these definitions tend to adopt the use of 'metabolomics' to discuss work on simple cell systems and intracellular metabolite concentrations and 'metabonomics' for the analysis of products of non-enzymatic reactions which interact with and influence metabolite formation (Daykin and Wulfert, 2006). However, it will be up to the reader to distinguish between those definitions. Both terms claim superiority, though ultimately it will be left for the user community to decide. The term metabolomics will be employed here with the realization that either term may be used interchangeably.

As the definition of the metabolome above suggest, in a metabolomics experiment the ultimate goal would be to capture and quantify all the metabolites in a cellular system

simultaneously without pre-selecting a specific pathway. However, metabolites consist of a diverse set of chemical (molecular weight, polarity, solubility) and physical (volatility) properties. Moreover, the metabolome extends over a wide concentration range, which is estimated to be between 7-9 magnitudes of concentration (pM-mM) (Dunn and Ellis, 2005). This diversity means that no one analytical technique is capable for all investigations. Thus, a number of strategies are employed, as listed below, with nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled to chromatography, being the most prevalent analytical techniques (Dunn *et al.*, 2011b): -

- **Metabolomics,** initially defined as the comprehensive analysis of the entire metabolome. The term is now used to describe one or more of the strategies defined below.
- **Metabolite profiling**, the holistic study of the metabolite complement of a biological system, often employing multiple analytical platforms, to encompass a wide coverage of the metabolome.
- **Metabolic footprinting**, analysis of the extra-cellular metabolome which is composed of metabolites consumed/not consumed from the environment or metabolites secreted from the intra-cellular volume.
- **Metabolite target analysis**, quantitative analysis of a small number of metabolites related to a specific metabolic reaction or pathway, for example; a particular enzyme system that would be affected by abiotic or biotic perturbation.

Whilst these strategies are not universally accepted, they are continuously evolving. Hence, there can be overlap in their definitions; however, the above classification illustrates the options available for monitoring the metabolome (Dunn and Ellis, 2005; Hollywood *et al.*, 2006).

Although metabolomics is complementary with transcriptomics and proteomics, it also has certain advantages. In particular, the metabolome is further downstream from gene to function, thus, any change in cell physiology (resulting from gene deletion or over expression) are amplified through the hierarchy of the transcriptome and

proteome (Goodacre *et al.*, 2004). It is also important to highlight those factors other than gene expression or single nucleotide polymorphisms, which can affect the system biology view of an organism; environmental factors such as diet, age, sex, ethnicity, lifestyle, and microfloral populations all have large influences and these factors need to be deconvolved (Lindon *et al.*, 2004). Hence, metabolic fluxes are not just regulated by gene expression but by both post-transcriptional and post-translational modifications and as such, the metabolome can be considered closer to the phenotype (Hollywood *et al.*, 2006).

Metabolomics has already been widely employed in the study of toxic insult (Lindon *et al.*, 2003b; Nicholson *et al.*, 2002), the study of disease and disease models (Brindle *et al.*, 2002), as a means of discovering novel biomarkers for early disease detection or providing new drug targets as well as providing insights into the disease process itself. Furthermore, metabolomics is increasingly being applied to the study of healthy individuals (e.g. HUSERMET project[1]), which aims to provide a metabolic 'reference map' of the normal population against which data from other studies can be drawn against. Currently, the samples most commonly analysed are plasma (Daykin *et al.*, 2002; Lenz *et al.*, 2003; Nicholson *et al.*, 1983; Zelena *et al.*, 2009) and urine (Connor *et al.*, 2004; Daykin *et al.*, 2005; Gika *et al.*, 2007), which are considered to be readily accessible and minimally invasive. However, analysis has been performed on other biofluids such as cerebrospinal fluid (Holmes *et al.*, 2006; Sweatman *et al.*, 1993), seminal fluid (Lynch *et al.*, 1994; Spraul *et al.*, 1994b), saliva (Takeda *et al.*, 2009), and eccrine sweat (Harker *et al.*, 2006) as well as tissues and tissue extracts (Dunn *et al.*, 2011a; Xu *et al.*, 2009).

## 1.2 Application of Techniques

The first requirement for metabolomics analysis is to have techniques available that are as comprehensive as possible. This is especially important, as metabolites are heterogeneous in nature, thus, isolating and measuring them all together ('true metabolomics') is extremely difficult. For practical reasons this is never achieved, hence, most metabolomic studies are really 'metabolic profiling' of a subset of

[1] http://www.husermet.org/

chemical classes (Kell, 2004). Indeed, specific platforms are not a prerequisite for metabolomic studies, so in theory, any technique capable of generating comprehensive data in a relatively short time frame can be used as a powerful means of generating multivariate metabolic data (Robertson, 2005). Thus, the choice of analytical tool is based on the level of chemical information required and the appropriate time frame in which the data must be obtained. However, one must remember that there will be introductions of chemical bias with respect to the method, which are highlighted in Table 1.1.

**Table 1.1** Consideration for metabolomics analysis. Reproduced with modifications (Hollywood *et al.*, 2006).

| Consideration | Approach | Comments |
|---|---|---|
| Chemical information | MS | $MS^n$ will provide some structural information. FT-ICR-MS can generate empirical formulae for *m/z* <500. |
| | NMR | Gives detailed structural information, particularly using 2-D NMR of isolated metabolites. |
| | Chromatography (GC, HPLC, CE) | On its own will not generally lead to metabolite identification. However, coupled with MS and NMR is very powerful for analyte identification. |
| | FT-IR, Raman | Provides limited structural information, but useful for identification of functional groups. |
| Chemical bias | GC-MS | Solvent extraction bias: non-polar versus polar analytes. Need for chemical derivatization. |
| | LC-MS | Solvent bias means it is usually more applicable to polar compounds (HILIC) or non-polar compounds (reverse phase $C_{18}$ or $C_8$ columns). |
| | NMR, FT-IR, Raman | Biased by concentration, available functional groups and where they resonate in the spectrum. |
| Speed | Chromatography (GC, HPLC, CE) | Very useful for separation but typically takes 30 min. |
| | NMR | Few minutes to hours. Depends on the strength of the magnet, sensitivity can be improved with cryo-probes. |
| | ESI-MS | 1-3 min flow-injection (direct infusion) mode. |
| | FT-IR | 10-60 s. |

The two most information-rich techniques that offer atom-specific molecular structural information are MS and NMR. The resolution, sensitivity and selectivity of MS based techniques can be enhanced through coupling to gas chromatography (GC) or liquid chromatography (LC). Furthermore, hyphenating individual systems, LC-NMR-MS, can maximise the information obtained, since the NMR will yield structural information and the MS can determine the mass of each component.

NMR spectroscopy has some specific advantages in the area of metabolomics over other analytical methods discussed in Table 1.1 and these are highlighted in Table 1.2. This is typically the case in regards to analysing biofluids, as it is rapid, non-destructive, requires little or no sample preparation and uses small sample sizes. NMR is based on the fact that nuclei such as $^{1}H$, $^{13}C$ and $^{31}P$ can exist at different energy levels due to the differences in nuclear spin, which can be measured by applying an external magnetic field. This means a comprehensive profile of metabolite signals can be produced without the need for pre-selection of measurement parameters or the use of separation or derivatisation procedures (Lindon *et al.*, 2004). Thus, the sample is left unperturbed, allowing rapid analysis of many biochemical molecules simultaneously. Moreover, the non-destructive nature of NMR is particularly useful when further analysis is required for identification of unknown metabolites or when using hyphenated techniques such as LC-NMR-MS (Corcoran and Spraul, 2003); unlike LC-MS where the sample is destroyed after analysis and derivatisation and optimisation procedures can often be expensive and time consuming. More recently, the technique of magic-angle spinning, where the samples are spun rapidly at 54.7° relative to the applied magnetic field has opened up the possibility of applying metabolomics to tissue samples (Lindon *et al.*, 2009; Sitter *et al.*, 2009). This would serve as a tool for linking biofluid changes to the mechanism of action in target tissues, particularly when products of metabolism dominate changes in the biofluids (Robertson, 2005). Further technological advances that have improved the resolution and sensitivity of NMR includes the introduction of cryogenically cooled probes whereby the magnet is super-cooled with cryogenic liquid. This enables the thermal noise of the system to be reduced, thereby, improving the signal-to-noise ratio by approximately four fold (Robosky *et al.*, 2007). This also enables smaller sample volumes to be measured or allows measurements of fixed sample concentrations with a weaker magnetic field strength, which will be more commercially affordable (Kovacs *et al.*, 2005). In comparison, MS also offers a number of advantages as highlighted in Table 1.3. These include an increase in sensitivity and when hyphenated with LC, can detect hundreds or thousands of metabolite features in a given sample as well as providing metabolite identification. Nonetheless, one thing is clear, these two complementary approaches (MS and NMR) will provide information

on different sets of metabolites, and integration of both techniques will provide a more comprehensive characterisation.

**Table 1.2** Summary of the main features of $^{1}$H NMR Spectroscopy. Reproduced with modifications (Daykin and Wulfert, 2006).

| Feature | Comments |
|---|---|
| Selectivity | No need for pre-selection of analytical conditions based on the chemical properties of the analyte, or postulation of the metabolites affected by a disease toxicological process. |
| Non-invasive | NMR spectroscopy is non-invasive, non-destructive and non-equilibrium perturbing, thus, allowing subsequent analysis of a sample with other techniques. |
| Speed | A typical single $^{1}$H NMR biofluid spectrum is obtainable in <10 minutes, thus, can allow >100 samples/day. |
| Sample volumes | With standard NMR tubes, a total volume of 600 µl is required for routine spectroscopy, or 5 µl for micro-volume NMR probe. |
| Sample preparation | Minimal sample preparation for 'global' metabolite profiling. Only the addition of deuterium oxide is required for analysis. However, with complex components (biofluids) physical separation maybe used to simplify spectra. |
| Dynamic information | Information can be obtained on dynamic biochemical processes in complex matrices and molecular interactions such as protein-ligand binding. |
| Structural information | Data provides qualitative structural information that can also be quantitative with addition of internal standards at known concentrations. |
| Reproducibility | NMR spectroscopy is an inherently reproducible analytical method. |
| Sensitivity | Inherently insensitive technique, realistically detection limits remain in the uM levels for complex samples such as biofluids, though this is improving with cryoprobes and increased field strength magnets. |

**Table 1.3** Summary of the main features of LC-MS.

| Feature | Comments |
|---|---|
| Selectivity | Dependent on the column, polar compounds (HILIC) and non-polar compound ($C_{18}$ or $C_8$). |
| Invasive | Biological fluids (minimally invasive) or tissue samples need to be collected prior to analysis. The sample is destroyed upon analysis. |
| Speed | LC-MS typically around 30 min per sample, though, DIMS ~1-3 min per sample. |
| Sample volumes | Typically 5 µl per injection. |
| Sample preparation | Derivatisation generally not needed. Requires sample extraction, followed by evaporation (overnight) before reconstitution. Also, pooled samples are required to generate QC samples for quality assurance. Targeted methodologies generally require the addition of internal standards. |
| Dynamic information | Post-translational modifications and single nucleotide polymorphisms in proteome analysis, structure elucidation, metal complex and disulphide bond interactions. |
| Dynamic range | Depending on mass analyser, can be between $10^4$-$10^6$ orders of magnitude. |
| Structural information | Depending on the mass analyser, can provide fragmentation profiles as well as empirical formula. Data can be both qualitative and quantitative. |
| Identification | LC-MS libraries are not as robust as GC libraries, thus, can be complex. |
| Reproducibility | Dependent on sample matrix, typically 120 injections per new column. |
| Sensitivity | Typically sub picomole concentrations when using targeted methodologies. |
| Size and cost | Relatively small and cheaper to maintain (ca. NMR). |

## 1.3 NMR Background and Theory

Since the introduction of NMR spectroscopy by Rabi and Breit (Breit and Rabi, 1931; Rabi, 1937) and its further development by Bloch and Purcell (Bloch *et al.*, 1946), NMR spectroscopy has become an invaluable tool for chemists and structural biologists and has increasingly been used in metabolic profiling over the last two decades.

NMR spectroscopy is based on the magnetic properties of the atomic nuclei. Many nuclei (most commonly $^{1}H$, $^{13}C$, $^{19}F$ and $^{31}P$) possess a property described as spin, which makes the nuclei behave like magnets, i.e. possesses a nuclear magnetic moment ($\mu$) described by the magnetogyric ratio ($\gamma$) for each particular nucleus. The nuclear spin is quantised and is described by the nuclear spin quantum number $I$, which has values of 0, ½, 1, 1½, etc. in units of $h/2\pi$, where $h$ is the Plancks constant. The actual value of the spin depends on the atomic number and mass number of the nucleus. However, only atoms with odd mass numbers, for example; $^{1}H$, will be considered.

A nucleus of spin $I$ has $2I + 1$ possible energy levels, which are defined by the magnetic quantum number $m_I$, which has values of $-I$, $-I + 1$,..., $I - 1$, $I$, i.e. for a nucleus of spin ½, $m_I = +½$ or $-½$. There is no difference in energy between nuclei spinning in different directions. However, in the presence of an external magnetic field ($B_0$), the magnetic dipoles align with or against the external magnetic field resulting in an energy difference between $m_I = +½$ and $-½$ spin states (see Figure 1.1).

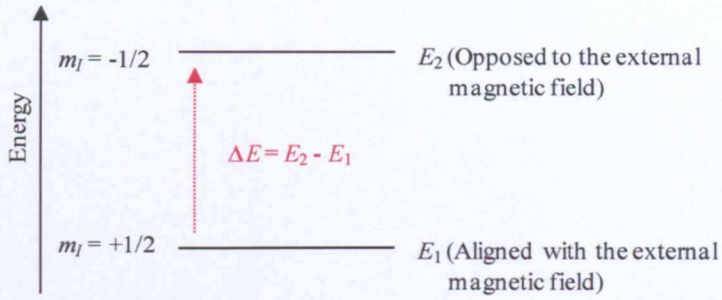



**Figure 1.1** Schematic representing the splitting of nuclear spin states in the presence of an external magnetic field.

The energy of this interaction is proportional to $\mu$ and $B_0$ and is explained in Equation 1.1.

$$\Delta E = \frac{\gamma h B_0}{2\pi}$$

**Equation 1.1**

The difference between these energy levels increase with an increase in $B_0$. The relative distribution of these populations is described by Boltzmann distribution (Equation 1.2)

$$\frac{N_{-1/2}}{N_{1/2}} = e^{\left(-\frac{\Delta E}{kT}\right)}$$

**Equation 1.2**

where $N$ is the fraction of the population of nuclei in each energy state, $T$ is the temperature, and $k$ = Boltzmann constant ($1.38 \times 10^{-23}$ J $k^{-1}$). The majority of the spins occupy the lower level, resulting in a weak net magnetisation aligned along the axis of $B_0$. It is this weak net magnetisation that is measured in NMR spectroscopy. The net magnetisation will start to precess around the direction of the $B_0$ (also termed as the z-axis) at the Larmor frequency ($\nu_0$) as depicted in Figure 1.2.

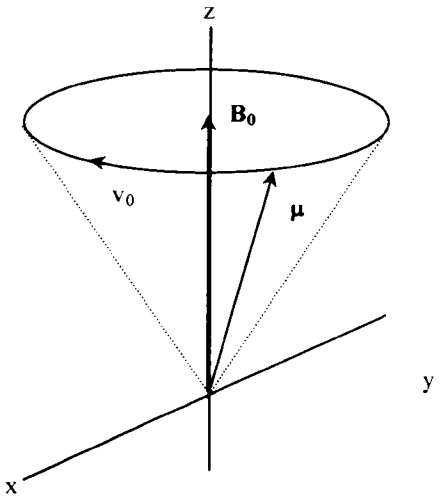



**Figure 1.2** Schematic to illustrate precessional orbit . The vector μ of the magnetic moment precesses in a static field with the Larmor frequency $\nu_0$ about the direction of the magnetic vector $B_0$.

A particular nucleus ($^1$H, $^{13}$C etc.) resonates at a characteristic frequency, i.e absorbs radio frequencies (RF) within a certain range, for example: $^1$H resonance frequency is 100 MHz. By applying RF pulses (also termed $B_1$) 90° to the z-axis at exactly the frequency of the nucleus, the net magnetisation can be flipped from the z-axis into the x-y plane, thereby creating an observable signal. Once the RF pulse is removed, the perturbed spin system will begin to relax back towards its equilibrium condition in the z-axis. The emissions from the net magnetisation decay are recorded by the spectrometer as a function of time in the form of free induction decay (FID). The signal is then Fourier transformed to obtain the spectrum in the frequency domain. For a more detailed description of NMR spectroscopy, a number of books are available (Abraham *et al.*, 1988; Claridge, 2008; Keeler, 2010).

### 1.3.1 1D $^1$H NMR Spectroscopy

1D $^1$H NMR spectrum contains chemical shift and J-coupling information for each proton in a molecule, which are indicative of the chemical environment (i.e. number of spin active neighbours, connecting bonds and geometrical relationships) and is independent of the applied field strength. Interpretation of this information is used for structural identification of compounds.

The chemical shift is a combination of the $B_0$ and the magnetic field at the nucleus; with the difference between these two termed nuclear shielding. Each chemical environment will precess at a slightly different frequency, giving rise to different signals in the spectrum, with an increase in chemical shift values resulting from more electronegative groups. Typically, chemical shift is always measured against a suitable reference, often trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TSP, $\delta = 0$), and is defined as: -

$$\delta = \frac{\nu_{sample} - \nu_{reference}}{oscillator\ \ frequency\ (Hz)} x10^6 (ppm)$$

**Equation 1.3**

Where $\nu_{reference}$ and $\nu_{sample}$ are the resonant frequency of their respective nuclei. This definition allows the resonance frequency of a signal to be expressed independently of the field strength. The chemical shift is expressed in parts per million (ppm) with the range being dependent on the nucleus being monitored, for example: $^1$H NMR range ~10 ppm.

J-coupling is the peak splitting pattern between adjacent nuclear spins due to the influence of bonding electrons or two nuclei in close proximity in which they interact with the spin-state of its neighbour. This is governed by the *n* number of equivalent coupled nuclei, each of spin $I = ½$, where the multiplicity of the signal will be split *n* + 1 times. The relative intensities of split peaks will be governed by Pascal's triangle, for example: a triplet has intensities of 1 : (1 + 1) : 1, and a quartet has intensities of 1 : (1 + 2) : (2 + 1) : 1. The distance between each of the signals is measured in Hertz.

### 1.3.2 2D NMR Spectroscopy

Biological samples produce complex $^1$H NMR spectra, often with heavily overlapping peaks in 3-4.5 ppm region. Thus, 2D NMR experiments (Table 1.4) can be used to increase spectral dispersion in the second dimension, allowing separation of overlapping peaks. Furthermore, 2D NMR experiments can be used to provide

unequivocal identification by obtaining information from correlations between homonuclear ($^1$H-$^1$H) and heteronuclear ($^1$H-$^{13}$C) coupling.

In comparison to 1D $^1$H NMR spectra, where the signal is obtained as a function of time, 2D NMR spectra, the signal is recorded as a function of two time variables, denoted as F1 and F2, with the resulting data Fourier transformed twice to yield a spectrum which is a function of two frequency variables. 2D data acquisition can be considered as an extension of a 1D NMR experiment. Initially, the sample is excited by an external magnetic field, which is allowed to evolve for a specific duration, F1. Further pulses then excite the nuclei, which is known as the mixing time, transferring the excitation to another nucleus, where the FID is recorded as function of F2 for each value of F1. The data is presented as a contour plot, where the interactions of the nuclei during the mixing time will result in diagonal cross- peaks arising from through bond (scalar) or through space (dipolar) interactions.

**Table 1.4** Summary of NMR experiments, which can be useful for assigning peaks in the $^1$H NMR spectra of complex mixtures (Aue *et al.*, 1976; Bax and Davis, 1985; Braunschweiler and Ernst, 1983; Cloarec *et al.*, 2005; Lindon *et al.*, 1996; Nagayama *et al.*, 1980; Pullen *et al.*, 1995).

| | |
|---|---|
| **Correlation Spectroscopy (COSY)** | The simplest type of 2D NMR experiment. It is used to establish connectivity between protons in a molecule or help simplify a spectrum of a complex mixture. |
| **Total Correlation Spectroscopy (TOCSY)** | Similar to COSY, but in theory gives a total correlation of all protons in an unbroken spin system. |
| **Statistical Total Correlation Spectroscopy (STOCSY)** | Takes advantage of the multicolinearity of the intensity variables in a set $^1$H NMR spectra to generate a pseudo-2D NMR spectrum that displays the correlation among the intensities of various peaks across the whole sample. |
| **J-Resolved Spectroscopy (J-Res)** | Used to establish the multiplicity of peaks in regions of heavy spectral overlap (particularly useful for sugars). |
| **$^1$H-$^{13}$C Heteronuclear Single Quantum Coherence Spectroscopy (HSQC)** | Used to establish correlation between $^{13}$C chemical shifts and $^1$H chemical shifts of compounds and hence lead to identification of compounds. |
| **HPLC-NMR-MS** | Used to simplify NMR spectra by physical separation and provide simultaneous mass spectra in the same chromatographic run. In theory it can be less labour intensive than off-line separation followed by NMR. |

## 1.4 Mass Spectrometry

Mass spectrometry was developed over a century ago by Thompson and Aston, which has been recently reviewed (Griffiths, 1997). In the period since, many advances have been observed into what it is today, a highly sensitive tool that is capable of analysing small and large molecules. The basis of a mass spectrometer is the measurement of an ion's mass-to-charge ratio (*m/z*). A mass spectrometer is made up of three main separate systems: the ion source, the mass analyser and the detector. Ions are generated by inducing either the loss or the gain of a charge from a neutral species. Once formed, the ions are electrostatically directed into the spectrometer where they can be separated according their *m/z* and finally detected. The result of ionisation, ion separation and detection is a mass spectrum that can provide molecular weight and structural information. For a more detailed description of MS, a number of books are available (Hoffmann and Stroobant, 2001; McMaster, 2005; Siuzdak, 1996).

### 1.4.1 Electrospray Ionisation

Electrospray ionisation (ESI) is the most common ionisation method used in LC-MS analysis for the production of gaseous ions from a liquid solution and has been described in several reviews (Gabelica and De Pauw, 2005; Gaskell, 1997; Kebarle and Verkerk, 2009). However, a number of other ionisation modes can be employed, such as electron impact (GC-MS), chemical ionisation (GC-MS) and APCI (LC-MS), with the latter two being less frequently used.

ESI is considered as a soft ionisation technique that results in little fragmentation and is generally suited to metabolites of high polarity. The Electrospray can be considered as a fine spray of highly charged droplets, which is generated by applying a strong electric field (typically +4 kV in positive ion mode and –4 kV in negative ion mode) under atmospheric conditions to the capillary tip, in which the eluent passes through. Assuming a positive potential is applied to the capillary tip, positive ions will accumulate at the tip to form a 'Taylor cone' which continuously produces positively charged droplets that are directed towards a counter electrode of lower potential, as depicted in Figure 1.3. Heated dry gas (typically nitrogen) is also injected coaxially in

order to promote solvent evaporation. As the solvent evaporates, the droplets reduce in size, resulting in an increase in charge density on the droplets surface. This continues until the so called 'Rayleigh limit', where 'Coulomb fission' occurs, resulting from the mutual repulsion between like charges exceeding the surface tension of the droplet. As the evaporation proceeds, the progeny droplets also undergo fission, which is repeated until a singly or multiply charged ion is produced, which is then directed into the MS. Two models have been suggested for the generation of gas-phased ions: the ion evaporation mechanism proposed by Iribarne and Thomson (Thomson and Iribarne, 1979) and the charged residue mechanism proposed by Dole (Dole *et al.*, 1968). Though the ESI mechanism is still in debate, it is generally accepted that small ions are produced from the ion evaporation mechanism and larger molecules such as globular proteins are produced *via* the charged residue mechanism (Gabelica and De Pauw, 2005).

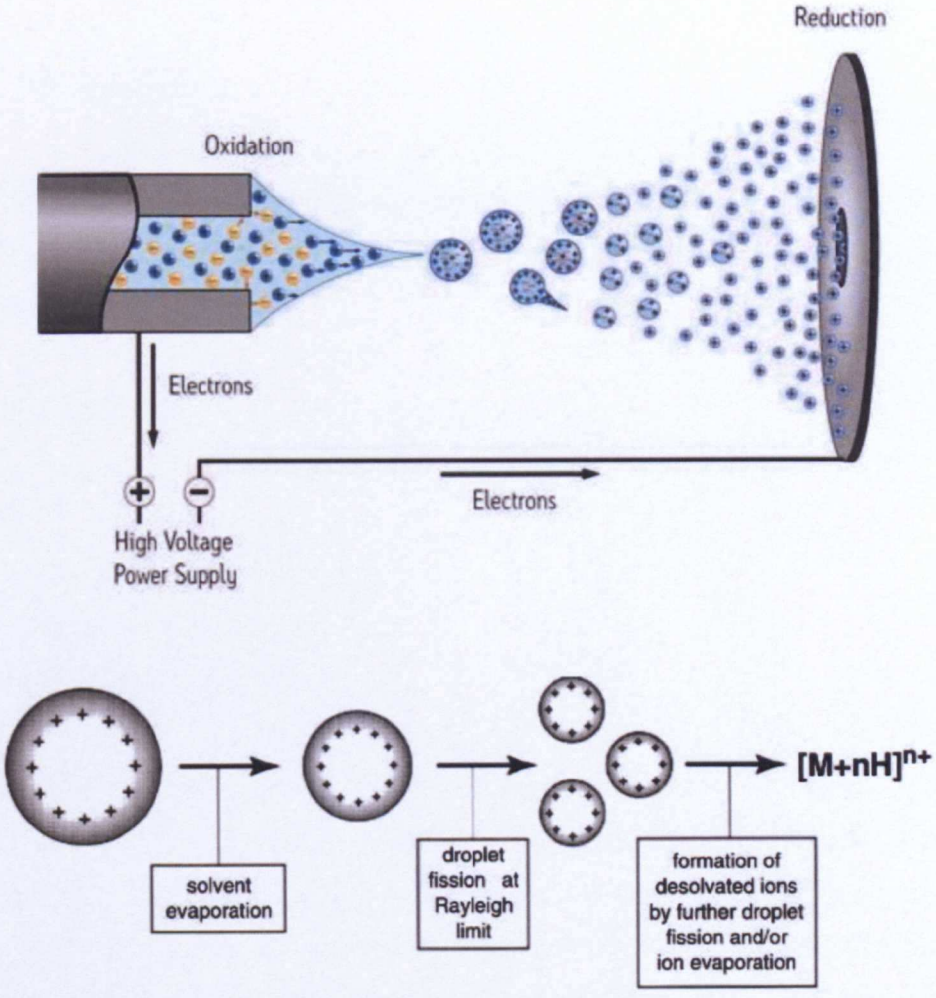



**Figure 1.3** Schematic representation of the generation of an electrospray and droplet formation in positive mode (modified from www.waters.com).

### 1.4.2 Mass Analysers

Mass analysers scan or select ions over a particular mass range. The key feature of all mass analysers is their measurement of *m/z*, which for a protonated molecule can be described as: -

$m/z = [M + nH] / nH$

Where

*m/z* – mass to charge ratio

M – molecular mass

nH – number of protons

However, other species such as sodium, ammonium or potassium adducts can also form. Furthermore, if the ion is multiply charged then the *m/z* will be significantly less than the actual mass. Multiple charging and the formation of adducts can yield many peaks that correspond to the same metabolite, which can make data analysis complex. Mass analysers have variations in their capabilities, such as accuracy, dynamic range and resolution, as summarised in Table 1.5. The resolution of a mass analyser is defined as the ability to distinguish between ions of different *m/z* ratios. Thus, greater resolution corresponds directly to the increased ability to differentiate ions (Siuzdak, 1996). The most common definition for resolution is given by: -

$\text{Resolution} = M / \Delta M$

where M and $\Delta M$ correspond to *m/z* and full width half maximum (FWHM) respectively. For example, an *m/z* of 500 and FWHM of one will have a resolution equal to 500. High resolution mass analysers allow separation of an ion's individual isotopes rather than a weighted average of all isotopes of each constituent element of the molecule, thereby, producing narrow peaks allowing more accurate determination of its position. Thus, the resolving power of the mass analyser to a certain extent determines the accuracy of the instrument.

**Table 1.5** General comparison of mass analysers (Hu *et al.*, 2005; Makarov *et al.*, 2006; McLuckey and Wells, 2001; Siuzdak, 1996).

| Mass Analyser | Quantity Measured | Mass Range (*m/z*) | Resolution | Dynamic Range | Mass Accuracy (ppm) | Advantages | Disadvantages |
|---|---|---|---|---|---|---|---|
| Quadrupole | Filters of *m/z* | 50-4000 | 4000 | $10^5$-$10^6$ | 100-1000 | Tolerant of high pressures, Suited for electrospray, Ease of polarity switching, Small size, and Cost | Mass range limited to m/z 3000, Low resolution |
| Ion trap | Frequency | 50 -2000; 200-4000 | 10 000 | $10^4$ | 100-1000 | Small Size, Medium resolution, Suited for tandom MS, Ease of polarity switching, and low cost | Limited mass range, Space charging effects, Precursor ion scanning and neutral loss scanning not available |
| Time-of-flight (TOF) | Flight time | No upper limit | 10 000 – 40 000 | $10^4$ | 5-10 | High mass range, fast scan speed, adaptation for MALDI, and low cost | Low resolution, Less accurate than an orbitrap |
| Orbitrap | Frequency | 50-2000; 200-4000 | 100 000 at mass 400 | $10^5$ | <5 | High resolution, More accurate than a TOF | High vaccum ($<10^{-7}$ Torr), Space charging effects, Instrument is large |

### 1.4.3 Quadrupole

Quadrupole mass analysers comprise four parallel rods with a fixed direct current (which can be positive or negative) and a superimposed radio-frequency (RF) potential being applied on opposing rods. The field on the quadrupoles functions as a mass filter by determining which ions are allowed to reach the detector. Ions entering this region will oscillate depending on their *m/z* ratio and depending on the applied radio frequency, only ions of a particular *m/z* will pass through. All other ions will have an unstable flight, causing the ions to be lost *via* contact with the rods.

### 1.4.4 Ion Trap

An ion trap analyser can conceptually be considered as a quadrupole bent on itself in order to form a closed loop, consisting of a ring electrode and two ellipsoid end caps that form a chamber. Ions with different masses entering the chamber are trapped together by an electric field and selectively expelled according to their mass by increasing the voltage on the ring electrode. There is a finite volume and capacity for the ions which limits the dynamic range, especially when analysing complex matrices. Furthermore, MS/MS fragmentation experiments are limited; for example, neutral loss scanning or precursor ion scanning cannot be conducted due to the ion trap being time-dependent rather than space-dependent, like quadrupoles. However, due to the structural similarities to quadrupoles, they are often hybridised (often called linear ion traps) to incorporate the functionalities of both, allowing on-the-fly experiments which would not be possible with either alone.

### 1.4.5 Time-of-Flight

Time-of-flight (TOF) is based on accelerating a set of ions to the detector (predetermined distance) with the same amount of kinetic energy, whereby the resulting velocity is characteristic of their *m/z* ratio. The smaller ions reach the detector first because of their greater velocity. More recent TOF instruments are combined with reflectors and ion pushers to further improve mass accuracy and resolution.

### 1.4.6 Orbital Trap

The orbitrap is a mass analyser that dynamically traps ions in an electric field formed between a central spindle electrode and two bell-shaped outer barrel electrodes (Hu *et al.*, 2005; Makarov, 2000). Electrostatic and centrifugal forces cause the ions to oscillate in both the axial and radial directions. The axial frequency is used to derive the *m/z* ratio because it is independent of the initial properties of the ions. The time domain transients are detected by the outer electrodes and converted to mass spectra through Fourier transformation. The orbitrap provides a higher mass resolution than most time-of-flight instruments (~10 000) and approaches that of FTICR (~100 000), to ensure higher mass accuracy and to enable confident discrimination between co-eluting, isobaric compounds in complex mixtures. The orbitrap analyser has also been coupled to a linear ion trap, allowing high resolution spectra while simultaneously conducting data-dependent MS/MS in the ion trap to aid identification of unknown metabolites (Hogenboom *et al.*, 2009) as $MS^n$ alone is not adequate for metabolite identification. A limited number of studies have reported the use of linear ion orbitrap in metabolomic analysis of microbes (Herebian *et al.*, 2009b), plants (Herebian *et al.*, 2009a), and animals (Dunn *et al.*, 2008; Zhang *et al.*, 2009).

### 1.4.7 Hybrid Instruments

The power of a given mass analyser can be increased by coupling to it another of the same or different type to obtain desirable performance characteristics. For example, QTOF or a hybrid quadrupole linear ion trap (QqQLit) (also referred to as QTRAP) mass spectrometer systems have special advantages in MS/MS studies, such as providing structural elucidation of metabolites. In the case of the QTOF, accurate mass measurements in the TOF can be performed on precursor or product ions. The QTRAP has the standard configuration of a QQQ mass spectrometer; however, Q3 can be operated as a linear ion trap on demand. This enables a number of scan functions; product ion scanning, precursor ion scanning (PI), natural loss scanning, and multiple reaction monitoring (MRM) as depicted in Figure 1.4. In addition to these scan functions common to a QQQ; the linear ion trap also includes Enhanced

MS (EMS), Enhanced Product Ion (EPI), $MS^3$, Enhanced Resolution (ER), Enhanced Multiply Charged (EMC), and Time Delay Fragmentation.

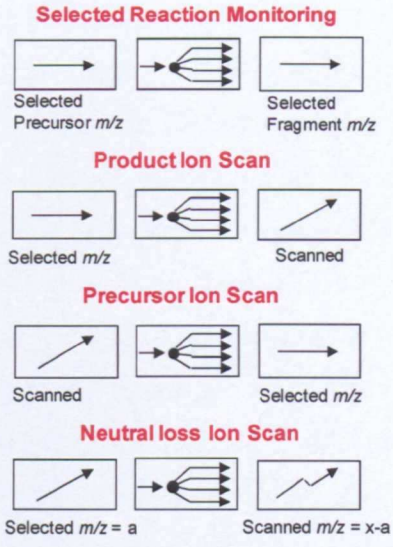



**Figure 1.4** Main MS/MS scan functions offered in tandem mass spectrometry.

The definitions of these scans are as follows: MRM (also referred to as single reaction monitoring (SRM)) consists of monitoring a fragmentation reaction, in which Q1 and Q3 are focused on selected masses. PI scanning consists of selecting a common fragment to monitor in order to determine the precursor ions. All the precursor ions that produce ions with the selected fragment mass in Q3 will be detected. EPI is generally coupled to an information dependent acquisition (IDA) function, whereby if the detected analyte signal meets a certain criteria (i.e. signal intensity), ions transmitted to Q3 are accumulated and scanned out to produce full ion spectra.

## 1.5 Multivariate Data Analysis

Multivariate analysis (MVA) can be broadly thought of as the application of mathematical and statistical methods for the aid of pattern recognition (PR) in chemical numerical data (Lindon *et al.*, 2004; Robertson, 2005). Metabolomics data, by definition, is multivariate since there are multiple measurements per sample, which typically exhibit correlated traits. Thus, MVA or PR is necessary for the investigation of such data (for example, spectra obtained from $^{1}H$ NMR spectroscopy or mass spectrometry), due to the fact that visual interpretation is impractical and the user will often miss the correlations between measurements, which will greatly reduce the amount of information that can be extracted from the dataset. Currently, the PR strategies typically pursued in metabolomics fall under the heading of 'unsupervised' and 'supervised', in order to generate the models depicted in Figure 1.5. Indeed, the general aim of PR is to classify an object or predict the origin of an object, based on the inherent patterns in a set of experimental measurements or descriptors in a way that will allow biological interpretation (Lindon *et al.*, 2004; Lindon *et al.*, 2003a). Like other 'omic' data, patterns provide one level of information, but the spectra can be drilled down to obtain the identification of individual components within the sample mixture (Robertson, 2005). Thus, MVA is an essential tool for investigating such data. However, there are obvious cost implications in terms of instrument time and 'man-hours' for the analysis of the data obtained. Though, through computational advancement in both hardware and software, these methods can now be achieved in a reasonable time frame.

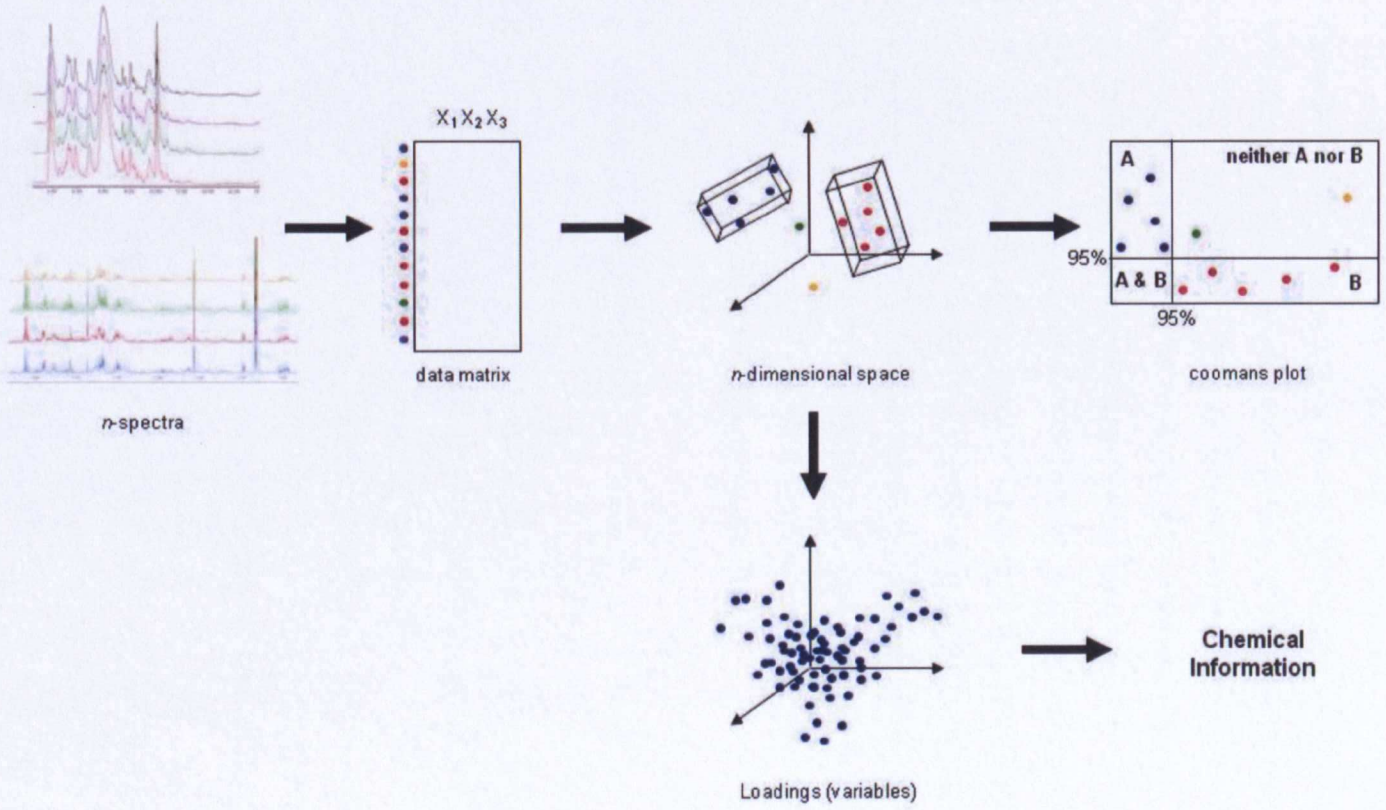



**Figure 1.5** Schematic diagram representing the process of assessing sample class from raw data obtained from either NMR or LC-MS. Reproduced with modifications (Holmes and Antti, 2002).

As mentioned previously, metabolomics data are essentially multivariate. Thus, the starting point for data analysis is a single matrix $N$ x $D$, where $N$ is the number of samples (either biological or technical replicates) and $D$ is the number of metabolites or variables. In the case of NMR data, the variables are often the chemical shift values, while for MS data this is most commonly represented as *m/z*. Each independent variable may be regarded as constituting a different dimension, thus, for $n$ variables, the object resides at a unique position in $n$-dimensional hyperspace (Goodacre *et al.*, 2007). Thus, the underlying theme of MVA is simplification or dimensionality reduction, for example, from several hundreds of peaks to a few coefficients, thereby facilitating in the aid of visualization of inherent patterns within the data. This may be achieved by unsupervised dimensionality reduction (through the use of projections), clustering (identifying groups of points which are similar to each), and supervised pattern recognition or machine learning (where knowledge about class membership is used to help discriminate between groups). It is beyond the scope of this thesis to describe in detail all the available methods on multivariate analysis; however, the following resources will provide a good overview (Beebe *et al.*, 1998; Duda *et al.*, 2001; Everitt, 1993; Hastie *et al.*, 2001).

### 1.5.1 Pre-processing of Data

Data pre-processing is arguably the most important stage of data analysis as the quality of any model produced will solely depend on the quality of the data presented (Daykin and Wulfert, 2006), i.e. garbage in, garbage out. The data initially collected by the analytical instrumentation is classed as raw data, which then can be exported in multiple different computer readable formats (generally for compatibility) before the pre-processing can be performed. The first stage of pre-processing is to reduce the file size, through reduction in data complexity, in order to make analysis less computationally extensive as well as presenting the data in a format suitable for a range of software packages such as SIMCA P (Umetrics, Umeå). The second stage is to remove or reduce any inaccuracies so that a single metabolite is reported as one feature. In terms of NMR data, one method would be to use integrals of a specific spectral peak. However, this is not only computational intensive, it can also be detrimental to the production of useful multivariate models because of any slight

variation of peak chemical shifts which can be brought about *via* changes in pH or ionic strength between samples. For example, citrate is particularly sensitive to these changes and whilst these shifts pose little problem by eye, from a data analysis perspective, if a certain peak is not consistently being expressed in the same position, the model will interpret the change as a different metabolite, making the model redundant. However, calculating the peak areas within specified segments, commonly referred to as 'bucketing' or 'binning' (Holmes *et al.*, 1997; Spraul *et al.*, 1994a), into bin widths of 0.04 ppm is a common tactic to solve this problem, as illustrated in Figure 1.6. This approach is adequate in most cases, though, it can be rather crude, as subtle metabolic differences between groups of data may be lost. For this reason, several authors prefer to align peaks with genetic algorithms, especially for high-resolution NMR data. Thus, a number of approaches have been proposed for this (Forshed *et al.*, 2003; Kassidas *et al.*, 1998; Lee and Woodruff, 2004; Stoyanova *et al.*, 2004; Vogels *et al.*, 1993). However, these are generally not feasible in metabolomic studies since the spectra can show either the complete loss of some peaks or appearance of new peaks. MS based data are generally converted from a 3D matrix of time *vs* mass *vs* intensity, to a matrix of chromatographic peaks (with associated retention time and accurate mass) and peak area. This deconvolution can be achieved either by using the manufacture's proprietary software (e.g. MarkerLynx supplied by Waters and SIEVE supplied by Thermo Scientific), or converting the raw data to produce a text-based file known as NetCDF (network common data format) (Rew and Davis, 1990), in order to use open-source software such as XCMS, Metalign, MZmine, MathDAMP (Baran *et al.*, 2006; Katajamaa and Oresic, 2005; Lommen, 2009; Nordstrom *et al.*, 2006).

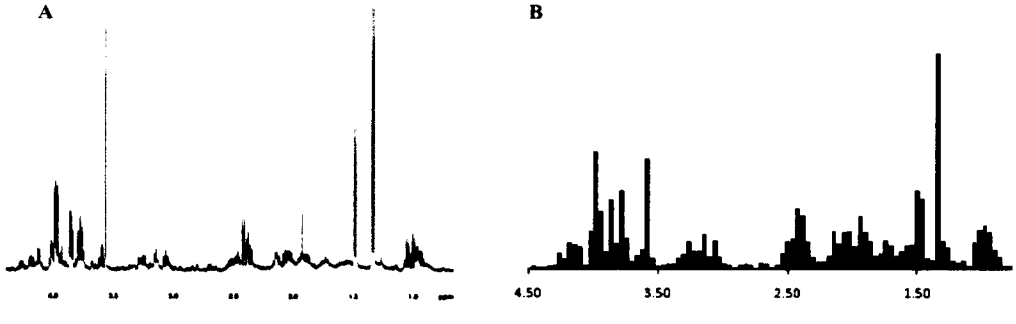



**Figure 1.6** An example of **A** NMR spectrum **B** segmented spectrum after bucketing to a width of 0.04 ppm. Own data obtained from the analysis of human apocrine sweat.

### 1.5.2 Data Pre-treatment Methods

Different pre-treatment methods can be used for generate MVA models, which can affect the level of biological information obtained. The two main methods currently used in metabolomics studies are mean centring and unit variance scaling (also known as auto scaling) (Eriksson *et al.*, 2001). Mean centring focuses on the differences in the data and not the similarities by removing the offset from the data to zero, instead of around the mean of each metabolite concentration. However, this can bias the model to the metabolites that are higher in concentration. Unit variance compares metabolites based on correlations, as all metabolites have a standard deviation of one (i.e. become equally important). Thus, large peaks that dominate the spectra are treated as equally as small peaks. However, this can have undesirable effects as the baseline noise is inflated. These data pre-treatment methods, as well as other less common scaling and transformation methods are described further by van den Berg (van den Berg *et al.*, 2006).

### 1.5.3 Principal Component Analysis

Probably one of the oldest and most used techniques of multivariate analysis is PCA, which allows the expression of most of the variance within the data set in a small number of factors or principal components (PCs). This is achieved by transforming the original set of variables into a new set of uncorrelated variables called PCs. These new variables are created from linear combinations of the initial descriptors with appropriate weighted coefficients. Indeed, this reduces the dimensionality of the data set while accounting for as much variation as possible. The properties of the PCs are such that each PC is orthogonal (uncorrelated) to each other and that each successive PC explains the largest variation of the data set not accounted for by the previous PCs. Thus, this will remove any 'noise' since after the first few PC, the axes will be due to random noise in the data set. Hence, the first few PCs will describe most of the variation, but the number is dependent upon the application e.g. for clinical applications you may use many more. Plotting two PCs summarises the observations in K-space by projecting each variable along each PC. The co-ordinate values on this plane are called scores, hence, plotting the projected configuration is known as a score

plot. Observations which are close to one another are similar in composition, whereas observations which are at opposite sides are significantly different. In order to determine which variables are influential or how the variables are correlated, a loading plot is used. The loadings define the orientation of the PC plane with respect to the original variables. Thus, variables which are inversely correlated are positioned in opposite sides of the plot and the distance from the origin represents how influential each variable is on the model.

### 1.5.4 Partial Least Squares Regression

There are a large number of supervised methods (i.e. class membership of the samples are included in the calculation). One of the most popular is PLS (Partial Least Squares), which is commonly used to determine whether a relationship exists between two matrices, **X** and **Y**. The **X** matrix usually comprises of spectral or chromatographic data, while, the **Y** matrix contains quantitative values for those samples (*a priori* knowledge), for example, concentration, drug dosage or storage time (Lindon *et al.*, 2006; Trygg *et al.*, 2007). These relationships are derived through the use of latent variables of the original matrix **X** (known as the predictor variable) and the observed vector **Y** (known as the response variable), which are acquired by an iterative procedure. These latent variables are used to create linear combinations of columns (**X** and **Y**) so that their covariance is maximised. When the first latent vector is found, it is subtracted from both **X** and **Y** and repeated until **X's** components tend to a zero value (Wold *et al.*, 1999). The resulting model can then be used to predict the analyte concentrations or examine the influence of time on a data set from the spectra of a new sample.

### 1.5.5 Principal Component Discriminant Analysis

PC-DA can be considered when more than two **Y** variables are being compared at any one time. It has the ability to summarise metabolite differentiation between-group variability, while overlooking within-group variation. Initially, the mean spectra for each class are calculated as per PCA, in order to construct significant limits for each specified class. Then the individual spectra, from each class, are projected onto the

PCA model, illustrating how close individual samples are to their respective means. However, unlike PLS, classification and prediction performance can only be qualitatively measured.

## 1.6 Identification of Components

Identification of individual metabolites from the obtained data is not always mandatory, while in other studies it is a critical part for the biological interpretation. For commonly analyzed biosamples, such as blood and urine, this can be achieved by comparison with spectral databases (see Table 5.2 on page 155), literature, and two-dimensional NMR spectroscopy. Moreover, for MS based approaches, a workflow summarised in Figure 1.7, can be followed in order to reduce the number of initial hits from databases in order to putatively identify unknown endogenous metabolites which can then be later confirmed by spiking the sample with internal standards. Nonetheless, for other samples, which are less well reported, these avenues of investigation will not result in positive metabolite identification. In this case, separation techniques such as LC-NMR-MS are required to gain full structural information. LC-NMR-MS can allow unequivocal identification of metabolites where neither LC-NMR nor LC-MS alone could generate all the necessary information. Once identified, the information can be stored in a database for future reference.

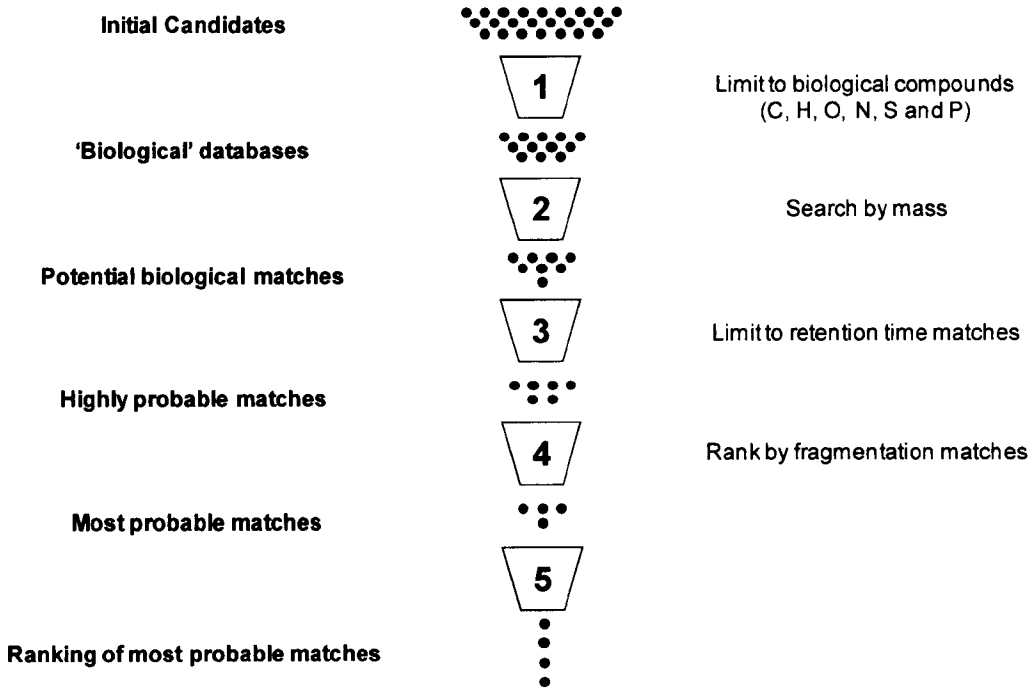



**Figure 1.7** Summary of the proposed workflow for reducing potential matches from databases searches.

## 1.7 Sweat Gland Biology

Skin is regarded as the most versatile organ of the human body (Wilke *et al.*, 2007) and accounts for 12-15% of body weight (Gallagher *et al.*, 2008). A vast amount of research has been published on the different roles, either from a barrier function or as a first-line immune response system (Menon and Kligman, 2009). However, relatively few studies have focussed on sweat glands or their biology even though regulation of core body temperature by sweating is a fundamental process for survival. Constant core body temperatures above 40°C result in protein denaturation and cell death, resulting in multiple organ failure. Hence, the most useful purpose of the sweating mechanism is to down-regulate the body core temperature by releasing thermal energy through the evaporation of water from the skin surface.

The nervous system ultimately governs the rate of perspiration to meet the requirement of the body and therefore production increases in proportion to the level of environmental stress and metabolic rate. This process is regarded as thermoregulatory sweating and involves eccrine glands, which are distributed over the whole body surface. Since thermoregulatory sweating serves as a system for temperature reduction, failure in this system will therefore lead to hyperthermia and death (Wilke *et al.*, 2007). The onset of thermal sweating is caused when the sum of the internal body temperature has a 10-fold increase over the mean skin temperature, i.e. the internal temperature increases by a significant amount to what is experienced on the surface. It is also important to note that sweating can be affected by many internal factors such as gender, physical fitness, menstrual cycle and circadian rhythm as well as external factors like humidity. Thus, the sweating rate can vary from each individual. The onset of sweating can also arise from emotional stress (emotional sweating) or through the consumption of highly spicy food (gustatory sweating). The former is caused by a physical reaction to emotive stimuli, for example; anxiety, fear or pain, and can occur over the whole body but are mainly confined to the palms, soles, and axillary region (Allen *et al.*, 1973; Eisenach *et al.*, 2005). Thus, in this instance, sweating occurs independently of ambient temperature and decreases during relaxation (Wilke *et al.*, 2007). However, emotional sweating does not occur until the

onset of puberty, which leads to the assumption that apocrine and apoeccrine sweat glands are involved, as these glands become functional during this development stage (Lonsdale-Eccles *et al.*, 2003). Gustatory sweating on the other hand is the direct or indirect thermal effect of ingestion of certain foods (e.g. hot and spicy substances) and is confined to the face, scalp, and neck. Its onset is usually triggered within a few seconds of food entering the mouth, rarely by smelling of food and is not triggered through food reaching the stomach, chewing inert substances, or thought of food (Bloor, 1969; Bronshvag, 1978). Ingestion of food also causes an increase in metabolism, which leads to an increase in body temperature and the onset of thermal sweating.

### 1.7.1 Sweat Glands

Sweat glands are cutaneous appendages like hair follicles or sebaceous glands, forming tiny coiled tubes embedded in the dermis or subcutaneous fat. They contain myoepithelial cells, which, when contracted, squeeze the gland to discharge the accumulated secretions *via* the duct to the outer skin surface. Glands are characterised through their morphology or by their mode of secretion. For example: sweat glands secrete eccrine (secretion is released from the cell as a liquid without disintegration) or apocrine (secretion occurs via pinch off of outer cell parts) (Lonsdale-Eccles *et al.*, 2003; Wilke *et al.*, 2007). Through this distinction there are said to be two types of sweat glands: eccrine (also known as merocrine sweat glands) and apocrine glands. The morphology of the eccrine and apocrine sweat glands is depicted in Figure 1.8, whereby, the green (DiI stained) and red (Nileblue stained) outlines the coil cellular boundaries and nuclei respectively. While immunolabelling with MFG, a marker for eccrine glands, or CD15, a marker for apocrine glands, is also shown. Wilke and co-workers have reported a number of other antibodies that can also be used to differentiate between eccrine and apocrine glands in order to describe their structural diversity (Wilke *et al.*, 2006). The apocrine gland coil is ~800 μm in diameter (ca. to eccrine gland coil ~500-700 μm), with an outer and internal tubule diameter of ~200 μm and ~100 μm respectively. The duct and secretary coil is a very short straight tube (ca. to eccrine duct which is generally a corkscrew channel ~2000 μm in length) and is located near to the hair follicle. The secretary cells consist of a single layer of

columnar shaped cells situated near to the basal membrane. In comparison, the eccrine outer and internal tubule diameter is ~120 μm and ~40 μm respectively.

Sato and co-workers reported the existence of a third type of sweat gland which could not be classified as either eccrine or apocrine gland and showed characteristics of both, hence, it was termed the 'apoeccrine' gland (also known as mixed-type gland) (Sato *et al.*, 1987). It is questionable however, as to whether this third type of sweat gland exists. Recently, Bovell and co-workers (Bovell *et al.*, 2007) investigated the axillary skin of ten volunteers, and tried to identify apoeccrine glands by a means of serial sectioning histology and immunofluorescence using CD15, CD44, S100 and HMFG-1 antibodies. They concluded that no evidence of apoeccrine glands was found either by histology or by immunofluorescence. Thus, the lack of distinct features does not comply with the definition proposed by Sato (Sato *et al.*, 1987). To the author's knowledge, this work has not been repeated, thus, to overcome this controversy further investigations are needed on normhydrotic and hyperhidrotic patients, in addition to the effects of dissection on glandular appearance, to reveal if there really is a 'third' type of sweat gland.

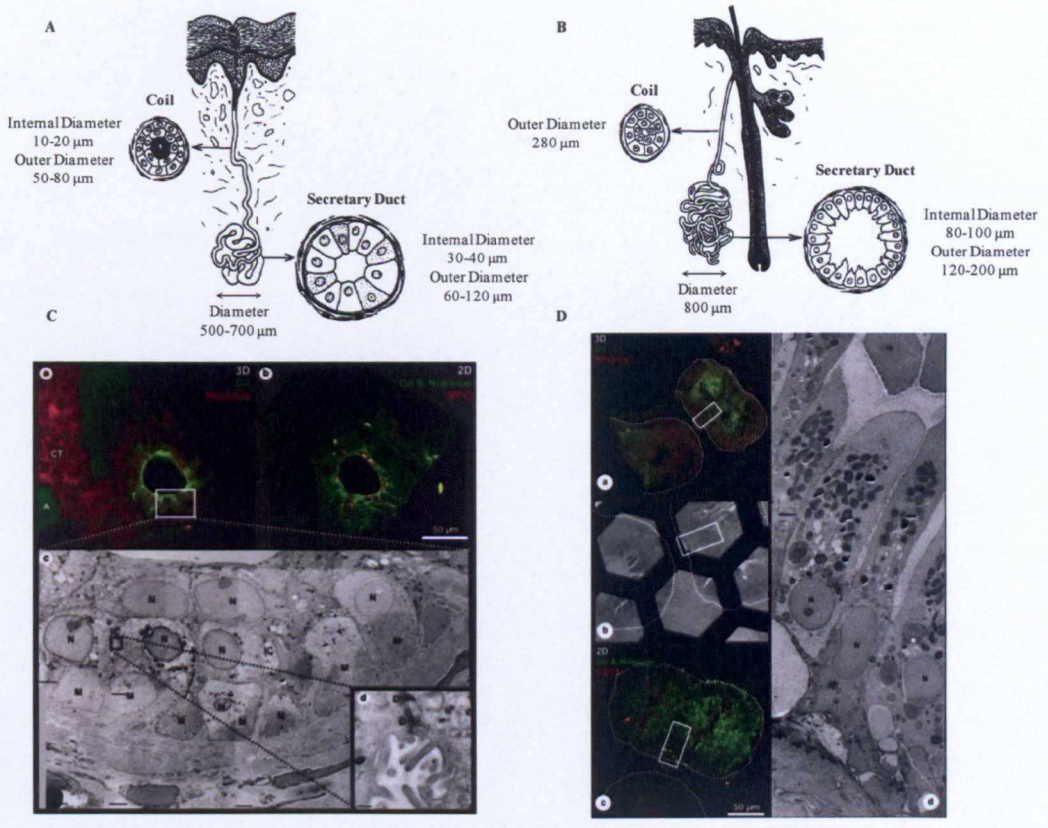



**Figure 1.8** Upper: Schematic diagram of **A** eccrine gland **B** apocrine gland showing both a cross-section of the coil and duct. Lower: Correlative microscopy of **C** eccrine coil **D** apocrine coil. Myoepithelial cells (M), Intracellular canaliculi (IC), Nuclei (N), and desmosomal contacts (D). Reproduced with modifications (Weiner and Hellmann, 1960; Wilke *et al.*, 2008).

### 1.7.2 Chemical Composition of Eccrine Sweat

Excreted sweat can vary in composition and electrolyte concentration depending on the secretary and reabsorptive properties at the coil and duct, respectively. When sweat is initially formed in the secretary coil it is near iso-osmotic (Sato *et al.*, 1987), and following excretion the sweat is relatively hypo-osmotic and acidic (Patterson *et al.*, 2000). This change in sweat osmolality and acidity can vary between skin regions (Sato and Dobson, 1970), as it is reliant on the functional capacity of the sweat gland, which is ultimately governed by the ductal transport processes. From an analytical perspective this adds further complexity to the study of human sweat as variation in sweat composition can vary significantly within an individual.

Sweat is primarily composed of water (99%), and is therefore well suited for evaporative cooling. A diverse array of organic and inorganic compounds can be found in sweat, many of which are found in plasma but at much lower concentrations (Harker *et al.*, 2006), thus, sweat glands appear to selectively secrete some substances and retain others (Robinson and Robinson, 1954). There are approximately 61 compounds reported in eccrine sweat with varying concentrations reported in the literature (see Table 1.6). To the author's knowledge, there is no reported literature on electrolytes and ionic constituents, or their respective concentrations in apocrine sweat. However, it is likely they would be of similar magnitude. Furthermore, it is important to establish chemical composition of eccrine sweat as this could provide useful information in identifying components present in apocrine sweat, which will be discussed in further detail later (see Section 1.7.5).

**Table 1.6** Median values of eccrine sweat constituents. Reproduced with modifications (Harvey *et al.*, 2010; Stefaniak and Harvey, 2006).

| Components | Molecular Formula | Median (min and max) concentration (M) |
|---|---|---|
| ***Electrolytes*** | | |
| Sodium | $Na^+$ | $3.1x10^{-2}$ ($1.1x10^{-4}$ - $3.9x10^{-1}$) |
| Chloride | $Cl^-$ | $2.3x10^{-2}$ ($1.7x10^{-5}$ - $2.8x10^{-1}$) |
| Calcium | $Ca^{2+}$ | $5.2x10^{-3}$ ($4.7x10^{-6}$ - $1.5x10^{-2}$) |
| Potassium | $K^+$ | $6.1x10^{-3}$ ($6.7x10^{-6}$ - $3.8x10^{-2}$) |
| Magnesium | $Mg^{2+}$ | $8.2x10^{-5}$ ($7.4x10^{-8}$ - $3.8x10^{-3}$) |
| Phosphate | $PO_4^{3-}$ | $3.1x10^{-4}$ ($2.3x10^{-5}$ - $1.1x10^{-3}$) |
| ***Inorganic Ions*** | | |
| Ammonium | $NH_4^+$ | $5.2x10^{-3}$ ($4.7x10^{-4}$ - $2.5x10^{-2}$) |
| Bicarbonate | $HCO_3^-$ | $3.0x10^{-3}$ ($2.6x10^{-4}$ - $2.0x10^{-2}$) |
| ***Ionic Species*** | | |
| Sulfate | $SO_4^{2-}$ | $4.2x10^{-4}$ ($7.0x10^{-5}$ - $2.0x10^{-3}$) |
| Sulfur | S | $2.3x10^{-3}$ ($2.2x10^{-4}$ - $2.3x10^{-3}$) |
| Fluoride | F | $1.1x10^{-5}$ ($5.8x10^{-8}$ - $9.5x10^{-5}$) |
| Phosphorous | P | $1.3x10^{-5}$ ($2.2x10^{-8}$ - $1.5x10^{-3}$) |
| Bromine | Br | $2.3x10^{-6}$ ($4.4x10^{-9}$ - $6.3x10^{-6}$) |
| Cadmium | Cd | $1.8x10^{-8}$ ($1.2x10^{-8}$ - $2.3x10^{-8}$) |
| Copper | Cu | $9.4x10^{-7}$ ($1.3x10^{-8}$ - $1.2x10^{-3}$) |
| Iodide | I | $7.1x10^{-8}$ ($7.9x10^{-11}$ - $7.5x10^{-5}$) |
| Iron | Fe | $9.8x10^{-6}$ ($1.5x10^{-8}$ - $1.1x10^{-3}$) |
| Lead | Pb | $1.2x10^{-7}$ ($3.5x10^{-9}$ - $2.0x10^{-7}$) |
| Manganese | Mn | $1.1x10^{-6}$ ($1.1x10^{-9}$ - $1.3x10^{-3}$) |
| Nickel | Ni | $4.2x10^{-7}$ ($1.7x10^{-9}$ - $8.3x10^{-7}$) |
| Zinc | Zn | $1.3x10^{-5}$ ($1.4x10^{-8}$ - $2.3x10^{-5}$) |
| ***Amino acids*** | | |
| Alanine | $C_3H_7NO_2$ | $3.6x10^{-4}$ (NA) |
| Valine | $C_5H_{11}NO_2$ | $2.5x10^{-4}$ ($1.3x10^{-4}$ - $3.8x10^{-4}$) |
| Leucine | $C_6H_{13}NO_2$ | $2.1x10^{-4}$ ($9.1x10^{-5}$ - $3.2x10^{-4}$) |
| Isoleucine | $C_6H_{13}NO_2$ | $1.7x10^{-4}$ ($7.6x10^{-5}$ - $2.8x10^{-4}$) |
| Phenylaline | $C_9H_{11}NO_2$ | $1.3x10^{-4}$ ($6.1x10^{-5}$ - $2.1x10^{-4}$) |
| Glycine | $C_2H_5NO_2$ | $3.9x10^{-4}$ (NA) |
| Threonine | $C_4H_9NO_3$ | $4.5x10^{-4}$ ($1.4x10^{-4}$ - $7.6x10^{-4}$) |
| Tyrosine | $C_9H_{11}NO_3$ | $1.7x10^{-4}$ ($6.6x10^{-5}$ - $3.0x10^{-4}$) |
| Aspartic acid | $C_4H_7NO_4$ | $3.4x10^{-4}$ (NA) |
| Glutamic acid | $C_5H_9NO_4$ | $3.7x10^{-4}$ (NA) |
| Histidine | $C_6H_9N_3O_2$ | $5.2x10^{-4}$ ($2.7x10^{-4}$ - $1.3x10^{-2}$) |
| Lysine | $C_6H_{14}N_2O_2$ | $1.5x10^{-4}$ ($9.6x10^{-5}$ - $2.2x10^{-3}$) |

*Table 1.6 continued*

| Components | Molecular Formula | Median (min and max) concentration (M) |
|---|---|---|
| Arginine | $C_6H_{14}N_4O_2$ | $7.8x10^{-4}$ ($3.3x10^{-4}$ - $4.4x10^{-3}$) |
| Tryptophan | $C_{11}H_{12}N_2O_2$ | $5.5x10^{-5}$ ($2.0x10^{-5}$ - $9.1x10^{-5}$) |
| Creatine | $C_4H_9N_3O_2$ | $1.5x10^{-5}$ (NA) |
| Ornithine | $C_5H_{12}N_2O_2$ | $1.5x10^{-4}$ (NA) |
| Citrulline | $C_6H_{13}N_3O_3$ | $4.0x10^{-4}$ (NA) |
| *p*-Aminobenzoic acid | $C_7H_7NO_2$ | $7.1x10^{-8}$ ($5.8x10^{-9}$ - $1.8x10^{-2}$) |
| ***Hydroxy/Keto Acids*** | | |
| Pyruvic acid | $C_3H_4O_3$ | $1.8x10^{-4}$ ($1.0x10^{-7}$ - $1.0x10^{-3}$) |
| Lactic acid | $C_3H_6O_3$ | $1.4x10^{-2}$ ($3.7x10^{-3}$ - $5.0x10^{-2}$) |
| ***Fatty Acids*** | | |
| Acetic acid | $C_2H_4O_2$ | $1.3x10^{-4}$ ($5.9x10^{-5}$ - $4.2x10^{-4}$) |
| Propionic acid | $C_3H_6O_2$ | $3.5x10^{-6}$ ($1.2x10^{-6}$ - $7.4x10^{-6}$) |
| Butyric acid | $C_4H_8O_2$ | $2.4x10^{-6}$ ($5.0x10^{-7}$ - $6.0x10^{-6}$) |
| Isobutyic acid | $C_4H_8O_2$ | $8.0x10^{-7}$ (NA) |
| Isovaleric acid | $C_5H_{10}O_2$ | $1.1x10^{-6}$ ($2.0x10^{-7}$ - $4.5x10^{-6}$) |
| Hexanoic acid | $C_6H_{12}O_2$ | $9.0x10^{-7}$ ($2.0x10^{-7}$ - $3.5x10^{-6}$) |
| ***Carbohydrates*** | | |
| Dehydroascorbic acid | $C_6H_6O_6$ | $1.1x10^{-5}$ ($7.6x10^{-9}$ - $8.6x10^{-4}$) |
| Glucose | $C_6H_{12}O_6$ | $1.7x10^{-4}$ ($5.6x10^{-6}$ - $2.2x10^{-3}$) |
| ***Amino Ketones*** | | |
| Urea | $CH_4N_2O$ | $1.0x10^{-2}$ ($1.8x10^{-}3$ - $4.6x10^{-2}$) |
| Creatinine | $C_4H_7N_3O$ | $8.4x10^{-5}$ ($8.8x10^{-6}$ - $2.0x10^{-3}$) |
| ***Purines and Purine Derivatives*** | | |
| Uric acid | $C_5H_4N_4O_3$ | $5.9x10^{-5}$ ($4.2x10^{-6}$ - $4.8x10^{-3}$) |
| ***Vitamins*** | | |
| Choline | $C_5H_{14}NO$ | $2.6x10^{-5}$ ($6.8x10^{-7}$ - $1.5x10^{-4}$) |
| Ascorbic acid | $C_6H_8O_6$ | $1.0x10^{-5}$ ($1.1x10^{-7}$ - $3.6x10^{-5}$) |
| Inositol | $C_6H_{12}O_6$ | $1.6x10^{-6}$ ($8.3x10^{-7}$ - 1.2) |
| Nicotinic acid | $C_6H_5NO_2$ | $4.1x10^{-1}$ ($1.4x10^{-7}$ - 3.7) |
| Pantothenic acid | $C_9H_{17}NO_5$ | $1.3x10^{-1}$ ($6.8x10^{-8}$ - 3.6) |
| Pyridoxine | $C_8H_{11}NO_3$ | $1.0x10^{-8}$ ($2.4x10^{-9}$ - $5.0x10^{-3}$) |
| Riboflavin | $C_{17}H_{20}N_4O_6$ | $2.0x10^{-2}$ ($1.3x10^{-8}$ - $8.0x10^{-1}$) |
| Folic acid | $C_{19}H_{19}N_7O_6$ | $1.6x10^{-8}$ ($1.2x10^{-8}$ - $2.0x10^{-8}$) |
| Thiamine | $C_{12}H_{17}ClN_4OS$ | $5.0x10^{-3}$ ($4.5x10^{-9}$ - 5) |

The major cations ($Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$) and anions ($Cl^-$, $PO_4^{3-}$, $HCO_3^-$) located in sweat are similar to that found in plasma. The most concentrated solute in sweat is NaCl, which ranges from 5 mM to as high as 148 mM (Robinson and Robinson, 1954). Any salt depletion produced by sweating can cause heat cramps and fatigue resulting from the reduction in extracellular fluid volumes due to the reduction in tonicity. Furthermore, a relationship between sweat $Na^+$ concentration and pH has been reported, whereby, the greater the concentration of $Na^+$ the greater the pH (Kaiser *et al.*, 1974). Hence there is considerable variation in the observed pH of sweat. In the literature, the observed pH ranges from 2.1-8.2 (median 5.3) for eccrine sweat (Stefaniak and Harvey, 2006), which has been found to be more acidic than that of apocrine sweat (Robinson and Robinson, 1954).

Potassium concentration tends to be lower than that of sodium or chloride averaging approximately 4.5 mM. It has been reported by Locke (Locke *et al.*, 1951), that potassium concentration varies inversely with sodium concentration and that the ratio of Na/K varies directly with sodium concentration. While Patterson and co-workers reported a positive relationship between lactate and $K^+$ concentration (Patterson *et al.*, 2000).

Lactic acid and pyruvate have both been measured in sweat secretion, values of which range from 4-40 mM and 0.1-0.8 mM, respectively (Robinson and Robinson, 1954), of which, both substances are more concentrated than that of blood or urine. Despite lactate being present in high concentrations, little attention has been made to its functioning role within the sweat gland. It has been suggested that lactic acid may come from the blood for a regulatory purpose in preventing an excess accumulation of blood and tissue lactate (Robinson and Robinson, 1954). However, it is debatable as to whether lactate present in sweat is derived from blood lactate. Others have suggested that lactate is produced from the gland's metabolism (Gordon *et al.*, 1971; Sato and Dobson, 1971; Weiner and Vanheyningen, 1952). In the literature, it is currently unclear as to why there is such a varied concentration range in both lactate and pyruvate with respect to changes in temperature, sweating rate, sweating duration and acclimatisation, although these factors have been studied in regards to sodium and chloride concentration of sweat. Nonetheless, there have been a few studies highlighting an inverse relationship between lactate concentration and exercise

intensity (Lamont, 1987; Meyer *et al.*, 2007). Although, this relationship is likely be due to a dilution effect resulting from the increase in sweat secretion (Buono *et al.*; Green *et al.*, 2004).

Nitrogenous constituents of sweat vary in concentration when compared to blood plasma. For example: ammonia is typically 100 times more concentrated in sweat; urea is about twice as concentrated in sweat; amino acid concentrations are similar or slightly lower than in plasma, although the concentrations of individual amino acids may differ; and both uric acid and creatinine are more dilute in sweat. Typical nitrogen concentrations of sweat reported in the literature vary from 0.5-8 mM (Sato *et al.*, 1989). Other amino acids, including cysteine, methionine, proline, and serine have also been detected in eccrine sweat, however, their quantitative concentration have not been reported (Coltman *et al.*, 1966; Liappis and Hungerla, 1972; Stefaniak and Harvey, 2006).

### 1.7.3 Axillary Malodour Formation

The human body generates a variety of different odours, for example; scalp, hair mouth, axilla, foot, and general skin surface all possess characteristic odours. Of all the human scents, axillary odour is probably the most powerful and easily recognisable (Hasegawa *et al.*, 2004).

Axillary odours possess a distinctive malodorous scent communally called "body odour" which originates from the axilla regions. These regions contain a dense arrangement of apocrine, eccrine, sebaceous, and apoeccrine glands (see Table 1.7 for an overview between the differences of the glands), and volatile substances evaporating from these areas make a key contribution to human body odour (Zeng *et al.*, 1996a). This has been examined and discussed from an analytical, biological and a behavioural-physiology point of view (Hasegawa *et al.*, 2004). Although, in today's society, body odour is deemed unpleasant, several studies have illustrated that axilla odour may contain chemical signals that affect the menstrual cycle (Natsch *et al.*, 2004), or that it may be involved in mate selection depending on the major histocompatibility complex (MHC)-allele (Natsch *et al.*, 2004). Axillary secretions

are an ideal source of pheromones, thus, are generally secreted into areas that often contain hair that can greatly increase the surface area for dispersion and aid in volatilization. Hence, axilla is a focal point for a multi-billion-dollar consumer product industry since the general consensus in today's society is to eliminate or mask these body odours (Gautschi *et al.*, 2007; Wysocki and Preti, 2004).

**Table 1.7** Difference between eccrine, apocrine and apo-eccrine sweat glands. Reproduced with modifications (Lonsdale-Eccles *et al.*, 2003; Sato *et al.*, 1989).

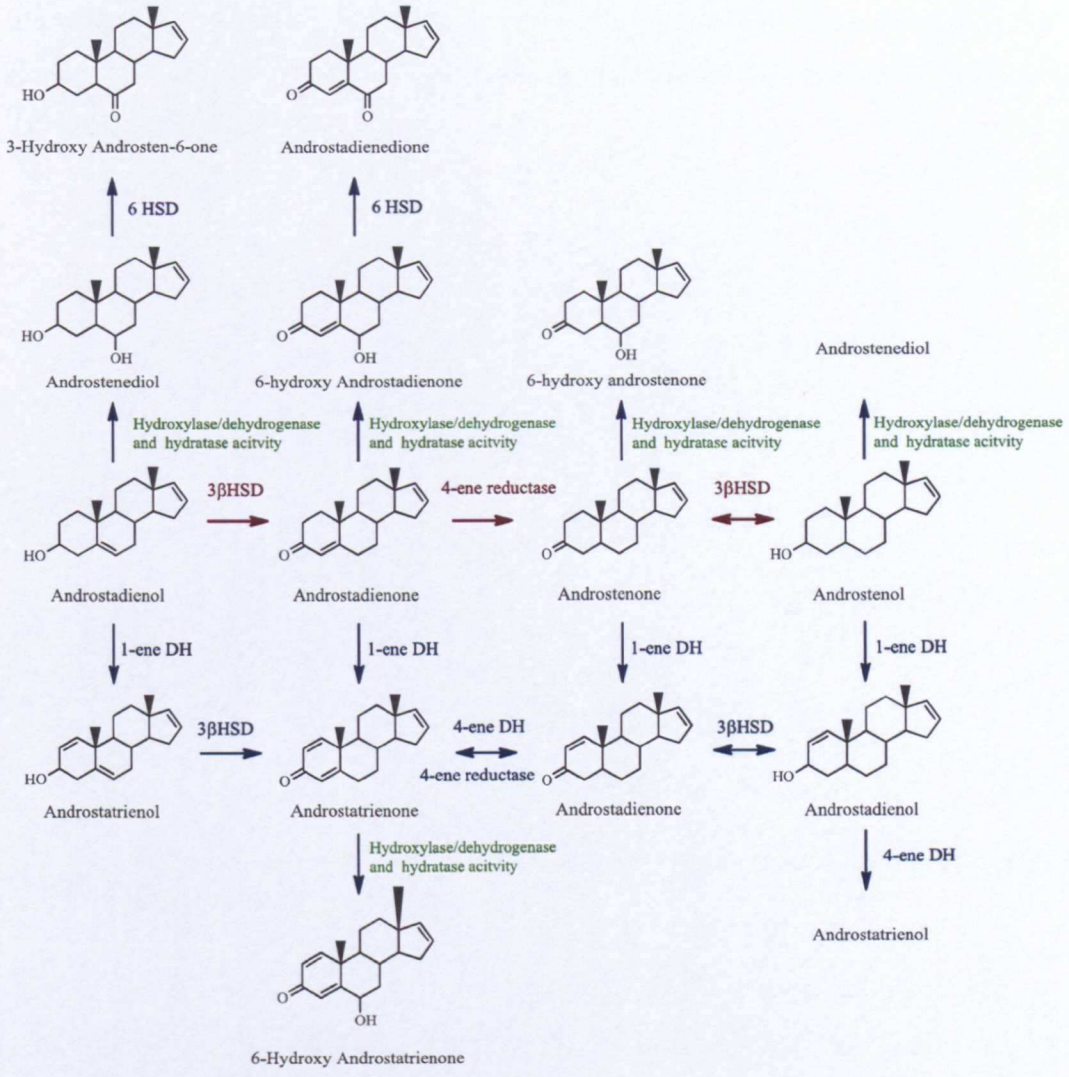
| | **Eccrine** | **Apocrine** | **Apo-eccrine** |
|---|---|---|---|
| Age of onset of gland activity | Birth | Adolescence | Adolescence |
| Distribution of sweat glands | Whole body – excluding auditory canal, clitoris and labia minora | Axilla, breast and labia | Hair-bearing area of axilla |
| Stimulation | Cholinergic >> Adrenergic | Cholinergic = Adrenergic | Cholinergic > Adrenergic |
| Gland size | Small | Large | Variable |
| Location of secretary coil | Mid-dermis | Deep-dermis/fat | Deep-dermis |
| Duct | Long – connecting directly to skin surface | Short – connecting to hair follicle | Long – connecting directly to skin surface |
| Cell type | Secretary (clear), dark and myoepithelial | Columnar secretary cell, myoepithelial | Dilated segments resemble apocrine gland and non-dilated resemble eccrine glands |
| Sweat secretion rate | Continuous – high output | Transient and intermittent – low volume | Continuous – very high output |
| Gland product | Water fluid | Turbid fluid – protein rich | Water fluid |

The ultimate source of axillary odour is apocrine sweat, which, when initially secreted is both sterile and odourless (Leyden *et al.*, 1981), and since the pioneering work of Shelly *et al.* (Shelley *et al.*, 1953), it is known that the action of skin bacteria is needed to generate the odoriferous components from the non-smelling molecules present within these secretions. Indeed, the axilla is a skin region supporting a dense bacterial population ($\sim 10^6$ cells $cm^{-2}$), which are dominated by two genera *Staphylococcus* and *Corynebacteria* (Austin and Ellis, 2003; Natsch *et al.*, 2003), both of which are best adapted for the conversion of fresh apocrine sweat to the 'classic male locker room smell' (Troccaz *et al.*, 2004).

Most individuals carry a flora that is dominated by either one of these two genera; however, strong correlations between high populations of *Corynebacteria* and strong axillary odour formation have been found. For example, certain strains of *Corynebacteria* have been associated with the clinical condition of plantar bromidrosis (acute offensive body odour) (Troccaz *et al.*, 2004). Thus, *Corynebacteria* and certain *Staphylococci* possess all the enzymatic machinery required to carry out biochemical conversions of proteins, lipids and steroids into odorous components (Natsch *et al.*, 2004; Troccaz *et al.*, 2004). As practical consequence of these findings, the cosmetic industry introduced halogenated antibacterials and aluminium preparations for reducing bacterial populations as their main active ingredient in deodorants over the last 40 years (Gautschi *et al.*, 2007; Natsch *et al.*, 2003). Considerable progress has been made since these initial observations, in terms of identifying odorous compounds, however, there has been less attention to the biochemistry of axillary odour formation. Hence there is only a limited amount of literature available regarding the structures of precursors isolated from axilla secretions, and no specific bacterial enzymes capable of generating these precursors have been isolated.

### 1.7.4 Chemical Nature of Body Malodour

The composition of sweat odour comprises of four principle components. First, a steroidal fraction comprising of four pungent steroids, 5α-androst-16-en-3-one, androsta-4,16-dien-3-one, and their respective alcohols, 5α-androst-16-en-3α-ol and androsta-4,16-dien-3α-ol. It has only been recently that the identities of these volatile steroids precursors present in apocrine sweat have been identified (Austin and Ellis, 2003). From their discoveries, Austin and Ellis proposed a new metabolic map (Scheme 1.1) of the biotransformations needed to generate odourous components from non-odourous precursors. They also illustrated that no single bacterium isolated was capable of carrying out the full complement of enzymatic transformations, thereby, generating a blend of 16-androstenes. Furthermore, the overall ability of *Corynebacteria* to biotransform odourous steroids from androsta -5,16-dien-3α-ol is low (Austin and Ellis, 2003).




**Scheme 1.1** Simplified schematic of the biotransformation of 16-androstenes by *Corynebacteria* (A) axillary isolates. It is important to note that no single isolate is capable of carrying out all the enzymatic reactions to produce all the steroidal malodour components. Key: HSD (hydroxysteroid dehydrogenase) and DH (dehydrogenase). Reproduced with modifications (Austin and Ellis, 2003).

Secondly, volatile fatty acids (VFAs) can be formed by the action of bacteria, typically *Corynebacterium* species (Figure 1.9). However, the *Corynebacterium* genus can be split into two groups depending on their ability to metabolise lipids, specifically free fatty acids (FFA). These groups have been named as *Corynebacterium* (A) and (B), where the latter are unable to utilise FFA as a carbon source (Austin and Ellis, 2003). Furthermore, the former, sub-group A, lack the ability to fully catabolise long chain fatty acids (LCFAs), and instead only partial degradation products are formed ($C_{6\text{-}11}$ acids). For example, methyl-branched fatty

acids such as isostearic acid (heterogeneous $C_{16-18}$ branched and unbranched fatty acids) are metabolised into a range of highly odourous 2-methyl-$C_{6-14}$ fatty acids (Troccaz *et al.*, 2004).

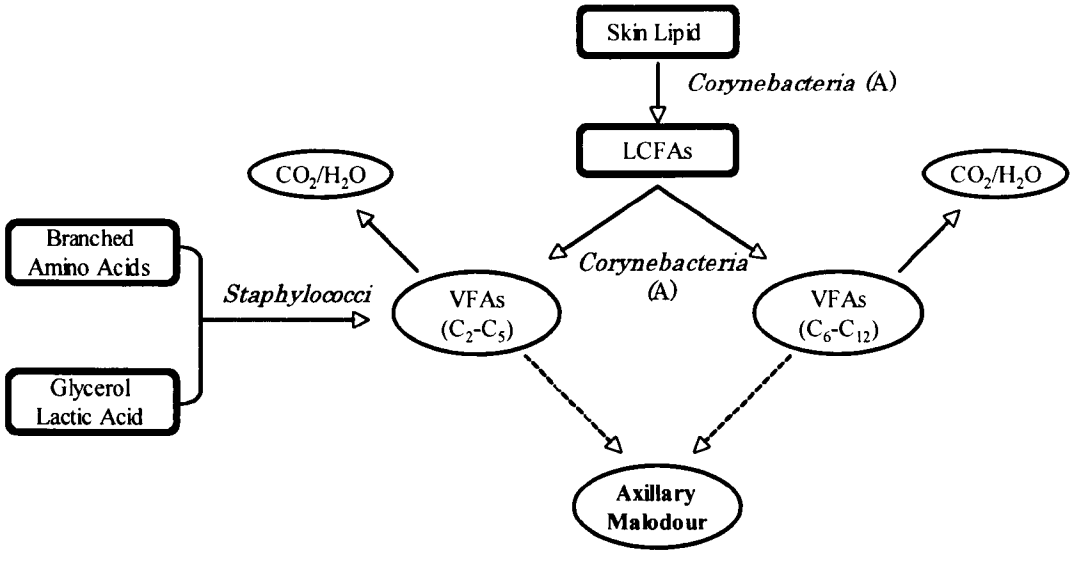
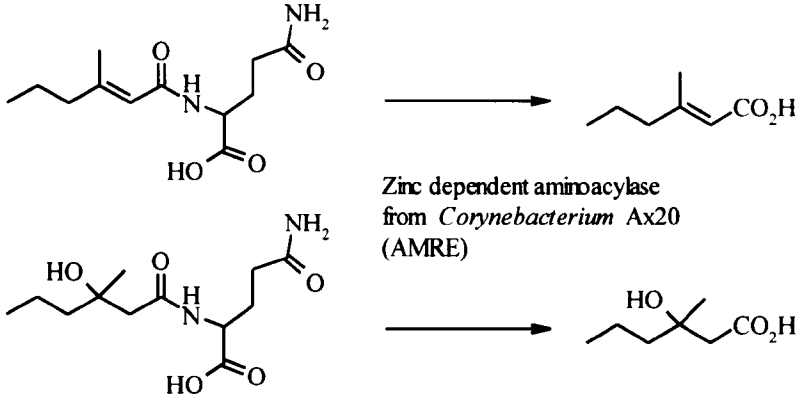



**Figure 1.9** Formation of VFA by Corynebacteria and Staphylococci. Reproduced with modifications from (James *et al.*, 2004).

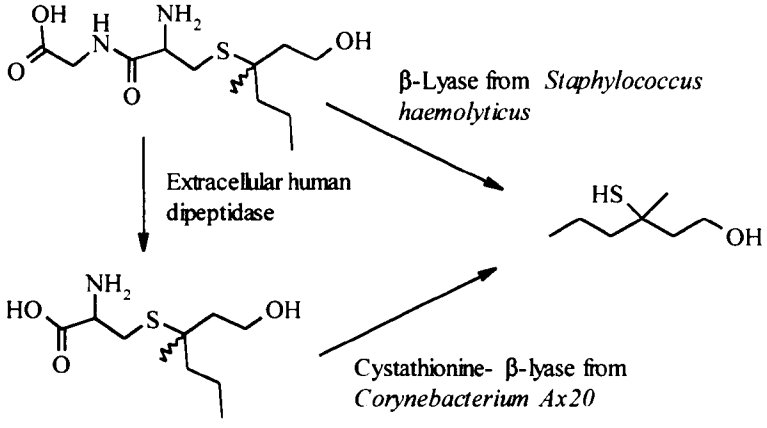
Thirdly, the predominant olfactory contributor in axillary sweat, (E)-3-methylhex-2-enoic acid (3M2H), is described as being the key odour component and was first presented by Zeng and co-workers (Zeng *et al.*, 1992; Zeng *et al.*, 1991). This compound had initially been found in the sweat of schizophrenic patients (Smith *et al.*, 1969) until Gordon and co-workers (Gordon *et al.*, 1973) showed that 3M2H was detectable in both normal population and schizophrenics, indicating there was no special relationship between 3M2H and schizophrenia (Akutsu *et al.*, 2006). In a later study, Spielmen and co-workers (Spielman *et al.*, 1995) illustrated that 3M2H was non-covalently associated with two proteins, which have been designated as apocrine secretion odour-binding proteins 1 and 2 (ASOB1, 45 kDa, and ASOB2, 26 kDa). The ASOB2 protein was identified as apolipoprotein D (apoD), a member of the lipocalin family of carrier proteins (James *et al.*, 2004; Zeng *et al.*, 1996a). However, Natsch and co-workers (Natsch *et al.*, 2003) produced a contradictory study, whereby, 3M2H and the chemically related 3-hydroxy-3-methylhexanoic acid (HMHA), are instead non-covalently bound to the carrier protein in the form of glutamine conjugates and

are released by the action of corynebacterial *N*-acylglutamine aminoacylase (Scheme 1.2). It was then postulated that once cleaved from the glutamine conjugate, the acids might be able to associate non-covalently with apoD (James *et al.*, 2004). More recently, Martin and co-workers described the relationship between axillary odorants and the *ABCC11* gene (Martin *et al.*, 2010). They reported that individuals who were AA homozygotic for single nucleotide polymorphism (SNP), 538G→A, leading to a G180R substitution, were found to have a significantly smaller amount of axillary odorants to those from AG and GG phenotypes. *ABCC11* gene has also been previously shown to function in the auditory canal, where, 538G→A SNP is associated with dry white ear wax phenotype that is predominant in Asians (80-95%) and rare in Africans or Europeans (0-3%) (Yoshiura *et al.*, 2006). This same SNP predominates in the Asian population, which nearly leads to a complete loss of typical body odour. The ABCC11 protein is expressed and localised in the apocrine sweat gland and appears to have an essential role in the secretion or formation of glutamine precursors. Furthermore, the work presented by Martin *et al* (2010) is similar to the work present by Jacoby *et al*, where they reported the concentration of ASOB2 to be low or undetectable in Asians, compared to non-Asian subjects, where it was readily detected (Jacoby *et al.*, 2004). Thus, the relationship between ASOB2 and ABCC11 protein and their regulation of odour production still needs to be assessed.




**Scheme 1.2** Release of sweat acids from glutamine. Reproduced from (Gautschi *et al.*, 2007).

Finally, sulfanylalkanols, sulfur containing thiols; have also been shown to have a key role in the perception of sweat malodour, possessing a sweat/onion-like character. This is derived from the action of pyridoxal phosphate-dependent β-lyase activity (probably related to cystathionine β-lyase) upon secreted sulfur-containing amino acids (Scheme 1.3) (Natsch *et al.*, 2003; Troccaz *et al.*, 2004). However, this has been proposed without data on the structure of the secreted precursor or isolation of the bacterial enzyme, as structure elucidation has proven to be more complex. Furthermore, certain bacteria have been associated with β-elimination activity for cysteine S-conjugates, primarily through the action of lipase, β-cystathionase (an enzyme involved in methionine biosynthesis), γ-cystathionase, tryptophanase (a transaminase) and β-lyases (again involved in methionine biosynthesis, whereby they cleave the intermediate (S)-cystathionine) (Natsch *et al.*, 2004; Troccaz *et al.*, 2004). In addition, sulfanylalkanols were recently found to be bound to the Cys-Gly dipeptide in axilla secretions (Natsch *et al.*, 2006).




**Scheme 1.3** Release of sulfanylalkanols from the Cys-Gly precursors Reproduced from (Gautschi *et al.*, 2007).

### 1.7.5 Natural Precursors/Apocrine Sweat Composition

Many years after the work of Shelley and co-workers (Shelley *et al.*, 1953), only speculations with respect to the chemical nature of apocrine sweat precursors were available. However, over the last decade a few authors have started to tackle this gap within the literature, mainly due to the analytical and computational advances, which enable the correct identification of these metabolites. Appendix A illustrates the full spectrum of compounds currently extracted from apocrine sweat (Natsch *et al.*, 2006; Natsch *et al.*, 2004; Troccaz *et al.*, 2004), while known odour precursors are summarised in Table 1.8.

**Table 1.8** Summary of reported odour precursors present in human apocrine sweat. See Appendix A for full complement of metabolites reported in apocrine sweat.

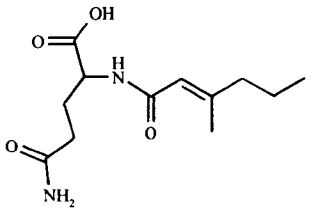
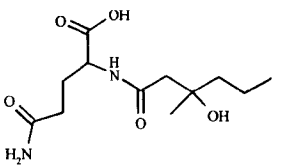
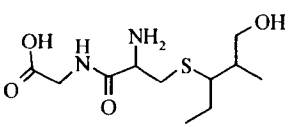
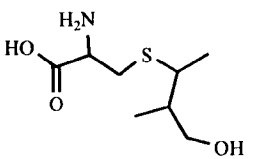
| Components | Structure | Molecule | Mass | M+H | Other Names |
|---|---|---|---|---|---|
| N-α-3-methylhex-2-enoyl-L-glutamine |  | $C_{12}H_{20}N_2O_4$ | 256.1423 | 257.1501 | Gln-conjugate + 3-Methylhex-2-enoic acid |
| N-α-3-hydroxy-3-methylhexanoyl- L-glutamine |  | $C_{12}H_{22}N_2O_5$ | 274.1529 | 275.1607 | Gln-conjugate + 3-Hydroxy-3-methylhexanoic acid |
| S-[1-(2-hydroxy-1-methylethyl)-2-methylethyl]-L-cysteinylglycine |  | $C_{11}H_{22}N_2O_4S$ | 278.1300 | 279.1379 | Cys-Gly-S-conjugate + 2-Methyl-3-sulfanylbutan-1-ol |
| S-[1-(2-hydroxy-1-methylethyl)-ethyl]-L-cysteine |  | $C_8H_{17}NO_3S$ | 207.0929 | 208.1007 | Cys-conjugate + 2-Methyl-3-sulfanylbutan-1-ol |

*Table 1.8 continued*

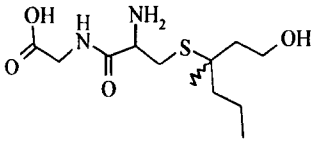
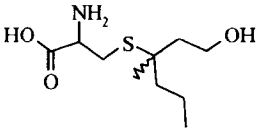
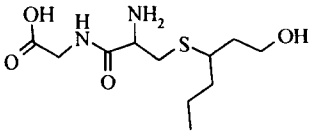
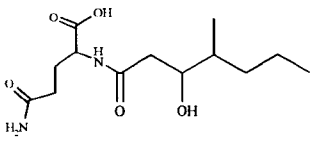
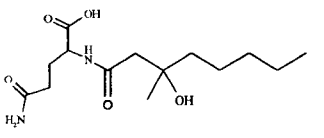
| Components | Structure | Molecule | Mass | M+H | Other Names |
|---|---|---|---|---|---|
| S-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cysteinylglycine |  | $C_{12}H_{24}N_2O_4S$ | 292.1457 | 293.1535 | Cys-Gly-S-conjugate + 3-Methyl-3-sulfanylhexan-1-ol |
| S-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cystein |  | $C_{10}H_{21}NO_3S$ | 235.1242 | 236.1320 | Cys-S-conjugate + 3-Methyl-3-sulfanylhexan-1-ol |
| S-[1-(2-hydroxyethyl)-butyl]-L-cysteinylglycine |  | $C_{11}H_{22}N_2O_4S$ | 278.1300 | 279.1379 | Cys-Gly-S-conjugate + 3-Sulfanylhexan-1-ol |
| N-α-3-hydroxy-4-methylhept anoyl- L-glutamine |  | $C_{13}H_{24}N_2O_5$ | 288.1685 | 289.1763 | Gln-conjugate + 3-Hydroxy-4-methylheptanoic acid |
| N-α-3-hydroxy-3-methyloct anoyl- L-glutamine |  | $C_{14}H_{26}N_2O_5$ | 302.1842 | 303.1920 | Gln-conjugate + 3-Hydroxy-3-methyloctanoic acid conjugate |

*Table 1.8 continued*

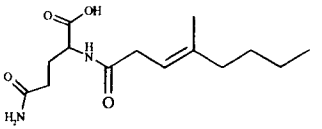
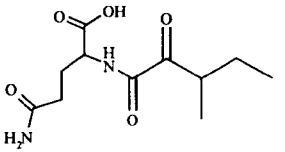
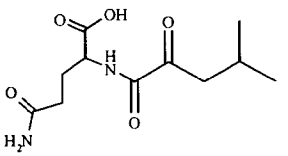
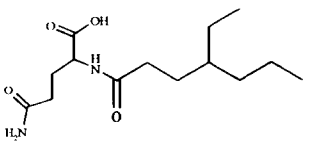
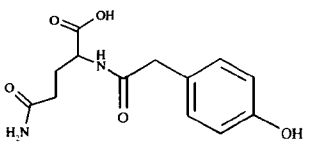
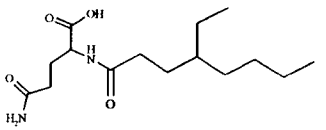
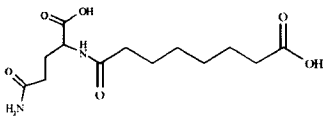
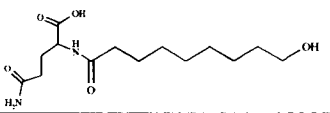
| Components | Structure | Molecule | Mass | M+H | Other Names |
|---|---|---|---|---|---|
| N-α-4-methyl-3-oct-enoyl- L-glutamine |  | $C_{14}H_{24}N_2O_4$ | 284.1736 | 285.1814 | Gln-conjugate + (E)-4-Methyloct-3-enoic acid |
| N-α-3-methyl-2-oxopent-anoyl- L-glutamine |  | $C_{11}H_{18}N_2O_5$ | 258.1216 | 259.1294 | Gln-conjugate + 3-Methyl-2-oxopentanoic acid |
| N-α-4-methyl-2-oxopent-anoyl- L-glutamine |  | $C_{11}H_{18}N_2O_5$ | 258.1216 | 259.1294 | Gln-conjugate + 4-Methyl-2-oxopentanoic acid |
| N-α-4-ethyl-hept-anoyl- L-glutamine |  | $C_{14}H_{26}N_2O_4$ | 286.1893 | 287.1971 | Gln-conjugate + 4-Ethylheptanoic acid |
| N-α-4-hydroxyphenyl-acetyl- L-glutamine |  | $C_{13}H_{16}N_2O_5$ | 280.1059 | 281.1137 | Gln-conjugate + Phenylacetic acid |

*Table 1.8 continued*

| Components | Structure | Molecule | Mass | M+H | Other Names |
|---|---|---|---|---|---|
| N-α-4-ethyl-oct-anoyl- L-glutamine |  | $C_{15}H_{28}N_2O_4$ | 300.2049 | 301.2127 | Gln-conjugate + 4-Ethyloctanoic acid |
| N- α -7-carboxy-hept-anoyl- L-glutamine |  | $C_{13}H_{22}N_2O_6$ | 302.1478 | 303.1556 | Gln-conjugate + Octanedioic acid |
| N-α-9-hydroxy- non-anoyl- L-glutamine |  | $C_{14}H_{26}N_2O_5$ | 302.1842 | 303.1920 | Gln-conjugate + 9-Hydroxynonanoic acid |

It has been speculated that body odour formation arises from common metabolic pathways of skin microflora and therefore is a by-product of bacterial metabolism that utilises these sweat secretions, which are essentially the by-products of the body's metabolism. Examples of bacterial metabolism include the catabolism of L-leucine into isovaleric acid (3-methylbutanoic acid) and the formation of short acids by the incomplete degradation of skin lipids (Natsch *et al.*, 2003). However, the recently identified glutamine conjugate (3M2H-Gln) is somewhat contradictory. Thus, it is more likely that these compounds are synthesised specifically to exert their action once secreted in the axilla region rather than just being a by-product of metabolism. The actual benefit of being secreted in precursor form instead of direct secretion of the acids could be many fold. For instance, the use of a precursor would lead to a controlled release and make the chemical signal longer lasting. More of a physiological approach would suggest that transporting low molecular weight acids is more achievable when they are in a water-soluble precursor form (Natsch *et al.*, 2003). This would indicate co-evolution between skin micro flora and humans, since the bacteria have adapted their enzymes to recognise the precursor structure of axilla secretions.

The work produced by Natsch (Natsch *et al.*, 2003) has illustrated that the aminoacylase is unique, whereby, it is very selective for the Gln residue but has a very broad substrate specificity regarding the acyl part. This would readily lend itself to being more malleable to other Gln-containing precursor structures. Furthermore, they confirmed the findings of Zeng's group (Zeng *et al.*, 1991), that other branched amino acids are also present in axilla secretions. Although, it was highlighted that further investigations would be needed to identify if these too are linked to Gln-conjugates.

Thiols and methylsulfanyl metabolites, in which sulfur is derived from the glutathione, have been identified in secretion products of xenobiotics. Typically, glutathione-(S)-conjugates undergo enzymatic hydrolysis to yield Cys-(S)-conjugates, firstly, by the cleavage of the γ-glutamyl moiety *via* γ-glutamyl *trans*-peptidase and then followed by the action of a carboxypeptidase (Starkenmann *et al.*, 2005). The formation of volatile sulfur compounds from Cys-(S)-conjugates have

been described in wine, passion fruit, *Allium* species and have been described as the substrates for β-lyase. Starkenmann group also postulated that the production of the Cys-(S)-conjugate takes place before excretion of the sweat onto the skin surface. Furthermore, this group speculated that the formation of 3-methy-3-sulfanlhexan-1-ol, 3-sulfanylhexan-1-ol, and 2-methyl-3-sulfanylbutan-1-ol are produced through their Cys-Gly-(S)-conjugates precursors.

Currently, there is lack of comprehensive analytical approaches to measuring the complex biochemicals excreted in apocrine sweat. Thus, this study exploits a range of LC-MS platforms and NMR techniques, coupled with a modern metabolomics approach, to provide further information in understanding the underlying physiological biochemistry of malodour. Global profiling *via* LC-MS, using high mass accuracy instrumentation such as QTOF or an orbitrap, and NMR will provide complementary metabolite information (Lanza *et al.*, 2010; Lenz *et al.*, 2004; Williams *et al.*, 2005a). This approach is typically used when biological knowledge is limited. A large number of metabolites are detected without *a priori* information, thereby, increasing the complexity of the data and as a result does not provide automatic chemical identification. Metabolite identification is currently one area that requires significant development, typically with LC-MS data. In comparison to NMR data, metabolite identification is less complex due to NMR databases being relatively well defined. The information obtained, coupled to MVA techniques, aims to identify inter- and intra-individual differences as well as providing further knowledge into the chemical composition of apocrine sweat. Targeted or semi-targeted LC-MS will provide information about metabolites of interest (either monitoring for specific metabolites using unique MRMs or a survey analysis for the same class of metabolites such as odour precursors). These strategies require the use of QQQ mass spectrometers which provide greater specificity compared to that of a QTOF or orbitrap spectrometers. The data obtained is often less complex then data obtained from global methodologies. Furthermore, targeted methodologies provide an increase in sensitivity (readily detecting sub picomolar concentrations) and provide quantitative information when using internal standards. Thus, the targeted methodologies will be able to screen for known odour precursors, while the semi-

targeted methodology using PI scanning provides the ability to screen for known and unknown odour precursors present in human apocrine sweat.

## 1.8 Aims of the Thesis

The overall aim of this project is to develop analytical methods that will ultimately allow the composition of axillary malodour to be identified, with particular reference to identifying new odour precursors. The project aims to achieve this by: -

- Developing and optimising, both NMR and LC-MS methodologies for the global analysis of human apocrine sweat secretions
- Developing and optimising mass spectrometry methodology for the analysis of conjugated fatty acids and thiols present in human apocrine sweat secretions
- Assessing inter-subject variability
- Assessing intra-individual differences between secretion obtained from the left and right arm
- Providing further knowledge to the chemical composition of human apocrine sweat

# Chapter 2

## 2 Development of $^{1}H$ NMR Spectroscopy Methodology for the Metabolomic Analysis of Apocrine Sweat

### 2.1 Introduction

NMR spectroscopy is one of the major technologies for metabolic profiling (Griffin, 2006; Lindon and Nicholson, 2008) due to its ability in providing comprehensive chemical information about unknown metabolites as well as providing information on a variety of dynamic processes, such as protein ligand binding (Liu *et al.*, 1997; Luo *et al.*, 1999; Medek *et al.*, 2000; Pellecchia *et al.*, 2002). Moreover, NMR spectroscopy benefits from being a specific and yet a non-selective technique (i.e. independent of the chemical properties of the metabolites being analysed). This means each observable resonance is specific to a particular metabolite, thereby, aiding identification of individual constituents. Moreover, the same observable nuclei (i.e. all $^{1}H$) have the same sensitivity, thus, in theory absolute concentration of metabolites can be determined when measured with an internal standard (e.g. TSP). The majority of biological samples analysed by NMR spectroscopy are measured in solution state, however, intact tissue samples using high resolution magic angle spinning (HR-MAS) can also be analysed (Sitter *et al.*, 2009). In addition, NMR requires minimal sample preparation, with only the addition of deuterated solvents such as $D_2O$ or $CDCl_3$ to provide a frequency lock of the magnetic field as well as being non-destructive allowing several analyses to be conducted on the same sample. Currently, no NMR-based study to date has attempted to qualitatively and quantitatively profile endogenous metabolites present in human apocrine sweat. Thus, NMR spectroscopy could provide a powerful tool for the analysis of human apocrine sweat. However, apocrine sweat is secreted in low volumes, typically 5 μl, with the majority of the metabolites present being lower concentrations than those found in plasma. Thus,

NMR methodologies need to be developed in order to compensate for the restricted sample volumes.

Over the last 50 years, the field of NMR has developed a sophisticated array of experimental capabilities (Braun and Berger, 2004). For example, 1D NOESYPR1D pulse sequence provides good solvent suppression while a 1D $^{1}H$ Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (Meiboom and Gill, 1958) can be used to reduce signals from large macromolecules such as phospholipids triglycerides and lipoproteins which give rise to broad signals while retaining those from smaller molecules such as amino acids and carbohydrates (Foxall *et al.*, 1993; Harker *et al.*, 2006; Nicholson *et al.*, 1995). However, compared to other common spectroscopic methods of molecular characterisation, NMR is by far the least sensitive as shown in Table 2.1 (Lacey *et al.*, 1999).

**Table 2.1** Limit of detection for common analytical techniques (Lacey *et al.*, 1999).

| Method | LOD (mol) |
|---|---|
| Fluorescence | $10^{-18}$ - $10^{-23}$ |
| Mass Spectrometry | $10^{-13}$ - $10^{-21}$ |
| Electrochemical | $10^{-15}$ - $10^{-19}$ |
| Radiochemical | $10^{-14}$ - $10^{-19}$ |
| UV-Vis absorbance | $10^{-13}$ - $10^{-16}$ |
| NMR | $10^{-9}$ - $10^{-11}$ |

NMR insensitivity stems from the fact that the energy levels of transitions are narrowly separated. Maxwell-Boltzmann statistics dictates that the population differences between the upper and lower energy states represents only a tiny fraction (<0.01%) of the nuclear spins from the total number of molecules (Lacey *et al.*, 1999). Thus, NMR spectroscopy can only detect and quantify metabolites present in relatively high concentrations, whereby, 20-40 metabolites are typically detected in tissues (Griffin *et al.*, 2001), 20-30 and 30-100 metabolites detected in blood plasma (Brindle *et al.*, 2002) and urine samples respectively (Connor *et al.*, 2004).

Historically, the need for relatively large sample volumes was dictated by the low sensitivity of the NMR instrument. However, modern advances in technology have brought about higher field strength magnets and a steady improvement of NMR probes, which have increased the overall sensitivity of the method (Eisenreich and Bacher, 2007; Schlotterbeck *et al.*, 2006). For instance, ultra high field NMR spectrometers (up to 900 MHz) and cryogenically cooled detector coils are available (Wishart, 2008). Nevertheless, analysis of micro volume samples, e.g. natural section of body fluid (apocrine sweat), still remains challenging (Schlotterbeck *et al.*, 2002).

The introduction of cryogenically cooled probes offers an increase in sensitivity by a factor of three to four compared to conventional probes by super cooling the receiver coil and preamplifiers to 25 K or below, resulting in a reduction of the thermal noise (Schroeder and Gronquist, 2006; Spraul *et al.*, 2003). However, the spectral dispersion of each signal remains the same (Schroeder and Gronquist, 2006). Nonetheless, in terms of cost implication, the cryoprobe offers considerable advantage over installing higher-field spectrometers, for example; 900 MHz only offers an increase in sensitivity by a factor of two as compared to at 600 MHz, therefore the increase in sensitivity is only modest in comparison (Rinaldi, 2004).

The simplest and cheapest approach to increasing the signal-to-noise without acquiring more expensive higher-field strength magnets or changing the RF coil is to use small sample tubes to decrease the volume required for acquisition. Typically, a total volume of 600 µl is required for sample acquisition when using 5 mm OD NMR tubes, as this volume is enough to 'fill' the coil of the probe so the field homogeneity will not be disturbed. Specially designed tubes such as capillary tubes or spherical inserts can be used to limit the sample volume to just the volume of the receiver coil, while a Shigemi tube compensates for less volume by using $D_2O$ matched glass. Thus, the aim of this chapter was to evaluate different NMR field strengths (400 and 500 MHz), probes (5 mm quattro nucleus probe (QNP), 1 mm micro-volume triple resonance inverse probe (TXI), and 5 mm TXI cryoprobe) and NMR tubes (e.g. standard tubes, Shigemi tubes, capillary tubes) in order to find the optimum analytical set-up which will ultimately be applied to the analyse of apocrine sweat which typically have 5 µl sample size volume.

## 2.2 Aims

- To create an artificial sweat matrix suitable for method development.
- Establish suitable NMR methodology to provide the optimum sensitivity for the analysis of low sample volumes, which, will be later used for the analysis of apocrine sweat.
- Use multivariate analysis to determine whether any correlation exists between the storage lengths of the artificial sweat matrix.

## 2.3 Materials and Methods

### 2.3.1 Chemicals

Creatine, $D_2O$, pyruvate, and trimethylsilyl [2,2,3,3,-$^2H_4$] propionate (TSP), was purchased from Fisher Scientific (Loughborough, UK). Citric acid, lactic acid ornithine, and potassium phosphate were purchased from Sigma Aldrich (Poole, UK). Creatinine was purchased from Alfa Aesor (Heysham, UK). Neocate was purchased from SHS International Ltd (Liverpool, UK). Neocate is composed of dried glucose syrup, non-hydrogenated coconut oil, high oleic sunflower oil, refined vegetable oils (canola, sunflower), arginine, aspartate, leucine, lysine acetate, glutamine, calcium phosphate dibasic, proline, valine, emulsifier (E472c), tripotassium citrate, isoleucine, glycine, threonine, tyrosine, phenylalanine, serine, histidine, alanine, cystine, tryptophan, sodium chloride, methionine, magnesium aspartate, high arachidonic single cell vegetable oil, choline bitartrate, magnesium chloride, tricalcium citrate, ascorbic acid, potassium chloride, myo-inositol, high docosahexaenoic acid single cell vegetable oil, taurine, ferrous sulphate, zinc sulphate, carnitine, nicotinamide, DL-alpha tocopheryl acetate, calcium D-pantothenate, antioxidants (E304, E307 and E306), manganese sulphate, copper sulphate, vitamin A acetate, pyridoxine hydrochloride, thiamine hydrochloride, riboflavin, potassium iodide, folic acid, chromium chloride, sodium molybdate, vitamin $K_1$, D-biotin, sodium selenite, vitamin $D_3$ and cyanocobalamin.

### 2.3.2 Sample Preparation

Apocrine sweat is a complex mixture with a wide range of metabolite concentration with different chemical properties. This diversity together with limited sample availability creates an analytical challenge when developing analytical methodologies. Thus, an artificial sweat matrix was developed for the purpose of developing analytical methodologies and was not intended to replicate apocrine secretions *per se*. An artificial sweat model (see Table 2.2) was proposed through personal communication with Dr Mark Harker (Unilever). However, due to the complex nature of blood plasma, the premise of the artificial sweat matrix was based on an amino acid hypoallergenic infant formula from Neocate (www.neocate.co.uk). The use of the Neocate as a biological matrix meant that the full quantitative composition of all the components were known (see www.neocate.co.uk/aaa_neocate/15999-ingredients.html) compared to a biological matrix from blood plasma, which is considerably more complex. Thus, Neocate provided a suitable matrix that was well-defined (in comparison with blood plasma), protein free (allowing comparative evaluation of the metabolites without complication from protein binding) and contains both low molecular weight metabolites and lipids. Additional metabolites that have been reported in eccrine sweat were also added. Each metabolite (see Table 2.3) was individually weighed out and spiked into the Neocate to create the desired concentration and made up to 1 ml with phosphate buffer (pH 6, 0.1 M). For storage purposes, the stock solution was then aliquoted into smaller sample volumes of 40 µl and stored at either -20°C or 4°C until required.

**Table 2.2** Artificial apocrine secretion model initially proposed for method development through collaboration with Unilever.

| Ingredient | Level in µg/ml |
|---|---|
| Cholesterol | 18.75 |
| Cholesterol esters | 0.25 |
| Wax esters (lanolin) | 0.9 |
| Squalene | 0.005 |
| Glycerides (tristearin) | 2.5 |
| Fatty acids (stearic acid) | 2.5 |
| Lysozyme | 40,000 |
| ApoD | 5,000 |
| Zn –α-glycoprotein | 5,000 |
| Amino acids/conjugates | 10,000 |
| Glutamine conjugate | Estimate 5 |
| Cysteine conjugate | Estimate 5 |
| Carbohydrate (glucose) | 1000 |
| Androsterone sulphate | 5 |
| Dehydroepiandrosterone sulphate | 5 |
| Synthetic plasma with phosphate buffer (pH 6) | To 1 ml |

**Table 2.3** Artificial sweat matrix composition used for NMR method development. The amount of Neocate used was based on the amino acid content to reflect that of the apocrine secretion model above.

| Metabolite | Level in mg/ml | Reference |
|---|---|---|
| Ornithine | 0.017 | (Coltman *et al.*, 1966; Liappis and Hungerla.H, 1972) |
| Creatinine | 0.061 | (Fukumoto *et al.*, 1988; Mosher, 1933; Stjohnlyburn, 1956) |
| Creatine | 0.0079 | (Stjohnlyburn, 1956) |
| Pyruvate | 0.086 | (Jin *et al.*, 2001) |
| Citric acid | 0.068 | (Leake, 1922) |
| Lactic acid | 3.90 | (Mosher, 1933; Patterson *et al.*, 2000; Stjohnlyburn, 1956; Weiner and Vanheyningen, 1952) |
| Neocate powder | 61.48 | |
| *Nutritional summary of Neocate* | | |
| Protein equivalent | 7.92 | |
| Total amino acids | 9.53 | |
| Carbohydrates | 33.12 | |
| Sugars | 3.01 | |
| Fat | 14.14 | |
| As saturates | 5.16 | |
| Monounsaturates | 5.53 | |
| Polyunsaturates | 2.77 | |

### 2.3.3 $^{1}$H NMR Spectroscopy

The preparation of the artificial sweat mixture for $^{1}$H NMR analyses varied depending on the type of NMR tubes being utilised. 5 µl of the actual amount of artificial sweat used remained constant, while the amount of $D_2O$ containing a primary reference standard, trimethylsilyl [2,2,3,3,-$^{2}H_4$] propionate (TSP, ~7.6 mM) varied as follows: -

- Standard 5 mm OD NMR tubes, 650 µl $D_2O$.
- Shigemi tube, 300 µl $D_2O$
- Capillary NMR tubes, 180 µl $D_2O$
- Spherical insert, 10 µl $D_2O$
- 1 mm Micro-volume probe, 3 µl $D_2O$

$^{1}$H NMR spectra were acquired on a Bruker Avance 400 spectrometer, operating at 400.13 MHz $^{1}$H observation frequency and equipped with a quadruple resonance ($^{1}$H, $^{13}$C, $^{31}$P, $^{19}$F) QNP probe with an internal probe temperature of 298 K, a TXI 1 mm micro-volume probe equipped with Z-gradient with a internal probe temperature of 298 K and a Bruker DRX-500 spectrometer, operating at 500.18 MHz $^{1}$H observation frequency, equipped with a triple resonance ($^{1}$H, $^{13}$C, $^{15}$N) cryogenically cooled TXI probe with an internal probe temperature of 303 K.

Spectra were acquired using a conventional solvent presaturation pulse sequence for solvent suppression based on the start of the NOSEY pulse sequence [RD-90°-$t_1$-90° - $t_m$-90° -acquire free induction decay], where 90° represents a non-selective 90° RF pulse, RD is a relaxation delay of 1.5 s during which the water peak was selectively irradiated, and $t_1$ corresponds to a fixed interval of 3 µs. Typically, 256 transients were collected into 32 K data points with a spectral width of 8000 Hz. Prior to Fourier Transform, exponential line broadening of 0.3 Hz was applied to FIDs which were zero-filled by a factor of 2. All spectra were manually corrected for phase and baseline distortions within TopSpin™ 2.1 (Bruker Analytische GmbH, Germany) and chemical shifts referenced to TSP standard at δ0.00 ppm.

### 2.3.4 Spectral analysis and metabolite quantification

Relative quantification of each identified metabolite peak was achieved by integration of $^1$H NMR peaks using Bruker AMIX program. $^1$H NMR can be used to analyse the relative concentrations of different metabolites because the area under each resonance is directly proportional to the number of nuclei giving rise to that resonance. Resonances from these metabolites were integrated along with those from the internal standard, TSP. The amount of each metabolite was then calculated in mmol/L using Equation 2.1 and then converted from molar concentration to mg/L afterwards.

$$C_m = \frac{I_m}{I_s} \times \frac{N_s}{N_m} \times C_s$$

**Equation 2.1**

where

| | |
|---|---|
| m | = Metabolite |
| s | = Standard |
| I | = Peak area integral |
| N | = Number of protons |
| C | = Concentration |

### 2.3.5 Data Pre-Processing

The $^1$H NMR spectra were reduced into consecutive integrated spectral regions of widths 0.04 ppm using AMIX software (Analysis of MIXtures, Bruker), also referred to as "bucketing". The region δ4.50-5.18, surrounding the water resonance was excluded from the analysis in order to remove the effects of variations in the suppression of the water resonance. The resulting data matrix was exported into Microsoft Excel 2007 and block-normalised by calculating the intensity of each bucketed spectral region as a percentage of the total spectral area, in order to minimise the effects of any concentration differences between samples.

### 2.3.6 Partial Least Squares Regression (PLS)

PLS regression was carried out using SIMCA-P version 11 (Umetrics, Umeå, Sweden) in order to determine the covariance between the NMR profiles and the observed storage time. PLS regression is a well established technique for modeling biochemical data and has been thoroughly described and explained by Geladi and Kowalski (Geladi and Kowalski, 1986). PLS builds a regressive model by maximizing the covariance between a set of variables **X** and a dependent variable **y** and it thus called a supervised method. The model is then used to predict values of **y** for a given set of **X** variables. The number of latent variables (LV) used to predict **y** is determined using cross-validation where the sample set is divided into a number of segments which in turn are excluded before re-entering into the model in order to estimate the prediction error. The optimal number of LV's used in the prediction model is chosen when the least error in prediction is observed. To evaluate the models performance, a random sub-set cross-validation was used where two thirds of the samples were used to create a training set and a third used as a test set.

## 2.4 Results and Discussion

The basic premise underlying the development was to obtain the highest possible S/N ratio from a limited sample volume (5 µl). This enhancement was also coupled with the desire to be cost effective and user-friendly. It has been well documented that decreasing the sample concentration by half requires quadrupling the number of transients to maintain a given S/N (Martin and Hadden, 1999). It was on this basis that the comparison between 5mm OD NMR tubes, 1.7 mm capillary NMR tube, Shigemi tube as well as the use of the 1 mm NMR micro-volume probe on a 400 MHz spectrometer and 500 MHz with a cryro-probe was conducted. This study compared the results obtained from $^{1}$H NMR spectra of artificial sweat matrix.

A typical 400 MHz $^{1}$H NMR spectrum of the artificial sweat mixture is given in Figure 2.1. At this stage, all peak assignments are tentative, based on comparison against spectra of authentic reference standards contained in a spectral database. Verification of these peak assignments will require the acquisition of 2D NMR

spectra such as correlation spectra (COSY), total correlation spectra (TOCSY) and J-Resolved spectra (J-Res). However, due the composition of the artificial sweat matrix being well defined this was not necessary.

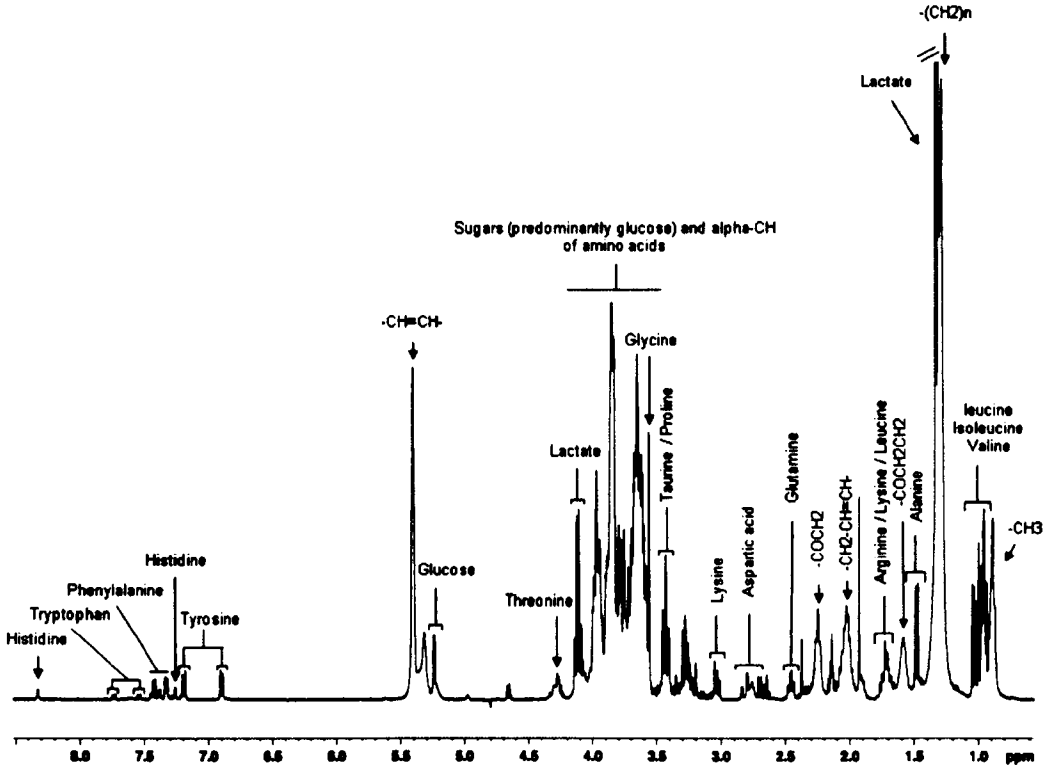



**Figure 2.1** Typical 400 MHz $^{1}$H NMR spectrum of artificial sweat mixture obtained using 1 mm micro-volume probe. The metabolites are tentatively assigned. Spectra were acquired using a standard 1D NOESYPRESAT sequence where ns = 256 scans.

### 2.4.1 Signal-to-Noise-Ratio

In NMR, the S/N is typically defined as the ratio of the height of any given peak in an NMR spectrum divided by two times the root mean-square of the noise floor (Lacey *et al.*, 1999). The sensitivity of an NMR experiment is governed by the properties of the probe for a given field strength and sample concentration. Other parameters such as the number of transients (ns) and total acquisition time ($t_{acq}$) can affect spectral results since the S/N ratio is proportional to the square root of the number of spectra

accumulated. Thus, the S/N ratio of a single scan can be increased by a factor of two by acquiring four scans, resulting in a four-fold increase in acquisition time. Similarly, the same S/N ratio may be obtained in one fourth of the time by doubling the sample concentration (Abraham *et al.*, 1988).

Table 2.4 highlights the mass sensitivity of the 5 mm QNP probe with the use of either capillary, Shigemi, and conventional 5 mm OD NMR tubes compared to that of a conventional 5 mm TXI cryo-probe and 1 mm micro-volume TXI probe.

**Table 2.4** Comparing the signal-to-noise of detectable metabolites obtained from 5 mm OD standard NMR tube (600 µl total volume), 2.5 mm OD capillary NMR tube (180 µl total volume), 5 mm Shigemi tube ( 300 µl total volume) and 1 mm micro-volume probe (8 µl total volume) at 400 MHz and 500 MHz.

| Chemical Shift (PPM) | Tentative Assignment | 400 MHZ | | | | 500 MHz Cryo-Probe | |
|---|---|---|---|---|---|---|---|
| | | 5mm Tube[a] | Capillary Tube[b] | Shigemi Tube[c] | 1mm Tube[d] | 5mm Tube[e] | Capillary Tube[f] |
| 0.9 | Lipid $CH_3$ | 22.0 ± 1.3 | 35.5 ± 3.8 | 69.9 ± 5.9 | 163.1 ± 4.0 | 193.5 ± 14.1 | 131.9 |
| 0.96 | Leucine/Isoleucine | 23.7 ± 0.7 | 26.6 ± 3.2 | 48.3 ± 5.2 | 173.4 ± 10.9 | 221.2 ± 6.8 | 124.4 |
| 1.04 | Valine | 15.3 ± 1.7 | 13.3 ± 1.9 | 17.7 ± 3.8 | 99.3 ± 6.3 | 110.6 ± 4.3 | 57.4 |
| 1.29 | Lipid $CH_2$ | 71.4 ± 3.6 | 122.4 ± 15 | 261.4 ± 17.5 | 560.6 ± 20.1 | 610.0 ± 46.7 | 433.7 |
| 1.33 | Lactate/Threonine | 189.8 ± 25.4 | 185.8 ± 23 | 338.0 ± 47.7 | 1178.9 ± 54.6 | 1335.3 ± 68.5 | 758.4 |
| 1.48 | Alanine | 15.9 ± 2 | 13.4 ± 1.6 | 20.4 ± 3.1 | 99.5 ± 6.1 | 105.4 ± 5.3 | 55.5 |
| 1.59 | Lipid $COCH_2CH_2$ | 6.9 ± 0.6 | 11.7 ± 1.4 | 26.4 ± 1.8 | 57 ± 1.9 | 67.8 ± 5.6 | 49.0 |
| 1.72 | Arg/Lys/Leu | 6.9 ± 0.4 | 8.5 ± 0.8 | 16.5 ± 1.4 | 55.9 ± 4.3 | 49.1 ± 2.2 | 29.1 |
| 1.92 | unknown/Arginine/Lysine | 28.2 ± 7.7 | 17.2 ± 0.4 | 24.2 ± 6.1 | 166.2 ± 14 | 170.2 ± 11.0 | 81.9 |
| 2.03 | Lipid $CH_2$-CH=CH | 9.5 ± 0.5 | 15.4 ± 1.8 | 33.1 ± 1.7 | 85.3 ± 0.6 | 83.8 ± 6.0 | 58.8 |
| 2.14 | Glutamine/Methionine | 13.7 ± 0.7 | 8.7 ± 0.6 | 16.0 ± 2.4 | 55.3 ± 4.3 | 113.4 ± 8.3 | 44.9 |
| 2.26 | Lipid $COCH_2$ | 9.6 ± 0.5 | 17.1 ± 2 | 33.0 ± 2.7 | 82.3 ± 1.8 | 100.7 ± 7.8 | 69.4 |
| 2.37 | Proline | 4.5 ± 0.9 | 4.3 ± 0.9 | 7.8 ± 1.3 | 49.0 ± 4.8 | 46.0 ± 2.2 | 23.7 |
| 2.45 | Glutamine | 4.9 ± 0.9 | 4.4 ± 1 | 7.6 ± 0.8 | 28.0 ± 1.3 | 36.0 ± 2.3 | 17.1 |
| 2.64 | Methionine | 0 ± 0 | 0 ± 0 | 0 ± 0 | 21.8 ± 1.0 | 17.8 ± 0.3 | 10.9 |
| 2.81 | Aspartic acid | 3.7 ± 0.5 | 3.6 ± 0.3 | 7.1 ± 0.4 | 24.5 ± 1.3 | 26.3 ± 0.9 | 14.8 |
| 3.02 | Lysine | 4.8 ± 0.5 | 5.2 ± 0.3 | 9.1 ± 1.4 | 34.3 ± 2.6 | 35.4 ± 2.1 | 20.8 |
| 3.42 | Proline/Taurine | 18.1 ± 0.3 | 19.1 ± 1.9 | 34.1 ± 5.1 | 131.0 ± 9.5 | 149.5 ± 5.2 | 84.2 |
| 3.55 | Glycine | 29.6 ± 5.4 | 23.9 ± 2.0 | 35.5 ± 5.7 | 219.4 ± 18.0 | 200.9 ± 7.5 | 107.3 |
| 4.11 | Lactate | 24.2 ± 5.0 | 18.8 ± 0.4 | 32.1 ± 6.6 | 164.6 ± 10.8 | 169.6 ± 9.9 | 94.4 |
| 4.28 | Threonine | 0 ± 0 | 4.4 ± 1.2 | 8.3 ± 0.7 | 24.4 ± 0.5 | 28.2 ± 3.2 | 19.3 |
| 5.23 | Glucose | 8.8 ± 0.5 | 10.2 ± 0.5 | 20.5 ± 0.9 | 58.3 ± 3.4 | 83.6 ± 2.4 | 50.9 |
| 5.4 | Lipid CH=CH | 43.4 ± 2.2 | 53.3 ± 6.0 | 106.4 ± 7.8 | 303.8 ± 21 | 399.3 ± 13.9 | 231.8 |
| 6.9 | Tyrosine | 3.0 ± 0.7 | 2.7 ± 0.2 | 3.5 ± 0.3 | 23.9 ± 2.8 | 20.5 ± 1.2 | 10.0 |
| 7.55 | Tryptophan | 3.9 ± 0.6 | 4.0 ± 0.6 | 5.1 ± 0.7 | 23.2 ± 2.2 | 25.8 ± 1.1 | 20.2 |
| 7.36 | Phenylalanine | 3 ± 0.2 | 3.1 ± 0.3 | 4.5 ± 0.2 | 20.0 ± 1.6 | 20.7 ± 0.5 | 12.5 |
| 8.01 | Histidine | 0 ± 0 | 0.8 ± 1.3 | 2.9 ± 0.4 | 10.7 ± 1.6 | 11.5 ± 1.7 | 9.4 |

Number of analytical replicates: **a,** n=3; **b,** n=3; **c,** n=2; **d,** n=3; **e,** n=3; **f,** n=1

From a practical point of view, the conventional NMR tubes (OD 5 mm) which are filled to a total volume of 600 µl, are user-friendly and with the use of the cryo-probe offer considerable advantage over the same tubes used on the 400 MHz spectrometer.

The simplest approach to potentially increasing the S/N without having to upgrade the field strength or probe design is to use smaller sample tubes (which may feature deuterium matched glass) or tube inserts to decrease the total volume required for an experiment. In theory, if the sample volume is decreased by a factor of four then the concentration can be increased by a factor of four, however, the number of spins ($^1$H of each atom) detected remains constant (Lacey *et al.*, 1999).

The Shigemi tubes (which have a total volume of 300 µl) are expensive and difficult to handle, and unlike the options available above, automation is not possible and therefore labour intensive and for this reason these were only acquired on the 400 MHz spectrometer. The results obtained are illustrated in Table 2.4. The use of solvent susceptibility matched shigemi tube should improve solvent suppression, RF homogeneity and overall performance resulting in a two-three fold increase in sensitivity (Schroeder and Gronquist, 2006). As illustrated in Table 2.4, the general increase observed was two to three-fold when compared to standard 5 mm OD NMR tubes. However, due to the expense and labour intensive nature of preparing the samples for acquisition this method was not considered as a viable option.

The capillary NMR tubes (OD 2.5 mm, L 178 mm) reduced the total volume required to 180 µl. However, as illustrated in Table 2.4, only offered a marginal increase in sensitivity over the conventional NMR tubes and proved to be less sensitive when acquired on the 500 MHz equipped with a cryo-probe. The lack of increase in sensitivity could be related to the fill factor as the RF field can be inhomogeneous with space, which is determined by the probe design (Jahnke, 1996). Since the capillary tube is further away from the $B_1$ field, compared to the standard tubes, the accuracy of the NMR measurements therefore diminishes causing line-broadening effects and reducing the overall sensitivity.

Alternatively, the sample can be contained in a spherical microcell bulb as depicted in Figure 2.2, reducing the volume to 18 µl (www.wilmad-labglass.com/group/2015).

The PTFE holder holds the microcell within the standard 5 mm OD NMR tube, and the positioning rod allows one to insert and remove the assembly from the tube. Both the PTFE holder and positioning rod are reusable while the inserts are intended for one time use. It must be stressed that locating the active region of the RF coil is not a trivial task. Moreover, shimming the sample is also difficult due to the air/glass interface within the NMR coil, resulting, in inhomogeneity of the sample, which again contributes to further line broadening effects. As a result, no useful data could be attained from using this method.

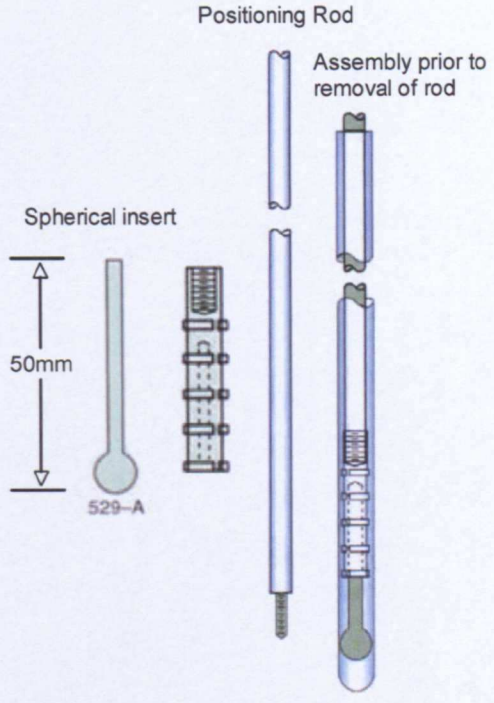



**Figure 2.2** Schematic diagram of the 5 mm micro-cell assembly.

Micro-volume probes allow samples as small as 5 µl to be handled successfully; however, quantitative transfer of a sample this small from the container to the NMR tube is still problematic. This is especially true when dealing with rare samples (e.g. sweat) rather than a diluted stock solution, although this problem could be minimised *via* the use of robotics. Another consideration, which would not be problematic for larger volume sizes, is the rate of moisture sorption. With the sample handling problems aside, there are additional advantages to using a micro-volume probes. For

example, signals from solvent impurities are much less prominent and 'solvent noise' from electrically conductive solvents (salt containing solutions) is reduced (Martin and Hadden, 1999). Another advantage of using small volumes is the reduction in the amount of expensive deuterated solvents (Schlotterbeck *et al.*, 2002) as well as the reduction in data acquisition time.

The results obtained are illustrated in Table 2.4. The comparative performance in the study demonstrates that the 1 mm micro-volume probe offered at least a 5-8 fold performance advantage over data acquired with a 5 mm OD NMR tube and offered a similar S/N ratio of the standard NMR tubes acquired with 500 MHz equipped with a cryo-probe. Thereby, using small sample volumes without the need for diluting the sample, offered a considerable advantage in sensitivity over the other techniques examined. Compared to the 5 mm tubes, the mean enhancement of 7.8 represents a decrease in data acquisition time of 60.84 ($7.8^2$) or the ability to perform an experiment in the same amount of time with 12.8% (1/7.8) of the sample mass.

The S/N value is directly proportional to the active volume of the probe ($V_{obs}$) for a concentration-limited samples. Thus, if an NMR tube is placed in the probe which has a two-fold decrease in its inner diameter; $V_{obs}$ is decreased four-fold, as well as the concentration sensitivity. Thus, to maximise the S/N, the tubes with the largest $V_{obs}$ (up to the maximum allowed by the probe) should be used, but at the expense of using additional sample. While a reduction in the diameter of the receiver coil increases the S/N ratio (Schroeder and Gronquist, 2006), a reduction in coil diameter inevitably results in a decrease in the sample volume, thus, micro-volume probes will only provide a sensitivity advantage where the mass-limited sample is fully soluble in the smaller volume. However, for concentration-limited samples, micro-volume probes will offer no competitive advantage as the small probe will accommodate less of the sample compared to that of conventional probes.

Cryogenic probes substantially increase the S/N ratio as shown in Table 2.4. In general these probes offer an advantage when the sample concentration is low, 2D or 3D experiments are required due to the low intrinsic sensitivity, and when the experiment time needs to be reduced.

### 2.4.2 Application with Mass Limited Samples

The spectra depicted in Figure 2.3 clearly show the power of reducing the sample volume size for metabolite identification. Using the 1 mm micro-volume probe we have been able to acquire $^{1}H$ NMR spectra from a limited sample volume of 5 µl which has comparable sensitivity to that of the 500 MHz equipped with a cryo-probe.

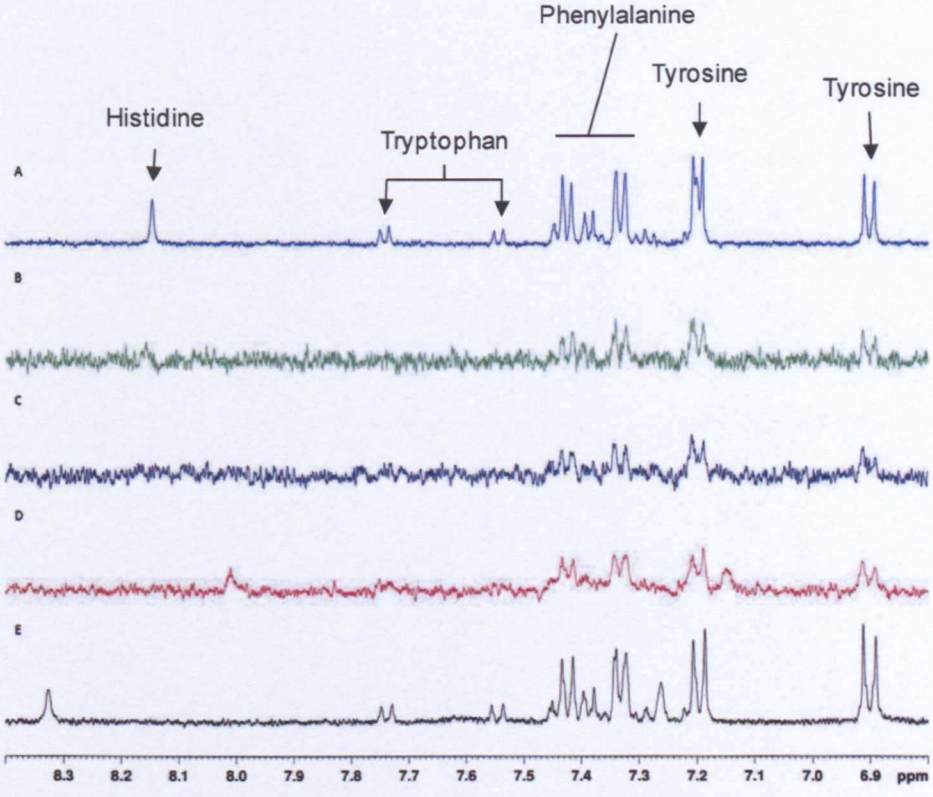



**Figure 2.3** Spectra comparison of the artificial sweat matrix obtained from **A** 5 mm OD NMR tube acquired on a 500 MHz equipped with a cryo-probe. **B-E** acquired on a 400 MHz spectrometer with **B** 5 mm OD NMR tube **C** Shigemi tube **D** capillary tube **E** micro-volume probe.

The limit of detection (LOD) and limit of quantification (LOQ) are defined as being 3 or 10 times the intensity of the noise respectively (Olson *et al.*, 1998). This is shown in Table 2.5 and was calculated by using Equation 2.1, substituting $I_m$ for the average noise intensity as previously reported (Savage *et al.*, 2011). The concentration LODs are in the low mg/ml range which may seem high, they correspond to nanomole amounts of sample, which is consistent with previous reports (Olson *et al.*, 1998). The ability to acquire high resolution spectra with 5 µl samples with improved mass sensitivity can further increase NMR popularity to biological applications in cases were only a limited volume of biofluid is available. The reduced volume also has the added advantage of reducing the background noise, as there is a smaller signal from the residual protonated solvent which is less detrimental to the quality of the spectrum obtained. Furthermore, there is a reduction of the "solvent noise" arising from electrically conductive solvents such as salt containing solutions. This has been shown by Olson and co-workers, where they reported a reduction in the S/N of <10% from a 30 mM sucrose concentration in the presence of 500 mM KCl (Olson *et al.*, 2004). An additional advantage of small-volume NMR probes is the reduction in the amount of expensive deuterated solvents (often by two orders of magnitudes).

**Table 2.5** Calculated limit of detection and limit of quantification of artificial sweat mixture. No adjustments have been made for differences in $T_1$ relaxation.

| Compound | 400 MHz | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 mm Tube | | | | Capillary Tube | | | | Shigemi Tube | | | | Micro-Vol Probe | | | |
| | LOD | | LOQ | | LOD | | LOQ | | LOD | | LOQ | | LOD | | LOQ | |
| | nmol | mg/L | nmol | mg/L | nmol | mg/L | nmol | mg/L | nmol | mg/L | nmol | mg/L | nmol | mg/L | nmol | mg/L |
| Ornithine | 4.08 | 0.83 | 13.62 | 2.77 | 2.45 | 1.80 | 8.17 | 6.00 | 3.53 | 1.56 | 11.78 | 5.19 | 0.83 | 13.72 | 2.77 | 45.72 |
| Creatinine | 2.72 | 0.47 | 9.08 | 1.58 | 1.63 | 1.03 | 5.44 | 3.42 | 2.36 | 0.89 | 7.85 | 2.96 | 0.55 | 7.83 | 1.85 | 26.09 |
| Creatine | 2.72 | 0.55 | 9.08 | 1.83 | 1.63 | 1.19 | 5.44 | 3.97 | 2.36 | 1.03 | 7.85 | 3.43 | 0.55 | 9.07 | 1.85 | 30.24 |
| Pyruvate | 2.72 | 0.37 | 9.08 | 1.23 | 1.63 | 0.80 | 5.44 | 2.66 | 2.36 | 0.69 | 7.85 | 2.30 | 0.55 | 6.09 | 1.85 | 20.31 |
| Citric acid | 4.08 | 1.19 | 13.62 | 3.96 | 2.45 | 2.57 | 8.17 | 8.58 | 3.53 | 2.23 | 11.78 | 7.42 | 0.83 | 19.62 | 2.77 | 65.39 |
| Lactic acid | 8.17 | 1.13 | 27.23 | 3.77 | 4.90 | 2.45 | 16.33 | 8.17 | 7.07 | 2.12 | 23.55 | 7.07 | 1.66 | 18.70 | 5.54 | 62.33 |
| Alanine | 2.72 | 0.37 | 9.08 | 1.24 | 1.63 | 0.81 | 5.44 | 2.69 | 2.36 | 0.70 | 7.85 | 2.33 | 0.55 | 6.16 | 1.85 | 20.55 |
| Valine | 2.72 | 0.49 | 9.08 | 1.64 | 1.63 | 1.06 | 5.44 | 3.54 | 2.36 | 0.92 | 7.85 | 3.07 | 0.55 | 8.11 | 1.85 | 27.02 |
| Leucine | 2.72 | 0.55 | 9.08 | 1.83 | 1.63 | 1.19 | 5.44 | 3.97 | 2.36 | 1.03 | 7.85 | 3.43 | 0.55 | 9.08 | 1.85 | 30.25 |
| Isoleucine | 2.72 | 0.55 | 9.08 | 1.83 | 1.63 | 1.19 | 5.44 | 3.97 | 2.36 | 1.03 | 7.85 | 3.43 | 0.55 | 9.08 | 1.85 | 30.25 |
| Proline | 4.08 | 0.72 | 13.62 | 2.41 | 2.45 | 1.57 | 8.17 | 5.22 | 3.53 | 1.36 | 11.78 | 4.52 | 0.83 | 11.95 | 2.77 | 39.83 |
| Methionine | 4.08 | 0.94 | 13.62 | 3.13 | 2.45 | 2.03 | 8.17 | 6.77 | 3.53 | 1.76 | 11.78 | 5.86 | 0.83 | 15.49 | 2.77 | 51.62 |
| Phenylalanine | 4.08 | 1.04 | 13.62 | 3.46 | 2.45 | 2.25 | 8.17 | 7.50 | 3.53 | 1.95 | 11.78 | 6.48 | 0.83 | 17.15 | 2.77 | 57.15 |
| Tryptophan | 8.17 | 2.58 | 27.23 | 8.59 | 4.90 | 5.58 | 16.33 | 18.61 | 7.07 | 4.83 | 23.55 | 16.10 | 1.66 | 42.57 | 5.54 | 141.91 |
| Glycine | 4.08 | 0.47 | 13.62 | 1.57 | 2.45 | 1.02 | 8.17 | 3.41 | 3.53 | 0.88 | 11.78 | 2.95 | 0.83 | 7.79 | 2.77 | 25.97 |
| Serine | 4.08 | 0.66 | 13.62 | 2.20 | 2.45 | 1.43 | 8.17 | 4.77 | 3.53 | 1.24 | 11.78 | 4.13 | 0.83 | 10.91 | 2.77 | 36.36 |
| Threonine | 8.17 | 1.50 | 27.23 | 4.99 | 4.90 | 3.24 | 16.33 | 10.81 | 7.07 | 2.81 | 23.55 | 9.35 | 1.66 | 24.73 | 5.54 | 82.42 |
| Cysteine | 4.08 | 0.76 | 13.62 | 2.54 | 2.45 | 1.65 | 8.17 | 5.50 | 3.53 | 1.43 | 11.78 | 4.76 | 0.83 | 12.58 | 2.77 | 41.92 |
| Tyrosine | 4.08 | 1.14 | 13.62 | 3.80 | 2.45 | 2.47 | 8.17 | 8.22 | 3.53 | 2.13 | 11.78 | 7.11 | 0.83 | 18.81 | 2.77 | 62.69 |
| Asparagine | 4.08 | 0.83 | 13.62 | 2.77 | 2.45 | 1.80 | 8.17 | 5.99 | 3.53 | 1.56 | 11.78 | 5.19 | 0.83 | 13.71 | 2.77 | 45.71 |
| Glutamine | 4.08 | 0.92 | 13.62 | 3.06 | 2.45 | 1.99 | 8.17 | 6.63 | 3.53 | 1.72 | 11.78 | 5.74 | 0.83 | 15.17 | 2.77 | 50.56 |
| Histidine | 8.17 | 1.95 | 27.23 | 6.50 | 4.90 | 4.22 | 16.33 | 14.08 | 7.07 | 3.65 | 23.55 | 12.18 | 1.66 | 32.21 | 5.54 | 107.36 |
| Lysine | 4.08 | 0.92 | 13.62 | 3.06 | 2.45 | 1.99 | 8.17 | 6.63 | 3.53 | 1.72 | 11.78 | 5.74 | 0.83 | 15.17 | 2.77 | 50.58 |
| Arginine | 4.08 | 1.09 | 13.62 | 3.65 | 2.45 | 2.37 | 8.17 | 7.90 | 3.53 | 2.05 | 11.78 | 6.84 | 0.83 | 18.08 | 2.77 | 60.27 |
| Glucose | 8.17 | 2.26 | 27.23 | 7.55 | 4.90 | 4.90 | 16.33 | 16.35 | 7.07 | 4.24 | 23.55 | 14.14 | 1.66 | 37.40 | 5.54 | 124.66 |

*Table 2.5 continued*

| Compound | 500 MHz | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 mm Tube | | | | Capillary Tube | | | |
| | LOD | | LOQ | | LOD | | LOQ | |
| | nmol | mg/L | nmol | mg/L | nmol | mg/L | nmol | mg/L |
| Ornithine | 0.61 | 0.12 | 2.03 | 0.41 | 0.38 | 0.28 | 1.28 | 0.94 |
| Creatinine | 0.41 | 0.07 | 1.35 | 0.24 | 0.26 | 0.16 | 0.85 | 0.54 |
| Creatine | 0.41 | 0.08 | 1.35 | 0.27 | 0.26 | 0.19 | 0.85 | 0.62 |
| Pyruvate | 0.41 | 0.05 | 1.35 | 0.18 | 0.26 | 0.13 | 0.85 | 0.42 |
| Citric acid | 0.61 | 0.18 | 2.03 | 0.59 | 0.38 | 0.40 | 1.28 | 1.35 |
| Lactic acid | 1.22 | 0.17 | 4.06 | 0.56 | 0.77 | 0.39 | 2.56 | 1.28 |
| Alanine | 0.41 | 0.06 | 1.35 | 0.19 | 0.26 | 0.13 | 0.85 | 0.42 |
| Valine | 0.41 | 0.07 | 1.35 | 0.24 | 0.26 | 0.17 | 0.85 | 0.56 |
| Leucine | 0.41 | 0.08 | 1.35 | 0.27 | 0.26 | 0.19 | 0.85 | 0.62 |
| Isoleucine | 0.41 | 0.08 | 1.35 | 0.27 | 0.26 | 0.19 | 0.85 | 0.62 |
| Proline | 0.61 | 0.11 | 2.03 | 0.36 | 0.38 | 0.25 | 1.28 | 0.82 |
| Methionine | 0.61 | 0.14 | 2.03 | 0.47 | 0.38 | 0.32 | 1.28 | 1.06 |
| Phenylalanine | 0.61 | 0.15 | 2.03 | 0.52 | 0.38 | 0.35 | 1.28 | 1.18 |
| Tryptophan | 1.22 | 0.38 | 4.06 | 1.28 | 0.77 | 0.88 | 2.56 | 2.92 |
| Glycine | 0.61 | 0.07 | 2.03 | 0.23 | 0.38 | 0.16 | 1.28 | 0.53 |
| Serine | 0.61 | 0.10 | 2.03 | 0.33 | 0.38 | 0.22 | 1.28 | 0.75 |
| Threonine | 1.22 | 0.22 | 4.06 | 0.74 | 0.77 | 0.51 | 2.56 | 1.70 |
| Cysteine | 0.61 | 0.11 | 2.03 | 0.38 | 0.38 | 0.26 | 1.28 | 0.86 |
| Tyrosine | 0.61 | 0.17 | 2.03 | 0.57 | 0.38 | 0.39 | 1.28 | 1.29 |
| Asparagine | 0.61 | 0.12 | 2.03 | 0.41 | 0.38 | 0.28 | 1.28 | 0.94 |
| Glutamine | 0.61 | 0.14 | 2.03 | 0.46 | 0.38 | 0.31 | 1.28 | 1.04 |
| Histidine | 1.22 | 0.29 | 4.06 | 0.97 | 0.77 | 0.66 | 2.56 | 2.21 |
| Lysine | 0.61 | 0.14 | 2.03 | 0.46 | 0.38 | 0.31 | 1.28 | 1.04 |
| Arginine | 0.61 | 0.16 | 2.03 | 0.54 | 0.38 | 0.37 | 1.28 | 1.24 |
| Glucose | 1.22 | 0.34 | 4.06 | 1.13 | 0.77 | 0.77 | 2.56 | 2.57 |

### 2.4.3 The Application of PLS Regression to Determine the Stability of Artificial Sweat Mixture during Storage

There were no obvious differences between the NMR spectra of the artificial sweat mixture acquired at varying storage time points when analysed by visual inspection. Thus, applying multivariate techniques through the use of projections will provide useful analysis to determine the relationship between multitudes of signals detected in the NMR spectrum over the storage period of one month. PCA is purely used as a visualisation method to highlight any major trends in the data (i.e. any variation in the NMR profiles) but gives no information how this is related to the amount of time being stored either at 4°C or -20°C. In comparison, PLS allows correlation with external variables to be evaluated in order to determine whether any correlation exists between the NMR profiles (**X**) and the storage period (**y**). To test the predictive power of the model, the data set was split into a training set containing the majority of the samples and a test set containing approximately one third of data. This equated to randomly removing whole days, because data included from replicate measurements in the training set will bias the model towards a better fit. To validate the predictions, five iterations were produced for both storage conditions to gain an accurate representation of the models in order to obtain stable prediction errors. In order to evaluate the performance of the predicative models the Root Mean Square Error (RMSE) in combination with the correlation coefficient (r) are used as a measure. RMSE is defined as follows:

$$RMSE = \frac{\sqrt{\sum (y_{pred} - y_{ref})^2}}{N}$$

**Equation 2.2**

where $y_{pred}$ is the predicted value, $y_{ref}$ is the measured value, and N is the number of samples. RMSEE is the root mean square error of the observations/training set and RMSECV is the root mean square error of cross validation which evaluates the stability of the current data set. If the model is good the RMSEE and RMSECV should be quite similar.

Three samples were omitted from both storage models due to poor NMR spectral quality. Leave one out internal cross validation of the training sets indicated two latent variables (LV) was optimal for the 4°C storage and one latent variable was optimal for the -20°C storage. The results from using five random subset cross validation procedures for both storage conditions are summarized in Table 2.6, while a representative model from both storage conditions are depicted in Figure 2.4, where the predicted versus observed values (days) are plotted for both the training set and test set.

**Table 2.6** Performance of PLS regression models from the artificial sweat matrix stored at 4°C and -20°C. $^{1}$H NMR spectra as **X** variable and storage day as **y** variable. Mean values are calculated from five iterations.

| Condition | LV[a] | $R^2X(cum)$[b] | $R^2Y(cum)$[b] | $Q^2(cum)$[c] | RMSEE[d] | RMSECV[e] | $R_{Predictive}$[f] | $R_{Training}$[f] |
|---|---|---|---|---|---|---|---|---|
| 4°C | 2 | 0.27 ± 0.02 | 0.87 ± 0.02 | 0.49 ± 0.15 | 2.94 ± 0.4 | 55.52 ± 73.22 | 0.44 ± 0.17 | 0.93 ± 0.01 |
| -20°C | 1 | 0.18 ± 0.02 | 0.74 ± 0.09 | 0.54 ± 0.16 | 3.77 ± 1.04 | 8.35 ± 3.31 | 0.61 ± 0.2 | 0.86 ± 0.05 |
| -20°C (no day 0) | 1 | 0.18 ± 0.03 | 0.71 ± 0.1 | 0.4 ± 0.26 | 3.85 ± 0.85 | 10.94 ± 5.06 | 0.37 ± 0.27 | 0.84 ± 0.06 |

[a] **LV** = Number of latent variables

[b] **$R^2$ (X or Y)cum** = Cumulative fraction of Sum of Squares (SS) of all the X's or Y's explained after each extracted component

[c] **$Q^2$cum** = The cumulative $Q^2$ for the extracted components

[d] **RMSEE** = Root mean square error of the fit for observations in the work set

[e] **RMSECV** = Root mean square error of cross validation of the prediction set

[f] **R** = Correlation coefficient

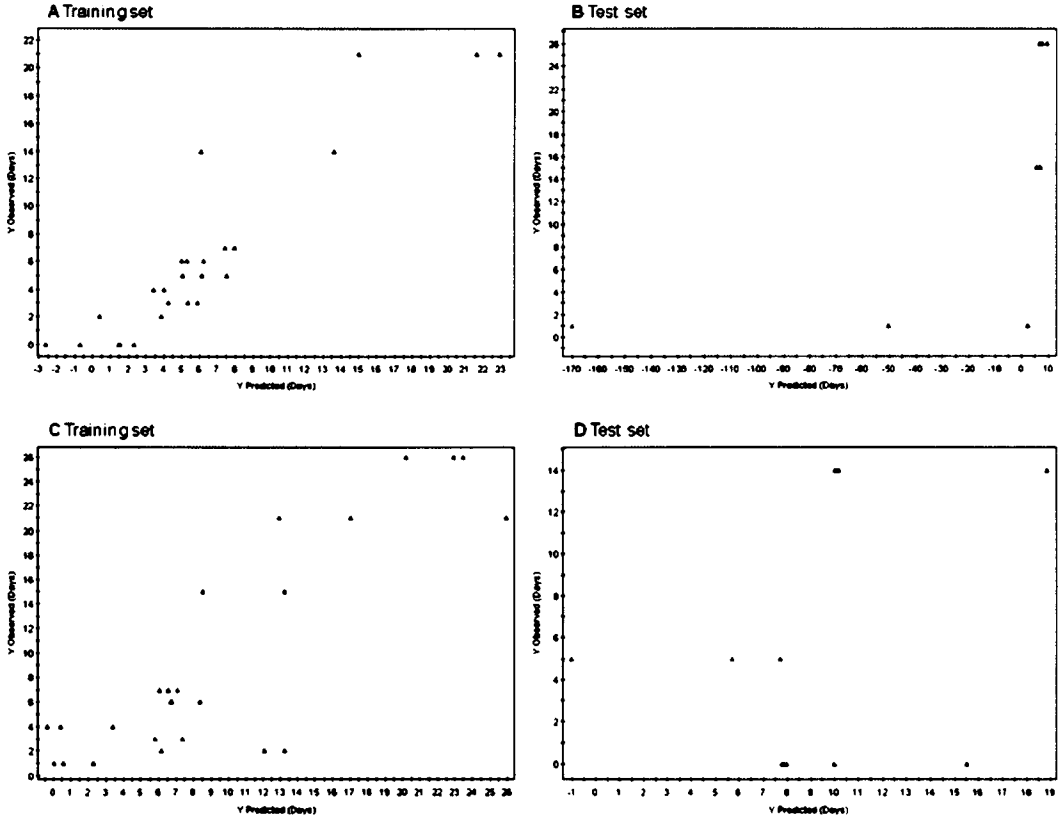



**Figure 2.4** PLS derived relationship between observed and predicted storage day using $^{1}H$ NMR spectra of the artificial sweat matrix. **A** and **B** PLS models from the artificial sweat stored at 4°C, **C** and **D** are the PLS models from the artificial sweat stored at -20°C. The predicted values of the test set are plotted against the observed storage day to visualise the predictive power of each model.

The results of the PLS models produced average $R^2X(cum)$ and $R^2Y(cum)$ values of 0.27 and 0.87 respectively and $Q^2(cum)$ was 0.49 for the 4°C storage condition and $R^2X(cum)$ and $R^2Y(cum)$ values of 0.18 and 0.74 respectively and $Q^2(cum)$ was 0.54 for the -20°C storage condition. Generally, $R^2X(cum)$, $R^2Y(cum)$ and $Q^2$ values should be well balanced as any differences larger than 0.2 indicate the presence of irrelevant model terms or a few outlying data points (Eriksson *et al.*, 2001). Together with a correlation coefficient (r) of 0.44 and RMSECV 55 days for the 4°C storage and r 0.54 and RMSECV 8 days for -20°C storage shows there is weak linear correlation between NMR profile and the number of days in storage and poor prediction ability for both models. A large standard deviation error of RMSECV indicates that the models are not stable. Furthermore, the r value of 0.9 and 0.44 for the 4°C storage condition and r 0.86 and 0.61 for the -20°C storage condition for the training set and predictive set respectively, indicate the model could be over fitted as these values are unbalanced. From this, it can be concluded that both storage

conditions don't significantly change over a course of a month and that any alterations in sample composition are likely to be random. Moreover, models with day 0 being omitted from the training set for the -20°C storage condition were produced to see if freezing the samples had an effect. As illustrated in Table 2.6, these models are similar and therefore can be concluded that freezing had minimal effect.

## 2.5 Conclusion

The aim of this chapter was to create an artificial sweat matrix, designed to broadly replicate apocrine gland secretions, in order to develop NMR methodology which will be suitable for the analysis of low sample volumes. The comparative performance assessment described in this study demonstrate that the 1 mm micro-volume probe offers an ~ 5.3 fold performance advantage over data acquired for the same mass limited sample using specialist tubes with a 5 mm sample probe. Furthermore, the micro-volume probe offers a similar enhancement to that offered from a 500 MHz instrument equipped with a cryoprobe. However, in terms of maintenance and cost, it is more viable to use the micro-volume probe. NMR spectroscopy-based studies on the whole are typically robust and reproducible and differences in solvent suppression are generally minor when compared to the effects caused by biological change. The stability study showed that there was minimal change over the storage of one month in both the samples stored at 4°C and -20°C. Thus, when comparing the reproducibility and sensitivity of different methods, storage conditions can be removed as an interfering factor. The high sensitivity offered by the 1 mm micro-volume probe opens many new potential avenues of investigation of limited volumes, weights, and low cell counts in biological sample, e.g. rodent CSF (Griffin *et al.*, 2002), urine and serum (Grimes and O'Connell, 2011) or apocrine sweat.

# Chapter 3

## 3 Development of Mass Spectrometry Methods for the Analysis of Key Metabolites in Apocrine Sweat

### 3.1 Introduction

Mass spectrometry is a mature and well established analytical technique which can be applied with or without chromatographic separation before detection. MS can offer a number of advantages over other analytical techniques, including increase in sensitivity, chemical identification capabilities *via* accurate mass measurements or mass spectrum interpretation, as well as the ability to quantitatively detect hundreds of metabolites in a given sample when combined with chromatography. In comparison to NMR spectroscopy, samples physically interact with the instrument which can change the response over short to medium periods of time, highlighting the importance of QC samples, as well as being destructive to the sample.

The analysis of apocrine sweat in the literature is predominantly focused on odour content, thus, the analytical strategies commonly used are GC-MS or variations of this (e.g. headspace analysis), which will be able to describe the volatile organic compounds that are present in apocrine sweat (Gower *et al.*, 1997; Natsch *et al.*, 2006; Penn *et al.*, 2007). However, these studies are mostly intended to characterise malodour rather than individual odour *per se*. Although the components of sweat have been studied, there is comparatively little attention to the biochemistry of axillary odour formation. Consequently there is limited literature available regarding the chemical structures of odour precursors isolated from axilla secretions, and no specific bacterial enzymes capable of recognising these precursors have been isolated. Furthermore, estimates for inter-individual variability or intra-individual consistency as well as the identities of individual odour need to be addressed.

A range of MS-based methods have been developed over recent years to undertake metabolic profiling of biological tissues, and the major approaches are summarised here.

### 3.1.1 Direct Injection Mass Spectrometry (DIMS)

The analysis of metabolite profiles from crude samples or sample extracts by DIMS can be performed in 1-3 min, providing a rapid, high throughput method, capable of screening up to 1000 samples per day (Rashed *et al.*, 1997). The samples can be introduced into the MS either through the use of the HPLC system (Kaderbhai *et al.*, 2003) or infused with syringe pumps (Goodacre *et al.*, 2002), resulting in one mass spectrum per sample, which is used for sample classification. Direct injection can avoid the disadvantages of LC system; noise levels, retention time shifts and high variability in signal intensities (Boernsen *et al.*, 2005). However, it is not without its pitfalls, as analysis is susceptible to ion suppression effects that arise from the competitive ionization with other compounds within the matrix. For example, the types of interference that can occur during acquisition are typically from ionic compounds such as salts, charged organic compounds, organic acids/bases, and hydrophobic compounds. However, through the use of a nano electrospray setup, these effects can be reduced due to the increase in ionization efficiency (Bedair and Sumner, 2008). Definitive metabolite identification is also a limitation with this technique due to the inability to distinguish between isomeric compounds.

### 3.1.2 Reverse Phase High Performance Liquid Chromatography Mass Spectrometry (RP-HPLC-MS)

MS based metabolomics are predominantly linked with GC (Fiehn *et al.*, 2000; Welthagen *et al.*, 2005), HPLC (Idborg-Bjorkman *et al.*, 2003; Jander *et al.*, 2004; Plumb *et al.*, 2002), or CE (Baidoo *et al.*, 2008; Sato *et al.*, 2004; Soga *et al.*, 2003), which can overcome many of the drawbacks of DIMS such as; increasing sensitivity due to reduction in background noise, reducing ion suppression caused by co-eluting compounds, and reducing isobaric interferences. For metabolomic purposes, HPLC-MS separations have primarily relied on using reversed-phase (RP) chromatography

coupled to electrospray ionization (ESI) in both positive and negative mode to obtain a comprehensive metabolic profile (Lenz and Wilson, 2007). RP-HPLC columns are composed of a non-polar stationary phase, typically silica, which has been modified with dimethylchlorosilane, containing a bulky alkyl group such as $C_{18}H_{37}$ or $C_8H_{17}$ to form the hydrophobic surface and an aqueous polar mobile phase. Thus, RP-HPLC separations are suitable for compounds of medium and low polarity, while more polar compounds (e.g. amino acids and sugars), are not well retained and non-polar compounds (e.g. lipids) are difficult to elute. These limitations can be partially overcome through the use of graphitized carbon columns (Tornkvist *et al.*, 2004), pre-column derivatization or through the use of ion-paring agents (e.g. perfluorinated carboxylic acids). Such approaches have not found widespread use because of practical difficulties in applying to biological extracts. Thus, these polar and ionic metabolites have traditionally been under represented in metabolomic studies.

HPLC-MS is capable of moderate to high throughput analysis with a reasonable dynamic range. However, unlike $^1$H NMR spectroscopy, HPLC-MS is chemically biased, as compounds need to be ionized in order to be detected as well as the column chemistry being selective to certain chemical species (typically non-polar metabolites, including lipids for RP-HPLC). Moreover, HPLC methods involve several parameters that need to be checked and validated such as sample handling, mobile phase composition, column chemistry, and gradient, which can be a time consuming process.

### 3.1.3 Hydrophilic Interaction Liquid Chromatography (HILIC)

An alternative technique for the separation of polar compounds is hydrophilic interaction liquid chromatography (HILIC), which was first introduced by Alpert for the separation of peptides, nucleic acids and other polar compounds (Alpert, 1990) and later used by Strege for drug research (Strege, 1998). HILIC is analogous to normal phase chromatography in that it utilizes a hydrophilic stationary phase, allowing the retention of polar analytes (Cubbon *et al.*, 2007). However, unlike normal phase, HILIC allows the use of aqueous/polar organic solvents, which are more compatible with ESI-MS system. In contrast to RP-HPLC, gradient elution

begins with low-polarity organic solvent and elutes polar analytes by increasing the polar aqueous content. However, the exact retention mechanism for HILIC is still open to debate within the scientific community. Alpert (Alpert, 1990) suggests that compounds are partitioned between a polar organic mobile phase and the water-enriched layer of the mobile phase that is partially immobilized on the polar stationary phase. Others have reported that separation is mainly governed by polar-polar interactions i.e., hydrogen bonding which depend on the acidity or basicity of the solutes, electrostatic interactions, dipole-dipole interactions, which rely on the dipole moments and polarizabilities of molecules (Wang *et al.*, 2008; Yoshida, 2004). In a recent review, Hemstrom and Irgum (Hemstrom and Irgum, 2006) considered the contribution from both the partitioning and adsorption processes and found that they fit better with the Synder-Soczewinski adsorption model (Snyder and Poppe, 1980) than the partitioning model (Wang *et al.*, 2008). The advantages of HILIC have been summarised (McCalley, 2007) as:

- Good retention of polar and ionic compounds compared to RP-HPLC.
- The order of elution of solutes is generally the opposite of that found in RP-HPLC, thereby, providing an alternative selectivity.
- Higher flow rates are possible due to the high organic content of typical mobile phases.
- ESI-MS sensitivity is enhanced in comparison to normal phase due to the high organic content in the mobile phase and the high efficiency of spraying and desolvation techniques.
- Good peak shape can be obtained for bases.

In HILIC mode, the mobile phase generally consists of between 5 and 50% water (Alpert, 1990), however, the composition is dependent on the polarity of both the stationary phases and the analytes to be separated. The water content must be low enough to achieve separation, but high enough for the mobile phase to dissolve the analytes and elute them in a reasonable timeframe. Typically, the more polar the stationary phase and the analyte, the higher the water content is needed for separation. The polar organic solvent of choice is generally acetonitrile (Churms, 1996) but other mixtures such as water-methanol (Valette *et al.*, 2004), dichloromethane-methanol

(Herbreteau *et al.*, 1992), isopropanol (Li and Huang, 2004), and ethanol have been used (Nguyen and Schug, 2008). Various hydrophilic columns have been employed in the HILIC mode, depending on the specific applications. Typically, nonbonded silica columns, polar-bonded silica phases have been employed in the literature. These include aminopropylamide-, poly(succinimide)-, diol-, cyanopropyl-, and sulfoalkylbetaine silica phases. The properties of these stationary phases have been discussed in a recent review by Hemstrom and Irgum (Hemstrom and Irgum, 2006). HILIC has shown promise in bioanalytical applications for a wide variety of polar and hydrophilic compounds which are summarised in Table 3.1. Generally, in HILIC mode, acetonitrile is used as the organic component with the addition of ammonium acetate, typically 5-20 mM, in addition to having long analysis times, typically 30 min.

To date no study has attempted to qualitatively and quantitatively determine the profile of human apocrine sweat. Thus, two additional MS analytical approaches will be developed to provide complementary information to the NMR data. Firstly, a global fingerprint of metabolites of medium to high polarity and secondly, identify specific odour precursors in a qualitative and quantitative manner. Moreover, the targeted based method will allow more definitive identification of unknown odour precursors, as although accurate mass can be obtained in the global methodology, this information does not provide any hits in biological databases such as HMDB or Lipid Maps. The former method will be optimised with an artificial sweat matrix and the complexity will then be increased using more representative biological sample (see chapter 4). The latter method will be developed with available standards.

**Table 3.1** Representative applications of HILIC reported in the literature

| Sample | Column | Mobile phase | Total run time (min) | Detector | Author |
|---|---|---|---|---|---|
| **Pharmaceutical drugs** | | | | | |
| Small polar compounds e.g. cytosine/aspirin | 4 columns (Zic-HILIC) | 85% MeCN, 20 mM ammonium acetate | 20 | UV 228 nm | (Guo and Gaiki, 2005) |
| Mildronate and related substances | 6 columns tested (zic-HILIC) | 90% MeCN + 0.1% formic acid or ammonium formate | - | ESI MS (+ve mode) | (Hmelnickis *et al.*, 2008) |
| Isoniazid in plasma | Hypersil silica | [A] 0.1% HOAC, 2.5 mM ammonium acetate [B] MeCN, 0.1% HOAC | 10 | ESI MS (+ve mode) | (Huang *et al.*, 2009) |
| Cyanobacterial toxins | TSK-gel amide | [A] 2 mM ammonium formate, 3.6 mM formic acid, pH 3.5 [B] 95% MeCN, 2 mM ammonium formate, 3.6 mM formic acid, pH 3.5 | - | API MS (+ve mode) | (Dell'Aversano *et al.*, 2004) |
| 3 Pharmaceuticals in human plasma | YMC Silica | 82% MeCN and 18% 10 mM ammonium acetate pH 5 | 2 | ESI MS (+ve mode) | (Apostolou *et al.*, 2008) |
| Aromatic, NH2, OH | YMC-Pack Diol-120-NP | 95% MeCN + 10 mM $NH_4Cl$ | 20 | UV 215 nm | (Wang *et al.*, 2005) |
| Oseltamivir in plasma and urine | ZIC-HILIC (SeQuant) | [A] 10 mM ammonium acetate, 1% formic acid [B] MeCN | | ESI MS (+ve mode) | (Lindegardh *et al.*, 2007) |
| Opioids and glucuronides | ZIC-HILIC (SeQuant) | [A] 90% MeCN, ammonium formate [B] 50/50 MeCN | 30 | UV (220 nm) and ESI MS (+ve mode) | (Vikingsson *et al.*, 2008) |
| **Plasma** | | | | | |
| Polar metabolites in plasma | Acquity silica UPLC BEH | [A] Water [B] 95% MeCN + 10 mM ammonium acetate | 15 | ESI MS (+ve/-ve mode) | (Cai *et al.*, 2009) |

*Table 3.1 continued*

| Sample | Column | Mobile phase | Total run time (min) | Detector | Author |
|---|---|---|---|---|---|
| Dasatinib, imatinib and nilotinib in mouse plasma | Silica/diol/pyridine/imidazole HILIC (Sepax) | [A] water + 0.1% formic acid/4 mM ammonium acetate [B] MeCN + 0.1% formic acid/4 mM ammonium acetate | 1 | ESI/APCI MS (+ve mode) | (Hsieh *et al.*, 2009) |
| Propofol in plasma | Atlantis HILIC | MeCN, water, 100 mM ammonium acetate, pH 5 (87:1:12) | 12 | ESI MS (-ve mode) | Cohen, 2007 |
| Miglustat in human plasma and CSF | Atlantis HILIC | MeCN/water/100 mM ammonium acetate pH 5 (75/10/15 *v/v*) | | ESI MS (+ve mode) | (Guitton *et al.*, 2009) |
| Doxazosin in human plasma | Atlantis silica | MeCN + 100 mM ammonium formate, pH 4.5 (93:7 *v/v*) | 3 | ESI MS (+ve mode) | (Ji *et al.*, 2008) |
| Carvedilol in human plasma | Atlantis silica | MeCN + ammonium formate (50 mM, pH 4.5) (90:10 *v/v*) | 2.5 | ESI MS (+ve mode) | (Jeong *et al.*, 2007) |
| Acetyl and carnitines | Atlantis silica | [A] 5% MeCN, 5 mM ammonium acetate [B] 95% MeCN, 5 mM ammonium acetate | 5 | ESI MS (+ve mode) | (Liu *et al.*, 2008b) |
| Arginine, plasma ADMA & SDMA | Luna silica | [A] MeCN, trifluoracetic acid, acitic acid (1000:25:10) [B] water, trifluroacetic acid, acitic acid (1000:25:10) | 5 | ESI MS (+ve mode) | (D'Apolito *et al.*, 2008) |
| Polar metabolites e.g. creatinine, uracil and hippuric acid | Supersphere Si | [A] 100 mM $NH_3$ [B] MeCN + 0.2% formic acid | 37 | ESI MS (+ve mode) | (Godejohann, 2007) |

*Table 3.1 continued*

| Sample | Column | Mobile phase | Total run time (min) | Detector | Author |
|---|---|---|---|---|---|
| Dacarbazine in plasma | TSK-gel amide | [A] MeCN<br>[B] 0.1% formic acid | 6 | ESI MS (+ve mode) | (Liu *et al.*, 2008a) |
| Methylmalonic acid in human plasma | PEEK Zic-HILIC (SeQuant) | 80% MeCN and 20% 100 mM ammonium pH 4.5 | 10 | ESI MS (-ve mode) | (Lakso *et al.*, 2008) |
| **Urine** | | | | | |
| Liver cancer | Acquity silica | [A] 100 mM ammonium formate + 0.1% formic acid<br>[B] MeCN | 35 | ESI MS (+ve mode) | (Chen *et al.*, 2009) |
| Urine | Luna silica | [A] 100% MeCN<br>[B] 13 mM ammonium acetate, pH 9.1 | 30 | ESI MS (+ve/-ve mode) | (Kind *et al.*, 2007) |
| Estrogen conjugates in urine | TSK-gel amide | 85% MeCN, 5 mM ammonium acetate, pH 6.8 | 25 | ESI MS (-ve mode) | (Qin *et al.*, 2008) |
| Urine | ZIC-HILIC (SeQuant) | [A] 5 mM ammonium acetate + 0.1% formic acid<br>[B] MeCN + 0.1% formic acid | 30 | ESI MS (+ve/-ve mode) | (Cubbon *et al.*, 2007) |
| Urine | ZIC-HILIC (SeQuant) | [A] 5 mM ammonium acetate, pH 4<br>[B] MeCN + 0.025% formic acid | 30 | ESI MS (+ve mode) | (Idborg *et al.*, 2005) |
| **Plant** | | | | | |
| Organic acids | TSK-gel amide | [A] 90% MeCN, 0.65 mM ammonium acetate, pH 5.5<br>[B] 60% MeCN, 2.6 mM ammonium acetate, pH 5.5 | 90 | ESI MS (+ve/-ve mode) | (Schlichtherle-Cerny *et al.*, 2003) |
| Plant compounds | TSK-gel amide | [A] MeCN<br>[B] 6.5 mM ammonium acetate, pH 5.5 | 60 | ESI MS (+ve/-ve mode) | (Tolstikov and Fiehn, 2002) |

*Table 3.1 continued*

| Sample | Column | Mobile phase | Total run time (min) | Detector | Author |
|---|---|---|---|---|---|
| Mixture metabolites: amino acids - bases | TSK-gel amide | [A] water, 0.1% v/v formic acid [B] 90% MeCN, 0.1% v/v formic acid | 30 | ESI MS (+ve mode) | (t'Kindt *et al.*, 2008) |
| Carbohydrate-related compounds | ZIC-HILIC (SeQuant) | [A] MeCN + 0.1% formic acid<br>[B] 5 mM ammonium acetate + 0.1% formic acid (pH 4) | 30 | ESI MS (-ve mode) | (Antonio *et al.*, 2008) |
| Melamine and cyanuric acid | ZIC-HILIC (SeQuant) | [A] 95% MeCN, 0.1% formic acid<br>[B] MeCN, 20 mM ammonium formate | 14 | ESI MS (+ve/-ve mode) | (Heller and Nochetto, 2008) |
| Ascorbic acid | ZIC-HILIC (SeQuant) | MeCN, 50 mM ammonium acetate, pH 6.8 | 5 | UV 268 nm | (Novakova *et al.*, 2008) |
| Phytosiderophores in plants | ZIC-HILIC (SeQuant) | [A] 90% MeCN, 10 mM ammonium acetate, pH 7.3<br>[B] 20% MeCN, 30 mM ammonium acetate, pH 7.3 | 60 | ESI MS (+ve/-ve mode) | (Xuan *et al.*, 2006) |
| DTC fungicide | ZIC-HILIC (SeQuant) | [A] MeCN<br>[B] 10 mM ammonium acetate | 23 | ESI MS (-ve mode) | (Crnogorac and Schwack, 2007) |
| **Breath** | | | | | |
| Exhaled breath | ZIC-HILIC (SeQuant) | [A] MeCN, 0.025% formic acid<br>[B] 5 mM ammonium acetate, pH 4 | 30 | ESI MS (+ve mode) | (Conventz *et al.*, 2007) |
| **Cells** | | | | | |
| Bacteria | ZIC-HILIC (SeQuant) | [A] 0.1% formic acid<br>[B] MeCN + 0.1% formic acid | 32 | ESI MS (+ve/-ve mode) | (Kamleh *et al.*, 2008) |

*Table 3.1 continued*

| Sample | Column | Mobile phase | Total run time (min) | Detector | Author |
|---|---|---|---|---|---|
| Cisplatin and its monohydrolyzed metabolite | ZIC-HILIC (SeQuant) | 70% DMF/20 mM ammonium acetate or 70% MeCN/30% 20 mM ammonium acetate | 30 | ESI MS (+ve mode) | (Nguyen and Schug, 2008) |
| Cell lysate acetylcholine | ZIC-HILIC (SeQuant) | 65% MeCN 35% 10 mM ammonium formate, 0.02% formic acid | 10 | ESI MS (+ve mode) | (Schebb *et al.*, 2008) |
| Long chain acylcarnitine | Atlantis silica | 200 mM ammonium formate, pH 3, MeCN, and Water (5:86:9) | 28 | ESI MS (+ve mode) | (Jauregui *et al.*, 2007) |
| **Human milk** | | | | | |
| N-linked glycoproteins in human milk | ZIC-HILIC (SeQuant) | 80% MeCN, 1% formic acid | - | MALDI and ESI MS (+ve mode) | (Picariello *et al.*, 2008) |
| **Others** | | | | | |
| Amino acids | TSK-gel amide | [A] 90% MeCN, 0.5 mM ammonium acetate, pH 5.5 [B] 60% MeCN, 2.5 mM ammonium acetate, pH 5.5 | | ESI MS (+ve mode) | (Langrock *et al.*, 2006) |
| Acetylcholine in subcutaneous tissue | ZIC-HILIC (SeQuant) | 70% MeCN, 10 mM ammonium formate, pH 4 | 15 | ESI MS (+ve mode) | (Fu *et al.*, 2008) |
| Amino acid analysis | ZIC-HILIC (SeQuant) | [A] 10 mM acetic acid [B] MeCN | 88 | ESI MS (+ve mode) | (Kato *et al.*, 2009) |
| Organoarsenicals | ZIC-HILIC (SeQuant) | [A] 70% MeCN [B] 125 mM ammonium acetate, pH 8.3 | 60 | ESI MS (+ve mode) | (Xie *et al.*, 2008) |
| N- and O-glycopeptides | Capillary ZIC-HILIC (SeQuant) | [A] 50% MeCN, 10 mM ammonium acetate [B] 80% MeCN, 10 mM ammonium acetate | 80 | ESI MS (-ve mode) | (Takegawa *et al.*, 2008) |

### 3.1.4 Analytical Strategies for Profiling Apocrine Sweat Metabolites

The following information was taken into account when developing methods in this chapter.

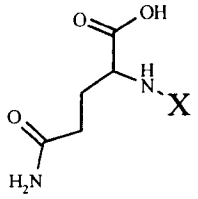
*Global approach*

In using DIMS or HPLC-MS in a comprehensive global approach, the intention is to measure as many of the small molecule components as possible in the sample. With regards to HPLC-MS, this intention puts large demands on the applied chromatographic separation capability. The stationary phase will also influence, in terms of retention properties of metabolites, the range of different chemical properties of the separated metabolites. Although the fingerprint regions would likely look different on different stationary phases, they are not expected to have a higher information content. Thus, the approach taken herein is to obtain a method that is capable of separating a range of polar compound in under 30 min.

*Gln, Cys and Cys-Gly -conjugate profiling*

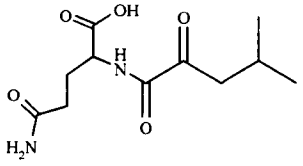
The general structures of the odour precursors all contain one of three common moieties (see Appendix A); Gln, Cys or a Cys-Gly residue, an example of which is depicted in Figure 3.1. Thus, a precursor ion scanning method was developed which would be later used to screen actual apocrine gland secretions in order to help identify all the amino acid conjugates present, including unknowns, in a single run without making prior assumptions of identity.

**Gln Conjugates: -**

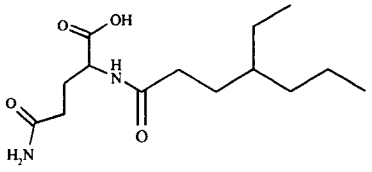


X= Fatty acid residue

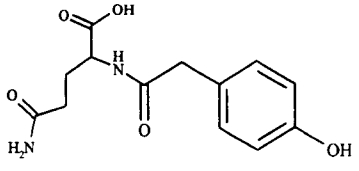
**Gln Conjugates Examples: -**



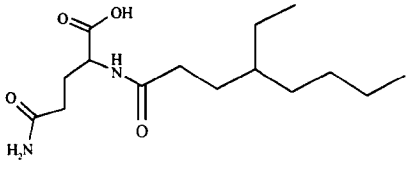
N-α-4-methyl-2-oxopent-anoyl- L-glutamine



N-α-4-ethyl-hept-anoyl- L-glutamine



N-α-4-hydroxyphenyl-acetyl- L-glutamine



N-α-4-ethyl-oct-anoyl- L-glutamine

**Figure 3.1** An example of common glutamine conjugates.

*Specific targeted analysis of odour precursors*

In a targeted approach, the main aim is to identify and quantify specific compounds of interest. As a consequence, the standard route of method development is to find the optimum chromatographic conditions to separate representative standards from one another as well as from matrix interferences. However, compromises will have to be made for the eluent composition to ensure sufficient analysis times, optimum peak capacity, and the stability of the sample profiles, in addition to the MS parameters, as one set of conditions is unlikely to suit all compounds.

### 3.1.5 Aims

- To establish an analytical method that would be capable of detecting and identifying a broad range of metabolites present in human apocrine sweat, either by DIMS or LC-MS.
- To establish an analytical method capable of identifying odour precursors from a common structure.
- To establish an analytical method to specifically identify odour precursors in human apocrine sweat using MRM analysis coupled with full product ion spectra.

## 3.2 Material and Methods

### 3.2.1 Chemicals

HPLC grade acetonitrile (far UV), methanol, ammonium formate, and glucose were purchased from Fisher Scientific UK Ltd (Loughborough, UK). HPLC grade water was obtained from a Millipore Milli-Q Gradient purification system (Bedford, MA). Formic acid, ammonium hydroxide, cholesterol, and stearic acid was obtained from Sigma Aldrich (Poole, UK) and stored at 4°C. Ammonium acetate was of ACS grade and tristearine from Fluka. L-alanine, L-valine, L-leucine, L-isoleucine, L-proline, L-methionine, L-phenylalanine, L-tryptophan, L-glycine, L-serine, L-threonine, L-cysteine, L-tyrosine, L-asparagine, L-glutamine, L-aspartic acid, L-glutamic acid, L-histidine, L-lysine, L-arginine were sourced from Sigma-Aldrich at a purity of 99%. Lanolin was sourced from Alfa Aesar (Heysham, UK). 3-methylhex-2-enoyl-L-glutamine (3M2H-Gln), N-α-3-hydroxy-3-methylhex-2-enoyl-L-glutamine (HMHA-Gln), S-[1-(2-hydroxy-1-methylethyl)-ethyl]-L-cysteine (2M3H-Cys) and S-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cysteine-glycine (3M3SH-Cys-Gly) were supplied by Dr Mark Harker (Unilever R&D, Bebington, UK).

### 3.2.2 LC-MS Columns

The $C_{18}$ Synergi Hydro-RP 80Å, (4 μm, 150 x 2 mm) was purchased from Phenomenex (Macclesfield, UK). An Atlantis $C_{18}$ column (3 μm, 100 x 2.1 mm) was purchased from Waters (Manchester, UK). A ZIC-HILIC (5 μm, 150 x 2.1 mm) column along with a guard column (20 x 2.1 mm) was purchased from SeQuant (Umeå, Sweden). All columns were equilibrated with mobile phase prior to use and washed as per manufacturer recommendations.

### 3.2.3 Sample Preparation

Apocrine secretion is a turbid fluid that is complex in nature, containing a wide range of compounds such as proteins, lipids and steroids (Labows *et al.*, 1979). The secretion of this fluid from the apocrine gland is transient and intermittent, with only low volume being produced. Obtaining this fluid from human volunteers is very costly, thus, an artificial sweat matrix was developed for the purpose of developing analytical methodologies, as depicted in Table 3.2, and not intended to replicate apocrine sweat secretions but to have chemical characteristics in common. Blood plasma (human venous blood collected from the Nottingham University Hospital and placed into vials containing lithium heparin as an anticoagulant) was used as the underlying matrix for the artificial sweat matrix, due to the plasma containing common metabolites, in particular the non-polar species like cholesterol, glycerides and fatty acids. The proteins present in the blood plasma were removed by precipitation, using a 3:1 acetonitrile before the addition of amino acids standards. Furthermore, the use of blood plasma as the matrix overcomes the difficulties of dissolving polar and non-polar components into a solution, due to many of the components being present in blood plasma. An amino acid standard mixture containing all the amino acids listed in Section 3.2.1 was also separately prepared at a concentration 40 μg/ml.

**Table 3.2** Artificial apocrine secretion matrix used for method development.

| Ingredient | Level in µg/ml | Ingredient | Level in µg/ml |
|---|---|---|---|
| Plasma | 500 µl | Threonine | 40 |
| Alanine | 40 | Cysteine | 40 |
| Valine | 40 | Tyrosine | 40 |
| Leucine | 40 | Asparagine | 40 |
| Isoleucine | 40 | Glutamine | 40 |
| Proline | 40 | Aspartic acid | 40 |
| Methionine | 40 | Glutamic acid | 40 |
| Phenylalanine | 40 | Histidine | 40 |
| Tryptophan | 40 | Lysine | 40 |
| Glycine | 40 | Arginine | 40 |
| Serine | 40 | | |

### 3.2.4 Global Profiling by LC-QTOF-MS

All experiments were conducted on a Shimadzu LC system (10AD VP) equipped with two Shimadzu binary pumps (LC-10AD), a vacuum degasser, a cooled autosampler (SIL-HTc) and a column oven (Shimadzu, Columbia, MD, USA). The HPLC system was coupled to a Waters QTOF Premier system (Water MS Technologies, Manchester, UK) equipped with an electrospray source operating in positive ion mode. The source temperature was set to 125°C with a cone gas flow of 56 l/h, a desolvation gas temperature of 350°C and a desolvation gas flow of 400 l/h. A capillary voltage and a cone voltage were set to 3.0 kV and 25 V, respectively. The MCP detector voltage was set to 1850 V. The QTOF acquisition rate was set to 0.3 s with a 0.01 s interscan delay. Tune page was used to regulate the sample cone voltage. Argon was employed as the collision gas at a flow rate of 0.4 ml/min. The mass spectrometer was mass calibrated prior to use with sodium formate (0.05 M sodium hydroxide and 0.5% formic acid in 90:10 2-propanol:water *v/v*) between *m/z* 50-1000 with a residual error $<1.79 \times 10^{-4}$ amu.

A 5 µl aliquot of artificial sweat mixture was injected onto a SeQuant ZIC®-HILIC column, 150 x 2.1 mm (5 µm particle size) along with a guard column (20 x 2.1 mm) operated at 25°C, with all the eluent from the LC column being directly transferred

into the ion source of the MS without post-column splitting. The final optimised mobile phase contained [A] 10 mM ammonium acetate and 90% acetonitrile at pH 5 and [B] 10 mM ammonium acetate. The gradient duration was 25 min at a flow rate of 250 µl/min. From the start to 2.5 min [A] was kept constant at 100% and linearly decreased to 50% in 7.5 mins and held constant for 3.5 mins. Finally, at the end of a gradient an 11.5 min re-equilibration period was employed. The mass spectrometric data was collected in full scan mode from *m/z* 50 to 1000 from 0-25 min.

### 3.2.4.1 Preliminary Development Work for LC-QTOF-MS

A range of different HPLC columns and mobile phase conditions were used during the method development and are listed below. The final optimised LC-QTOF-MS method is noted above (Section 3.2.4).

*Initial Column Selection Conditions*

$C_{18}$ Synergi Hydro-RP 80Å, (4 µm, 150 x 2 mm): - Mobile phase [A] consisted of 100% water and 0.1% formic acid [B] acetonitrile and 0.1% formic acid. The gradient duration was 10 min at a flow rate of 250 µl/min as follows: an isocratic stage of 0% [A] for 2 min, followed by a linear raise to 50% [A] in 8 min.

ZIC-HILIC column (5 µm, 150 x 2.1 mm): - Mobile phase [A] consisted of 90% acetonitrile and 5 mM ammonium acetate [B] 5 mM ammonium acetate, pH 6.2. The gradient duration was 10 min at a flow rate of 250 µl/min as follows: an isocratic stage of 0% [B] for 2 min, followed by a linear raise to 60% [B] in 8 min, and at 10 min decreased back to the initial 0% [B] for 5 min to re-equilibrate the column.

*Isocratic Elution*

Mobile phase consisted of 80% acetonitrile and 10-30 mM ammonium acetate, pH 5 or 70-90% acetonitrile and 20 mM ammonium acetate, pH 5. The elution duration was 50 min at a flow rate of 250 µl/min.

*Effect of Ionic Strength*

Mobile phase [A] consisted of 100% acetonitrile or 100% acetonitrile and 0.1% formic acid [B] 10-80 mM ammonium acetate, pH 5. The gradient duration was 40 min at a flow rate of 250 µl/min as follows: an isocratic stage of 5% [B] for 6 min, followed by a linear raise to 40% [B] in 14 min, further linear rise to 60% [B] in 10 min and at 30.0 min decreased back to the initial 5% [B] for 10 min to re-equilibrate the column.

Mobile phase [A] consisted of 90% acetonitrile and 0.5 mM ammonium acetate, pH 5 [B] 60% acetonitrile and 1-10 mM ammonium acetate, pH 5. The gradient duration was 45 min at a flow rate of 250 µl/min as follows: 5% [B] and increasing linearly to 60% over a period of 28.5 min, further increase to 95% [B] in 5 min and at 34 min decreased back to the initial 5% [B] for 10 min to re-equilibrate the column.

*Effect of pH*

Mobile phase [A] consisted of 95% acetonitrile and 10 mM ammonium acetate [B] 10 mM ammonium acetate. The pH was varied in both mobile phases between 4-8. The gradient duration was 40 min at a flow rate of 250 µl/min as follows: an isocratic stage of 0% [B] for 6 min, followed by a linear raise to 35% [B] in 14 min, a further linear rise to 55% [B] in 10 min and at 30.0 min decreased back to the initial 0% [B] for 10 min to re-equilibrate the column.

### 3.2.5 Global Profiling by Direct Injection Mass Spectrometry

Direct injection mass spectrometry was acquired with the same MS conditions and setting as noted in Section 3.2.4. The HPLC system was used to load 5 µl of the sample into the LC sample loop using LC solvents (50:50 *v/v* mixture of acetonitrile and water containing 0.1% formic acid) and pumped from 100-500 µl/min for one min. All the eluent directly transferred into the ion source of the MS. Three blanks were run between each sample to reduce any carry over from the HPLC-tubing.

### 3.2.6 Targeted Profiling by LC-MS/MS

Analysis was conducted on a Shimadzu LC system (10AD VP) equipped with two Shimadzu binary pumps (LC-10AD), a vacuum degasser, a cooled autosampler (SIL-HTc) and a column oven (Shimadzu, Columbia, MD, USA). The HPLC system was coupled to a Applied Biosystems 4000 QTRAP® (QqQLit) hybrid triple quadrupole linear ion trap (Foster City, CA, USA) equipped with a TurboIon electrospray source operating in positive ion mode.

#### 3.2.6.1 Optimisation of compound dependent parameters for MRM Analysis

Analyses were performed using multiple reaction monitoring (MRM) for 3M2H-Gln ($C_{12}H_{20}N_2O_4$, MW: 256.14), HMHA-Gln ($C_{12}H_{22}N_2O_5$, MW: 274.15), 2M3H-Cys ($C_8H_{17}NO_3S$, MW: 207.09) and 3M3SH-Cys-Gly ($C_{12}H_{24}N_2O_4S$, MW: 292.15). The optimisation of the precursor to product ion dissociation was performed by infusion into the MS of separate standard solutions (10 µg/ml) in 50:50 *v/v* of [A]/[B] mobile phase (see below) for each analyte at a flow rate of 10 µl/min. The probe capillary voltage (IS) was optimised at 5000 V; temperature of the turbo gas (TEM) 350°C; nebulizer gas (GS1), auxiliary gas (GS2), curtain gas (CUR) were set to 20, 10, and 10, respectively (arbitrary values). Nitrogen was used for collisionally induced dissociation (CID). Optimal declustering potential (DP) and collision energy (CE) values were then used for the determination of 14 MRM transitions, each representing an individual odour precursor (see Table 3.4). Each transition was performed with a 25 ms dwell time. DP and CE voltages for those conjugate precursors for which standards were unavailable, were predicted from those obtained from a closely related standard. The mass spectrometer was also set to use the information dependent acquisition (IDA) function, where the instrument utilises an MRM as survey scan to trigger a consecutive enhanced product ion (EPI) scan (MS/MS) for structural confirmation (Figure 3.2). IDA criteria were set to select those MRM transitions above a signal intensity of 5000 counts. Dynamic exclusion was set to 15 sec. Full product ion spectral data as well as retention times of each of the standards were used for structural and peak identification, respectively.

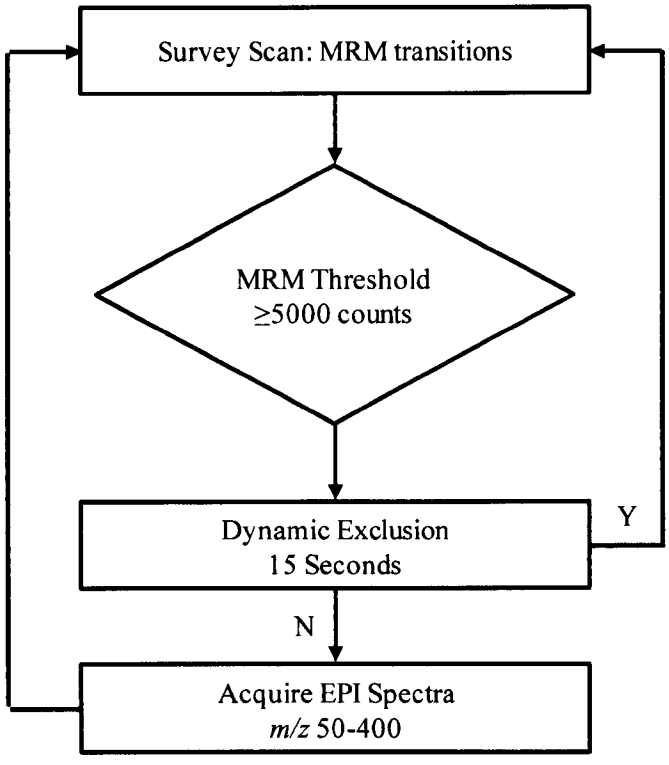



**Figure 3.2** Schematic of the IDA driven acquisition procedure.

### 3.2.6.2 Conjugate Profiling using Precursor Ion Scanning

Based on the evidence in the literature and available standards, Gln, Cys, and Cys-Gly conjugates, all fragmented under CID to produce their respected amino acid residue, *m/z* 147, 179 and 105 respectively. Thus, these ions were chosen as the signature product ions for the precursor ion scan. Subsequent ion trap scans were triggered when the threshold of the precursor ion scan reached $2x10^4$ counts. Each precursor ion scan ranged from *m/z* 200-400 over a scan time of 1.2 s and EPI MS/MS spectra were obtained over a range *m/z* 50-400 by ramping DP and CE from 25-35 V and 15-30 eV respectively. The quadrupoles were set to unit resolution with the mass range covering the range of analytes expected. PI scan was used as a filter to select candidates of interest on-the-fly, once the selection criteria are satisfied, mass spectra are obtained by fixing the first quadrupole (Q1) for the *m/z* of the pseudomolecular ion which is then passed through to a collision cell and scanned through the appropriate mass range in Q3. The mass accuracy of the spectra obtained is based on

the limitations of the quadrupole. However, such error can be corrected for by the use of EPI scan that follows each survey scan. When EPI spectra are collected, Q3 is acting as a linear ion trap rather than being scanned out, thus, ions are stored for a predetermined amount of time before being expelled and recorded.

#### 3.2.6.3 Chromatographic conditions

A 5 µl aliquot of each standard was injected onto a Atlantis $C_{18}$ column (3 µm, 100 x 2.1 mm) operated at 40°C, with all the eluent from the LC column being directly transferred into the ion source of the MS without post column splitting. The elution solvents were [A] water containing 0.1% formic acid and [B] methanol containing 0.1% formic acid. The gradient profile started at 15% [B] (0.5 min), increased to 90% (6 min) and maintained at 90% [B] for 5 min.

### 3.2.7 $MS^n$ for Fragmentation Confirmation

Fragmentation confirmation was performed with a LTQ Velos dual pressure linear ion trap (Thermo, Hemel Hempstead, U.K.). The analyses on the Velos instrument were performed using heated electrospray ionization (h-ESI) in positive mode at sheath, auxiliary and sweep gas flows of 5, 1 and 0.2 respectively (arbitrary units). The capillary and source heater temperatures were set to 240 and 40°C respectively. The ion spray voltage was adjusted to 2.5 kV. Analysis of the 2M3H-Cys, 3M3SH-Cys-Gly, HMHA-Gln, and 3M2H-Gln standards at 10 µg/ml were carried out by direct infusion at a flow rate 5 µl/min into the ion source as well as the mobile phase flow rate of 250 µl/min to produce a stable signal.

### 3.2.8 Data Analysis

QTOF – Results obtained from LC-MS were analyzed by the Micromass MarkerLynx v4.1 application manager (Waters, UK); the retention time ($t_r$) and *m/z* data pair for each peak were detected by the software.

QTRAP – Results obtained from LC-MS/MS analysis were processed by Analyst v1.4 (Applied Biosystems).

## 3.3 Results and Discussion

The strategy undertaken for method development was three-fold. Firstly, a method was developed that would be capable of identifying metabolites on a global scale (to complement the NMR data) either *via* DIMS or LC-MS. This global approach was initially developed with the use of an artificial sweat matrix with qualitative assessments being made in order to highlight optimum conditions for further investigation. The optimum conditions would then be further tested by increasing the complexity of the sample to that of apocrine sweat produced from cell lines (see chapter four) in addition to being more representative of the actual sample type under investigation. Secondly, a LC-MS/MS method was developed that was capable of specifically screening for known and unknown odour precursors by exploiting their common structural similarities. Finally, a LC-MS/MS method was developed that can qualitatively and quantitatively identify known odour precursors.

### 3.3.1 Direct Injection Mass Spectrometry

DIMS provided a sensitive high throughput method without chromatographic separation, with the speed of analysis usually less than 5 min. A typical DIMS mass spectrum of the amino acids present in the artificial sweat matrix is depicted in Figure 3.3. It is evident that a broad range of metabolites can be readily detected, including all the amino acids as well as lipophilic metabolites. The higher mass ranges (i.e. lipid content) are the most intense metabolites detected. Furthermore, when analysing amino acid standards in the artificial sweat matrix, ion suppression was observed across all analytes, in particular alanine, glycine, serine, cysteine, tyrosine, asparagine, and glutamic acid. Ion suppression effects (also known as matrix effect (King *et al.*, 2000)) can be viewed as a competition for ionisation between molecules that have simultaneously been introduced into the MS source. The ion suppression effect has a dramatic effect on the reproducibility between samples as depicted in Figure 3.4, in which the average coefficient of variation (CV) for the amino acids

present in the pseudo sweat mixture 43.9%. In contrast to LC-MS, the CV is expected to be less than 15% for an optimised method (FDA, 2001). Furthermore, in-source fragmentation could be observed for leucine/isoleucine and valine, which could be tentatively assigned to the product ions *m/z* 86 and *m/z* 72 respectively. This fragmentation is due to the loss of $H_2O$ and CO leading to immonium ions $[Im]^+ = [R\text{-}CH{=}NH_2]^+$, where R is the residue of the amino acids (Petritis *et al.*, 2000; Qu *et al.*, 2002). Conversely, this approach would allow a variety of metabolites (e.g. lipids and organic acids) to be detected in one brief analysis and have been used in microbial (Goodacre *et al.*, 1999; Vaidyanathan *et al.*, 2002) and plant (Goodacre *et al.*, 2003; Mauri and Pietta, 2000) studies.

However, this approach cannot distinguish between isomeric compounds such as leucine/isoleucine. In order to distinguish between isomers and reduce ion suppression effects, separation through chromatographic means will need to be in place. Moreover, analytical separation would result in an increase in sensitivity and MS data quality due to the reduction in background noise.

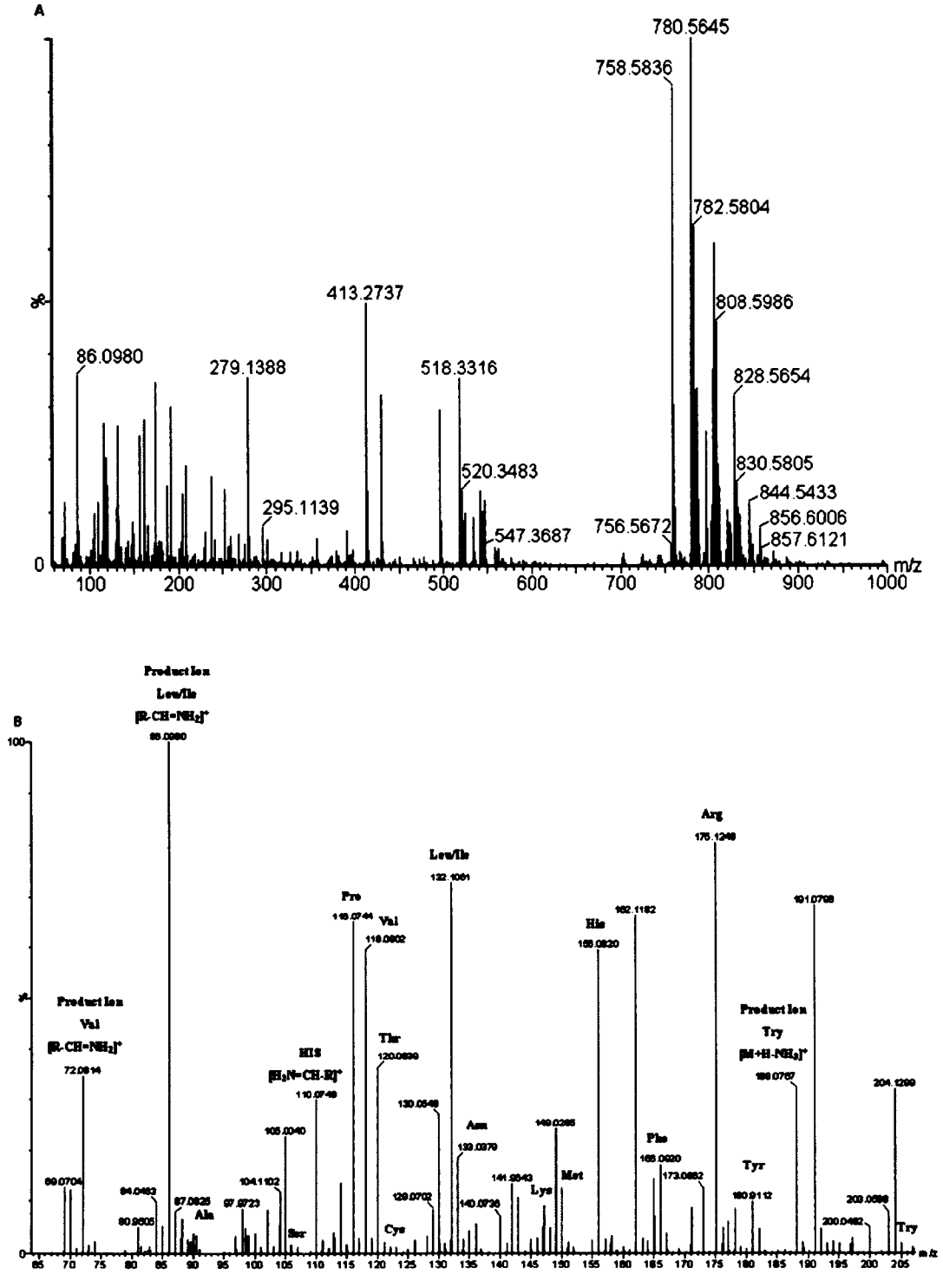



**Figure 3.3** DIMS mass spectra in positive mode **A** whole profile **B** range *m/z* 65-205 of artificial sweat matrix (amino acids content is at 40 μg/ml).

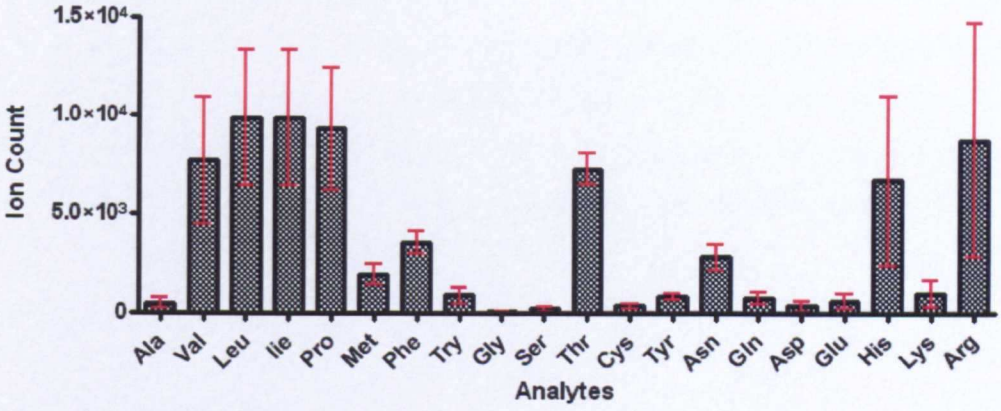



**Figure 3.4** DIMS reproducibility of the amino acid content present in artificial sweat matrix. The error bars represent the standard deviation ($n$= 5).

In conclusion, this approach would allow information-rich ESI-MS spectra from human apocrine samples to be generated without the need for chromatographic separation. However, the poor precision reported from this methodology suggests this method is suited to more of a screening approach rather than a quantitative method. The absence of chromatographic separation can be detrimental to mass accuracy arising from peak overlap with monisotopic differences of less than 0.025 Da (Dunn and Ellis, 2005) as well reducing the overall sensitivity arising from competitive ionisation (Mas *et al.*, 2007; Villas-Boas *et al.*, 2005). Furthermore, structural isomers have the same monisotopic mass and would require LC-MS to be detected separately. In addition, the classification of in-source fragmentation or adduct formation is complicated. In the literature, application of DIMS is mainly focused in the microbial (Castrillo *et al.*, 2003) and plant areas (Overy *et al.*, 2005), and when used in disease diagnostics, a MS/MS approach is taken (Rashed, 2001). Thus, this approach will no longer be pursued as it is not suitable to provide adequate identification of unknowns.

### 3.3.2 Global Profiling: HILIC-QTOF

As previously mentioned, the majority of the work published on apocrine sweat is predominately GC-MS based, focussing on volatile fatty acids (VFA). The limited LC-work that has been published is based on RP-HPLC systems (Emter and Natsch, 2008; Martin *et al.*, 2010; Natsch *et al.*, 2003; Troccaz *et al.*, 2009) leading to large

knowledge of non-polar metabolites. Thus, this work focuses on the developing a global method for the analysis of the more polar metabolites that are typically not well retained on a $C_{18}$ column. Systematic changes to the column, mobile phases, gradients, flow rates, and total analysis times were made in order to optimise the separation of the more polar compounds present in the pseudo sweat mixture.

### 3.3.2.1 Column Selection

Column selection depends largely on prior knowledge on the type of analyte, i.e. range of target compounds of interest. Thus, to evaluate the retention of polar metabolites in order to identify which type of column would be most suited for development, the amino acids content in the artificial sweat matrix were used. Amino acids represent a diverse range of polarities, thus, covering an adequate polarity range suitable for a global method development of polar to medium polar metabolites (Table 3.3). Initially, two columns were evaluated; a ZIC-HILIC column (5 µm, 150 x 2.1 mm) and a $C_{18}$ Synergi Hydro-RP 80Å, (4 µm, 150 x 2 mm) to identify which would be most suited for this application. The initial results obtained are illustrated in Table 3.3. The amino acids were poorly retained on the $C_{18}$ column, with the exception of Leu, Ile, Phe Try, and Tyr due to these particular analytes being more hydrophobic in nature. In comparison, the HILIC column was able to retain all the amino acids, which typically eluted around 5-8 min. The elution order from the HILIC column was generally from least polar to polar, which is opposite of traditional RP-LC. However, the basic amino acids, His, Lys, and Arg, were relatively less retained on the HILIC compared to the polar, non-polar or acidic amino acids, which eluted around 2-3 min. Thus, from this preliminary study, the $C_{18}$ column will be omitted from further development due to poor retention of the amino acids, while the HILIC column will be subjected to further development.

**Table 3.3** Retention time of standard compounds at 40 μg/ml on a HILIC and $C_{18}$ column.

| Name | Molecule | $pK_a$ of α-COOH Group | $pK_a$ of α-$NH_3^+$ Group | $pK_a$ of Ionizing Side Chain | Log P | MW | *m/z* (M+H) | HILIC | $C_{18}$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | RT/min | RT/min |
| *Nonpolar* | | | | | | | | | |
| Alanine | $C_3H_7NO_2$ | 2.3 | 9.7 | - | -2.77 | 89.09 | 90.0555 | 7.39 | - |
| Valine | $C_5H_{11}NO_2$ | 2.3 | 9.6 | - | -2.29 | 117.15 | 118.0868 | 5.36 | 1.40 |
| Leucine | $C_6H_{13}NO_2$ | 2.4 | 9.6 | - | -1.72 | 131.17 | 132.1024 | 3.84 | 3.15 |
| Isoleucine | $C_6H_{13}NO_2$ | 2.4 | 9.7 | - | -1.80 | 131.17 | 132.1024 | 4.96 | 3.15 |
| Proline | $C_5H_9NO_2$ | 2.0 | 10.6 | - | -2.62 | 115.13 | 116.0711 | 5.41 | 1.39 |
| Methionine | $C_5H_{11}NO_2S$ | 2.3 | 9.2 | - | -2.10 | 149.21 | 150.0589 | 4.57 | 1.45 |
| Phenylalanine | $C_9H_{11}NO_2$ | 1.8 | 9.1 | - | -1.44 | 165.19 | 166.0868 | 3.72 | 7.35 |
| Tryptophan | $C_{11}H_{12}N_2O_2$ | 2.4 | 9.4 | - | -1.15 | 205.09 | 205.0977 | 4.50 | 7.85 |
| *Uncharged polar* | | | | | | | | | |
| Glycine | $C_2H_5NO_2$ | 2.3 | 9.6 | - | -3.00 | 75.07 | 76.0398 | 8.09 | 1.15 |
| Serine | $C_3H_7NO_3$ | 2.2 | 9.2 | - | -3.00 | 105.09 | 106.0504 | 8.23 | 1.19 |
| Threonine | $C_4H_9NO_3$ | 2.6 | 10.4 | - | -2.83 | 119.12 | 120.0660 | 7.62 | 1.22 |
| Cysteine | $C_3H_7NO_2S$ | 1.8 | 10.8 | 8.3 | -2.55 | 121.16 | 122.0276 | 9.69 | 1.29 |
| Tyrosine | $C_9H_{11}NO_3$ | 2.2 | 9.6 | 10.1 | -2.11 | 181.19 | 182.0817 | 6.66 | 4.25 |
| Asparagine | $C_4H_8N_2O_3$ | 2.0 | 8.8 | - | -3.48 | 132.12 | 133.0613 | 8.30 | 1.19 |
| Glutamine | $C_5H_{10}N_2O_3$ | 2.2 | 9.1 | - | -3.11 | 146.14 | 147.0769 | 7.90 | 1.26 |
| *Acidic* | | | | | | | | | |
| Aspartic acid | $C_4H_7NO_4$ | 2.1 | 9.8 | 3.9 | -3.61 | 133.10 | 134.0453 | 8.60 | 1.21 |
| Glutamic acid | $C_5H_9NO_4$ | 2.2 | 9.7 | 4.2 | -3.51 | 147.13 | 148.0610 | 8.36 | 1.26 |
| *Basic* | | | | | | | | | |
| Histidine | $C_6H_9N_3O_2$ | 1.8 | 9.2 | 6.0 | -2.85 | 155.15 | 156.0773 | 3.09 | 1.11 |
| Lysine | $C_6H_{14}N_2O_2$ | 2.2 | 9.0 | 10.0 | -3.77 | 146.19 | 147.1130 | 2.17 | 1.05 |
| Arginine | $C_6H_{14}N_4O_2$ | 2.2 | 9.0 | 12.5 | -3.79 | 174.20 | 175.1195 | 2.12 | 1.13 |

Log P (Partition coefficient) of amino acids taken from Tayar and co-workers(Tayar *et al.*, 1992).

### 3.3.2.2 Solvent and Buffer Selection

Reviews of the relevant literature (Table 3.1) revealed that acetonitrile was the most effective solvent for the separation of polar/semi-polar compounds using HILIC; hence, mobile phase method development was based on this solvent (Alpert, 1990; Alpert *et al.*, 1993; Olsen, 2001; Schlichtherle-Cerny *et al.*, 2003; Valette *et al.*, 2004).

The buffers typically used for HILIC are either ammonium acetate or formate (5-20 mM) due to their compatibility with MS, solubility in high organic solvent and pH range from 3-8. Initially, binary solvent systems were evaluated as this overcomes some of the difficulties associated with retention time shifts due to column equilibration. The acetonitrile content or ammonium acetate concentration was separately altered between 70-90% and 10-30 mM respectively. As depicted in Figure 3.5 A, the organic content had a profound effect on retention, with increasing organic content resulting in longer retention of all amino acids, which is consistent with the literature (Guo and Gaiki, 2005; McCalley, 2010; Novakova *et al.*, 2008). Separation could be achieved with the use of high organic content, however, this would result in a long analytical run time (>40 min) due to the basic amino acids being well retained, which is considered unfavourable. In comparison, the change in salt concentration slightly increased the retention of all except the basic amino acids (Figure 3.5 B). A binary solvent system is not desirable, thus, a gradient profile was sought after in order to reduce the overall analytical runtime.

The effect of ionic strength and pH were then independently investigated through the use of gradient elution conditions. Initially, the ammonium acetate was varied between 1-80 mM as depicted in Figure 3.6 A-C. High salt concentrations resulted in a marginal increase in retention across all the amino acids, except the basic amino acid residues, His, Lys, and Arg, which decreased in retention. This observed effect is consistent with the HILIC mechanism (Bicker *et al.*, 2008; Lammerhofer *et al.*, 2008; Wu *et al.*, 2008) and the current literature (McCalley, 2010; Novakova *et al.*, 2008). The increase in salt concentration potentionally reduces the electrostatic repulsion,

since the sulfonate groups on the sulfobetaine stationary phase are negatively charged and prevent the carboxyl groups from interacting with the positively charged quaternary amine groups located closer to the silica surface (Guo *et al.*, 2007). Thus, as the salt concentration increases, the retention of basic compounds on the negatively charged sites are reduced, while the repulsion of acidic compounds from the same sites is reduced, thereby increasing the retention of the acidic metabolites.

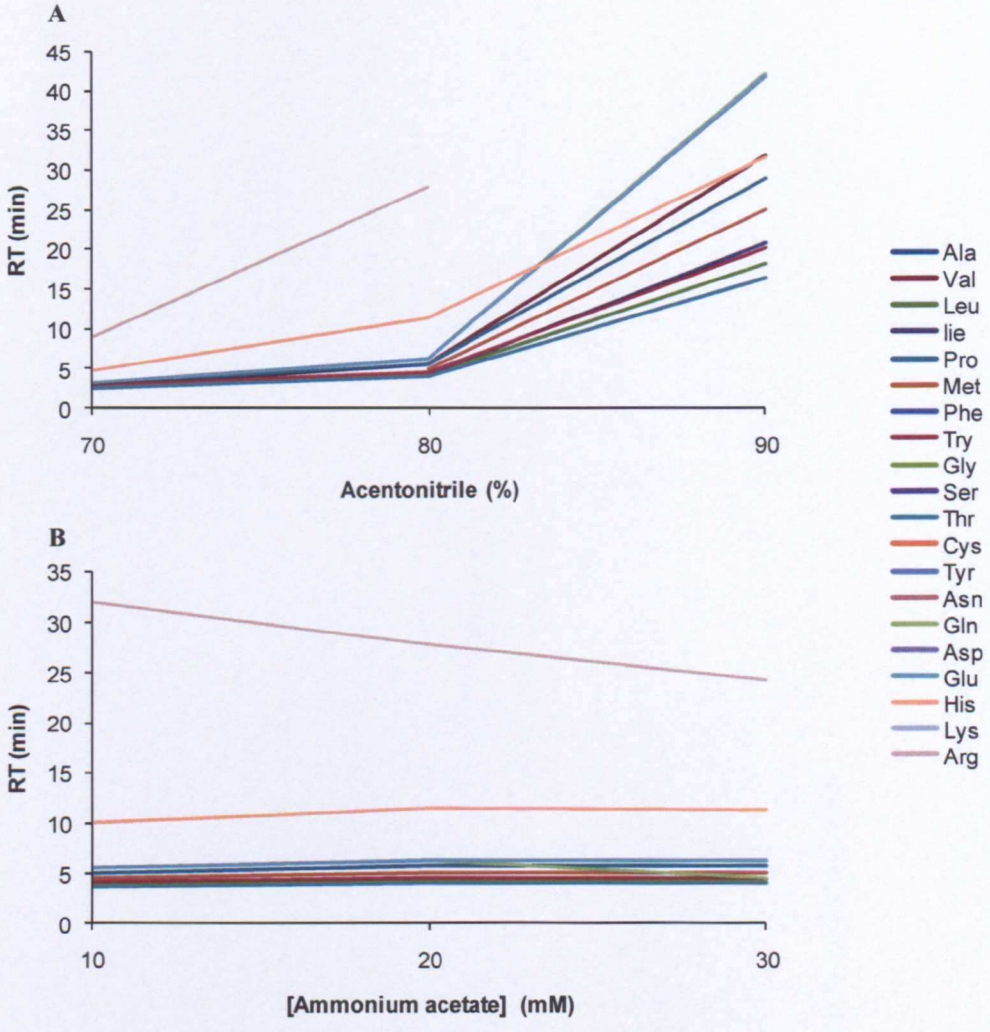



**Figure 3.5** Effect of **A** increasing organic content **B** salt concentration on amino acid retention time in a binary solvent system.

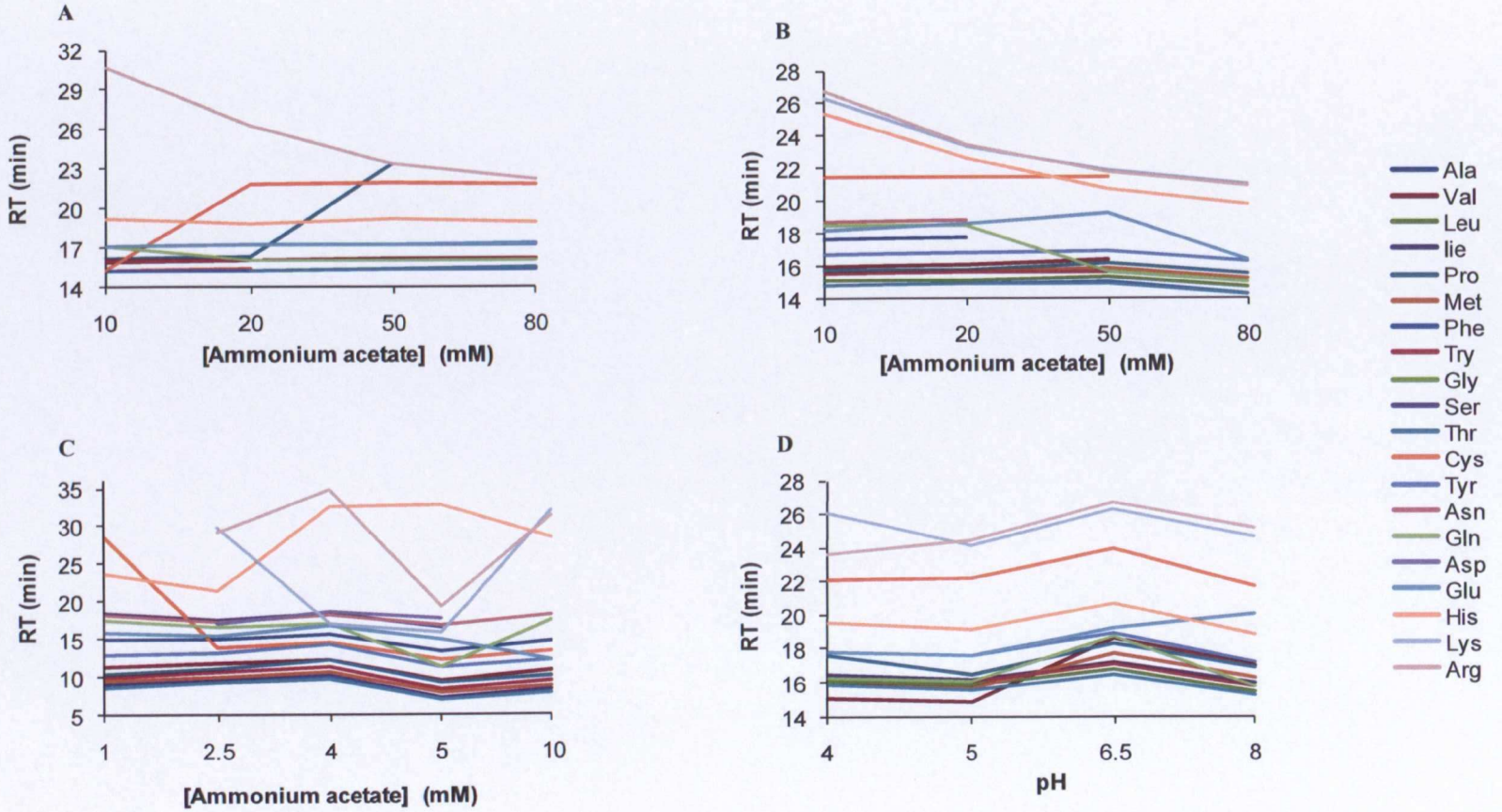



**Figure 3.6** Effect of salt concentration or pH on amino acid retention time. **A** and **B** Mobile phase [A] 100% acetonitrile or 100% acetonitrile + 0.1% formic acid respectively [B] 100% water with various concentration of $NH_4Ac$, **C** Mobile phase [A] 90% acetonitrile + 0.5 mM $NH_4Ac$ [B] 60% acetonitrile with various concentration of $NH_4Ac$, **D** Mobile phase [A] 95% acetonitrile + 10 mM $NH_4Ac$ [B] 100% water +10 mM $NH_4Ac$. pH change was consistent in both [A] and [B].

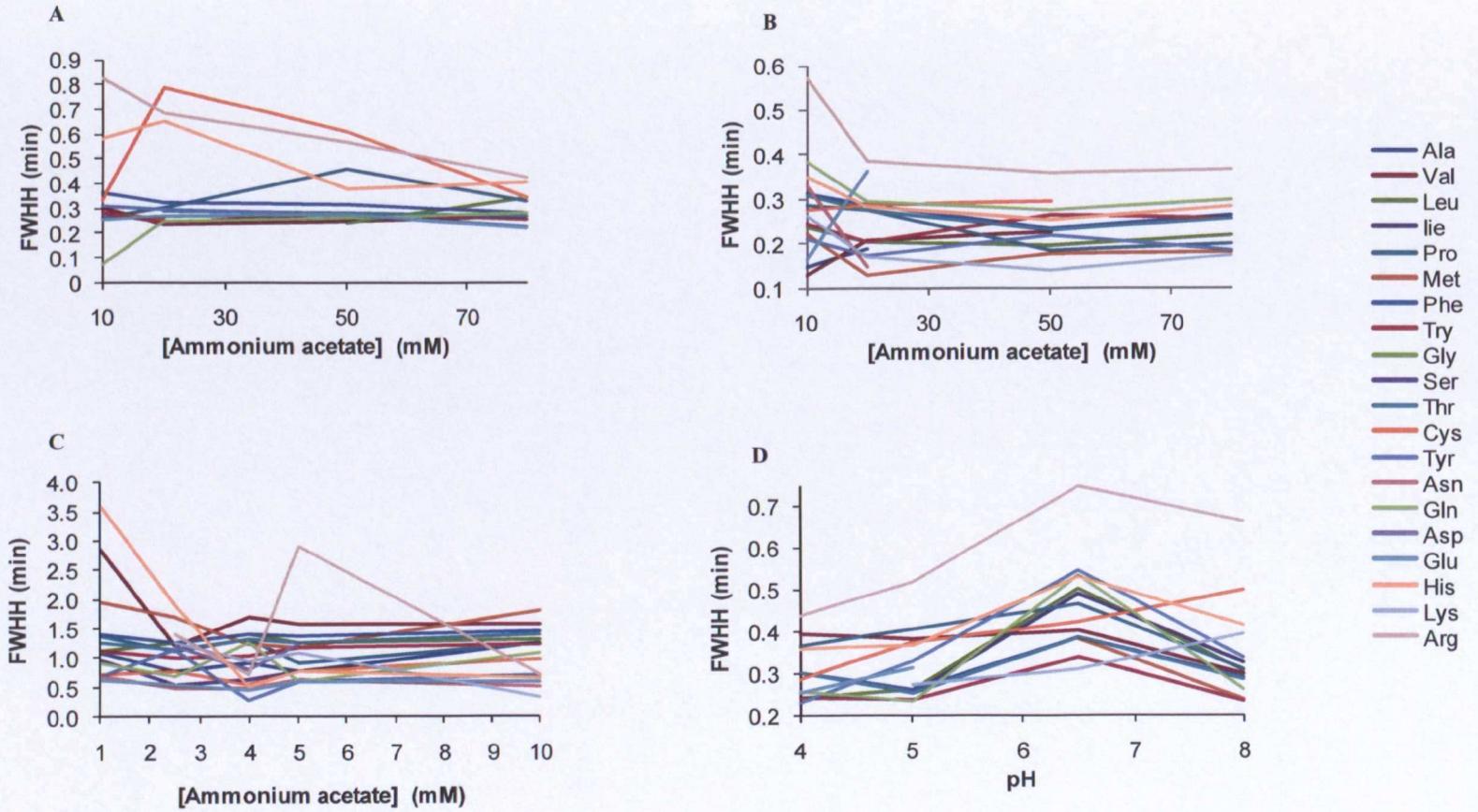



**Figure 3.7** Effect of salt concentration or pH on the amino acids peak width at half height. **A** and **B** Mobile phase [A] 100% acetonitrile or 100% acetonitrile + 0.1% formic acid respectively [B] 100% water with various concentration of $NH_4Ac$, **C** Mobile phase [A] 90% acetonitrile + 0.5 mM $NH_4Ac$ [B] 60% acetonitrile with various concentration of $NH_4Ac$, **D** Mobile phase [A] 95% acetonitrile + 10 mM $NH_4Ac$ [B] 100% water +10 mM $NH_4Ac$. pH change was consistent in both [A] and [B].

The effect of the mobile phase pH on the RP-HPLC is known to have a significant effect on the retention properties due to changes in the ionisation properties of the analyte. For example, a weak acid with a pKa value ~ 3 will be ionised at two pH units above or non-ionised two pH below, which will increase or decrease the retention respectively. Further changes in greater than two pH units either side of the pKa values will have minimal effect on retention because the ionisation of the analyte will remain unchanged (Snyder *et al.*, 1988). The effect of pH on HILIC separation was therefore investigated between pH 4-8, by altering stock salt concentration before mixing with acetonitrile. The absolute pH of the mobile phase was not directly measured and would presumably deviate from that of the stock solution (Barbosa and Sanznebot, 1994). Figure 3.6 D summarise the effect of altering pH on the HILIC retention of amino acids. The pKa values of the amino acids are summarised in Table 3.3. The COOH group and $NH_3$ group of all the amino acids will be ionised between pH 3-8. Only the ionisation state of the Asp, Glu, and His side chain will change under the experimental pH conditions. As depicted in Figure 3.7 D, the retention time essentially remained unchanged, with all the amino acids behaving in similar way. Thus, it is likely that the ion exchange effect is contributing to the overall retention, thereby, masking any pH effect. This is similar to Guo and co-workers (Guo and Gaiki, 2005) were they illustrated that pH had a minimal effect on retention when analysing salicylic acid, cytosine and cytidine, however, the retention of aspirin gradually decreased when reducing the pH.

The effect of ionic strength and pH on the peak width (FWHH), which is an indication of peak sharpness and, in general, an indication of column efficiency, is depicted in Figure 3.7. A change in pH was shown to have a marginal effect on FWHH. When the pH was increased to 6.5, FWHH tended to get broader across all the amino acids. In contrast to the salt concentration, peak width remained relatively constant, except in Figure 3.7 A, in which an increase in salt concentration reduced FWHH. This is likely due to the pH of the mobile phase under these conditions, which would be more basic as formic acid was not added to mobile phase [A].

It was concluded from these studies that 10 mM ammonium acetate at pH 5 would be the optimum conditions. Lower buffer concentrations are more favourable for MS detection and an acidic pH value resulted in an increase in peak efficiency. Figure 3.8

A and B represents extracted ion chromatograms of the amino acids standards and amino acids from artificial sweat matrix on a ZIC-HILIC column obtained from the optimum conditions, which ultimately will be used for the analysis of apocrine gland secretions. Not all peaks were fully resolved or contain a Gaussian peak shape (i.e. good chromatographic peak shapes). The basic amino acids, typically in the artificial sweat matrix, were more difficult to detect. This is most likely due to the poor peak shapes eluting over a longer periods of time and ion suppression effects from co-eluting metabolites. However, overall a good compromise between the broad ranges of polarities have been achieved within a 25 min runtime.

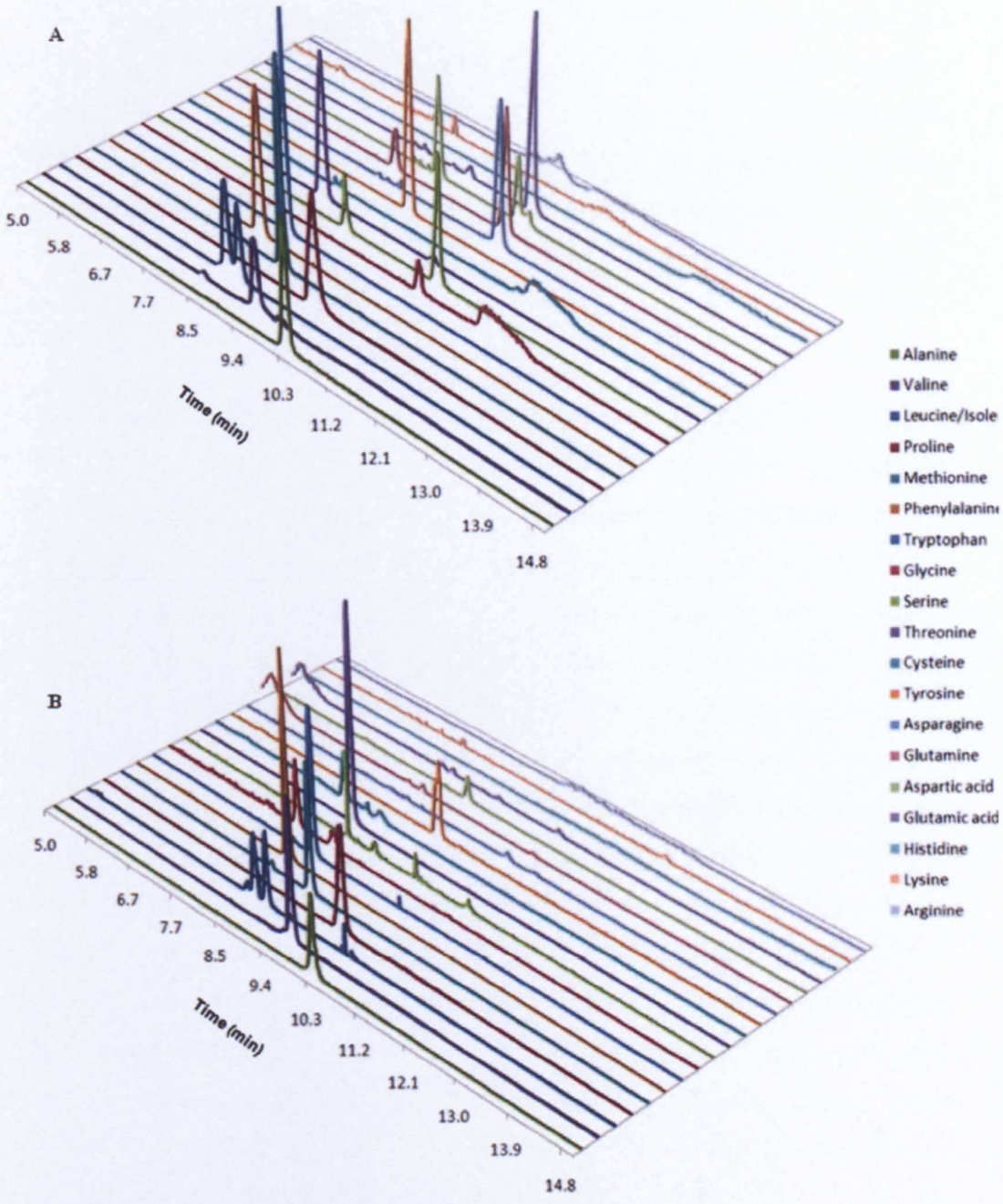



**Figure 3.8** 3D extracted ion chromatogram of **A** amino standards **B** amino standards present in the pseudo sweat mixture.

In summary, the HILIC method shows a good alternative in providing retention of polar metabolites compared to that of RP-HPLC. In contrast to the nature of and amount of organic modifier, both the investigated ranges of salt concentration and pH were found to have little effect compared to the amount of water present. This indicates considerable stability of the elution conditions for this stationary phase, which is desirable for retention time stability in metabolomics analysis.

### 3.3.3 Targeted LC-MS/MS

By employing the QTRAP® instrument and exploiting its unique scan capabilities (Hager, 2002; Hager and Le Blanc, 2003) it was possible to devise an analytical strategy capable of obtaining quantitative and qualitative data of metabolites in complex matrices as well as specifically tailoring for the survey of common structural moieties. The survey scans are generally based on pre-defined selection criteria of either a common precursor ion or a common natural loss which is processed "on-the-fly" in order to determine the pseudomolecular ion from a single injection. Once the selection criteria are satisfied, an enhanced product ion (EPI) scan is triggered to produce spectra which give full structural information. An enhanced resolution (ER) scan can also be trigged before acquisition of the EPI scan, in order to confirm the *m/z* value of the unknown pseudomolecular ion. However, when analysing complex mixture, the pseudomolecular ion might not be the most intense peak during that scan cycle resulting in the wrong ion being selected for EPI scan, thus, this function was removed. Nonetheless, three types of scans can be generated in a single cycle time that is very much lower than conventional triple-quadrupole (Q-q-Q) mass spectrometers and when the QTRAP® is switched to operate as an ion trap, higher quality spectra are obtained compared to that of a conventional Q-q-Q mass spectrometers.

### 3.3.3.1 Optimization of the Declustering Potential and Collision Energy

The MRM optimisation procedure involves a sequence of experiments where the voltages of the various ion optics parameters (i.e. DP and CE) are ramped to determine the maximum signal intensity for each ion. The optimum DP for each standard was obtained by direct infusion while systematically increasing the DP from 0 to 120 V. Optimum values for each of the standards were around 30 V. The CE was ramped between 5 and 100 V with the intensity of the amino acid product ion *m/z* 147 (Gln), *m/z* 105 (Cys) and *m/z* 179 (Cys-Gly) being plotted with each of the standards producing a similar maximum of around 20 V as summarised in Table 3.4.

**Table 3.4** Retention times and optimised declustering potential and collision energies for the odour precursor standards.

| Standard | Retention Time (min) | Precursor Ion (*m/z*) | Product Ion (*m/z*) | Declustering Potential (V) | Collision Energy (eV) |
|---|---|---|---|---|---|
| 2M3H-Cys | 3.2 | 208.1 | 105.1 | 40 | 20 |
| 3M3SH-Cys-Gly | 4.9 | 293.1 | 179.1 | 30 | 20 |
| HMHA-Gln | 5.2 | 275.1 | 147.1 | 30 | 25 |
| 3M2H-Gln | 6.5 | 257.1 | 147.1 | 30 | 20 |

### 3.3.3.2 LC-MS/MS Analysis of Odour Precursor Standard Compounds

*MS Production Fragmentation*

MS/MS transitions were generated as a result of the selective determination of the pseudomolecular ion and the most abundant product ion for each available amino acid conjugate standard. The literature is currently limited with respect to MRM analysis of odour precursors, and the LC-MS work that has been published is focused on single ion monitoring (SIM) (Troccaz *et al.*, 2009), with the majority of published work being with GC-MS. For the odour precursors for which there are no standards available, theoretical transitions were used based on fragmentation patterns from standards. Any co-eluting compounds were detected by defining a time in which the transition was excluded (dynamic exclusion) after acquiring the EPI scan. Figure 3.9 shows the EPI spectra of the four standards while their respective chromatographic separation is depicted in Figure 3.10. Source-dependent parameters optimised for

MRM remain the same for EPI scans. However, the DP and CE were ramped during the EPI scan cycle from 25-45 V and 15-30 V respectively. This was in order to obtain full fragmentation profiles of each standard to aid identification. It was experimentally observed that the mass accuracy of the EPI scan was usually within either 0.2 or 0.1 amu of the expected value. These EPI spectra are helpful in the confirmation of structural identification of amino acid conjugates in a complex biological sample. To my knowledge, this is the first work that presents full product ion spectra of any of the amino acid conjugates.

The elucidation of fragmentation mechanisms was not the main aim of this project, hence, fragmentation confirmation was only carried out on the available standards with the use of the $MS^n$. Confirmation of the fragmentation behaviour will provide additional information which will be later used when analysing data obtained from the PI survey scanning methodology. The 2M3H-Cys conjugate (*m/z* 208) contained fragments *m/z* 191, 105, and 87 (see Figure 3.9 A). The fragment at *m/z* 191 is consistent with a loss of OH, and *m/z* 105 is interpreted as cysteine residue $[M+H-NH_3]^+$ which can be further broken down to form *m/z* 87 $[105-H_2O]$ as well as coming from 2-methyl-3-sulfanylbutan-1-ol with the loss of $H_2S$ (*m/z* 34). The collisionally activated dissociation of the amino acid $[M+H]^+$ ions have been observed by other ionisation methods and the mechanisms of their formation has been explained (Biemann and McCloskey, 1962; Bouchonnet *et al.*, 1992; Dookeran *et al.*, 1996; Kulik and Heerma, 1988; Petritis *et al.*, 2000).

The 3M2H-Gln conjugate (*m/z* 257) contained fragments *m/z* 240, 147, 129 and 111 (see Figure 3.9 B). The fragment at *m/z* 240 was consistent with the loss of OH and can further fragmented to produce fragments of *m/z* 130 and 111 which are interpreted as the loss of glutamine $[M+H-NH_3]^+$ and 3-methylhex-2-enoic acid respectively. The peak at *m/z* 147 is considered as glutamine residue $[M+H]^+$ and can further be fragmented to produce an *m/z* 130 through a loss of $NH_3$. The peak at 129 is also interpreted as glutamine residue $[M+H-H_2O]^+$ and in turn can be further fragmented to form *m/z* 83 $[Im-NH_3]^+$.

The 3M3SH-Cys-Gly conjugate (*m/z* 293) contained fragments *m/z* 179, 162, 144, and 116 (see Figure 3.9 C). The fragment at *m/z* 179 was consistent with the loss of

Cys-Gly residue. The fragment of *m/z* 162 and 144 was consistent with the loss OH and a combined loss of OH from $NH_3$ respectively, from the Cys-Gly residue. The peak at *m/z* 116 is considered as the 3M3SH with the loss of $H_2S$ (*m/z* 34).

The HMHA-Gln conjugate (*m/z* 275) contained fragments of *m/z* 257, 240, 147, 130, and 111 (see Figure 3.9 D). The fragment of *m/z* 257 and 240 is consistent with a loss of OH and a combined loss of OH and $NH_3$ from 3M2H and Gln respectively. The peak at *m/z* 147 and 130 is considered as glutamine residue $[M+H]^+$ and $[M+H-NH_3]^+$ respectively.

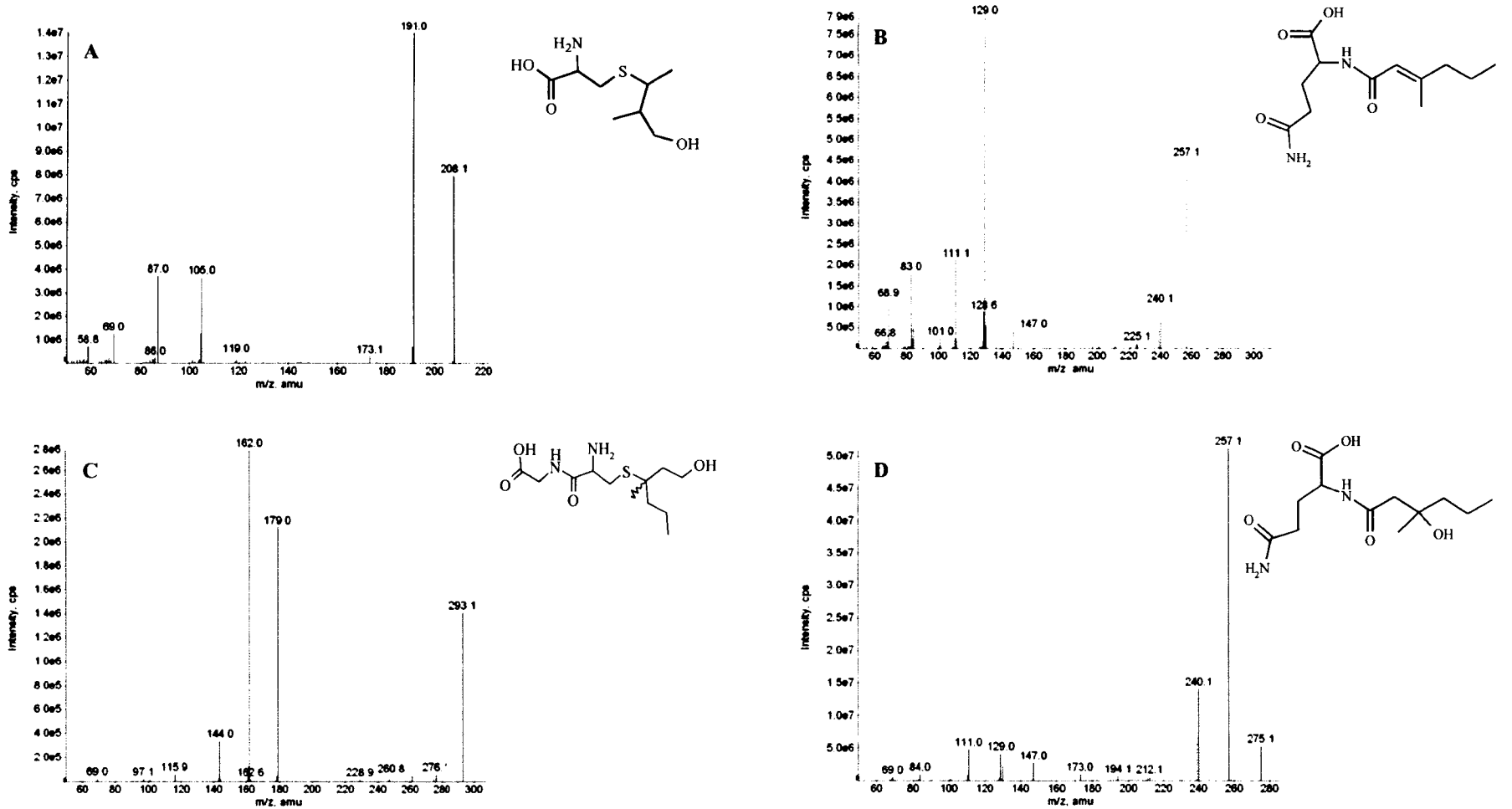



**Figure 3.9** EPI spectra of **A** 2M3H-Cys **B** 3M2H-Gln **C** 3M3SH-Cys-Gly **D** HMHA-Gln conjugate obtained using MRM as a trigger.

*LC-MS/MS Analysis of the Conjugates*

The analytical method was initially optimised with four odour precursors (3M2H-Gln, HMHA-Gln, 2M3H-Cys and 3M3SH-Cys-Gly) which were purchased *via* custom synthesis from Unilever (Bebington, UK). The chromatographic method was initially modified from the work published by Troccaz and co-workers (Troccaz *et al.*, 2009). The racemic odour precursors were prepared at a concentration of 1 µg/ml and injected on to the HPLC column, while the MS was in MRM mode. Figure 3.10 shows a typical extracted ion chromatogram of the optimal chromatographic conditions, with an elution profile of 2M3H-Cys conjugate (3.2 min), 3M3SH-Cys-Gly conjugate (4.9 min), HMHA-Gln conjugate (5.2 min) and 3M2H-Gln conjugate (6.5 min). The diastereoisomers of Cys conjugate at 3.2 min showed peak splitting which previously have been reported to co-elute (Troccaz *et al.*, 2009). This was confirmed by full MS/MS product ion scanning, which produced identical fragmentation patterns. Modifying the pH of the mobile phase affected the intensity of each standard with minimal effect on the peak splitting of the 2M3H-Cys conjugate. Experiments comparing methanol and acetonitrile as the organic mobile phases resulted in methanol providing improved retention of the 2M3H-Cys conjugate.

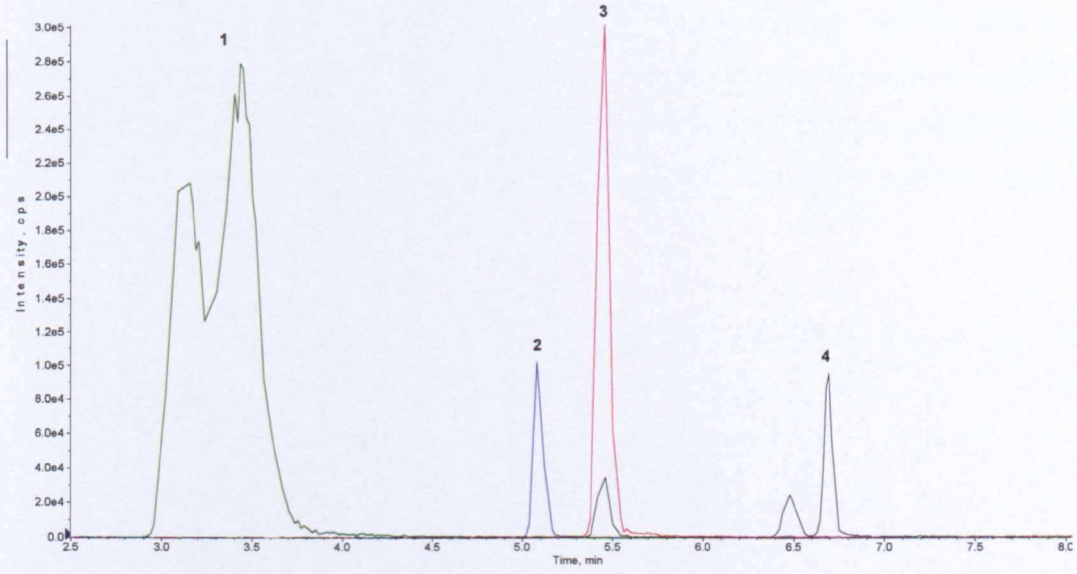



**Figure 3.10** Extracted ion chromatogram of **1)** 2M3H-Cys **2)** 3M3SH-Cys-Gly **3)** HMHA-Gln **4)** 3M2H-Gln conjugate reference standards containing 1 µg/ml of each precursor.

### 3.3.3.3 LC-MS/MS profiling of Odour Precursors using Precursor Ion Survey Scan

As depicted in Figure 3.11 - Figure 3.13, the PI scanning approach was able to detect each of the standards at the correct retention time compared with the verified LC-MS/MS targeted analysis and were positively identified using the EPI spectra. As noted previously in Section 3.3.3.2, 2M3H-Cys conjugate has a double peak (see Figure 3.11), where each peak produces similar EPI spectra as previously mentioned. Figure 3.12 also shows a double peak in the extracted ion chromatogram; both peaks produce a similar EPI spectrum suggesting that one peak may arise from an impurity present in the synthetic standard. The EPI spectra obtained from PI scanning were similar to that produced in the targeted MS/MS approach (see Figure 3.9), thereby, allowing identification of odour precursors without making assumptions of their absolute identity. Nonetheless, there were limitations to this scanning method. The EPI threshold was set higher than that of the LC-MS/MS method by an order of magnitude. This was to allow high quality spectra to be obtained at the expense of detecting conjugates at a low concentration range as well as limiting false positives. Furthermore, this method is not as sensitive as MRM analysis by at least one order of magnitude. This is due to compromises in the CE and DP being made in order to cover a range of metabolites, longer scan times (s instead of ms) resulting in fewer data points across any one peak, and an increase in background ions. However, any peaks of interested that have been detected can be added to the MRM methodology in order to increase the sensitivity.

In summary, the precursor ion scan coupled with full product ion spectra has the potential to identify common structural moieties of the odour precursors in order to identify possible new conjugates in biological extracts. Once new compounds have been identified by the precursor ion approach, then precursor and product ion can be used to monitor for these new conjugates in a biological extract.

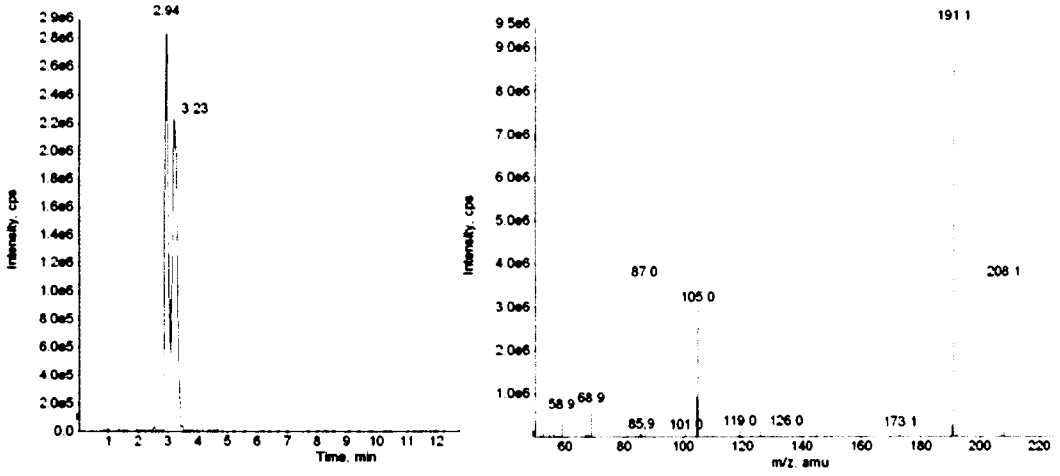



**Figure 3.11** Extracted ion chromatogram of 2M3H-Cys (*m/z* 208) and EPI spectrum of the peak at 3.2 min from an EPI triggered PI scan of *m/z* 105. Both peaks produce similar EPI spectra.

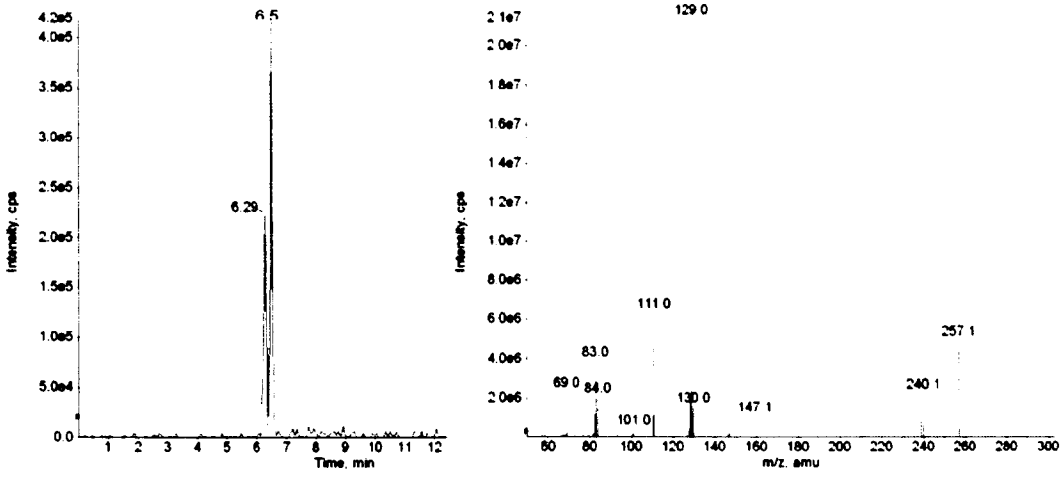



**Figure 3.12** Extracted ion chromatogram of 3M2H-Gln (*m/z* 257) and EPI spectrum of the peak at 6.5 min from an EPI triggered PI scan of *m/z* 147.

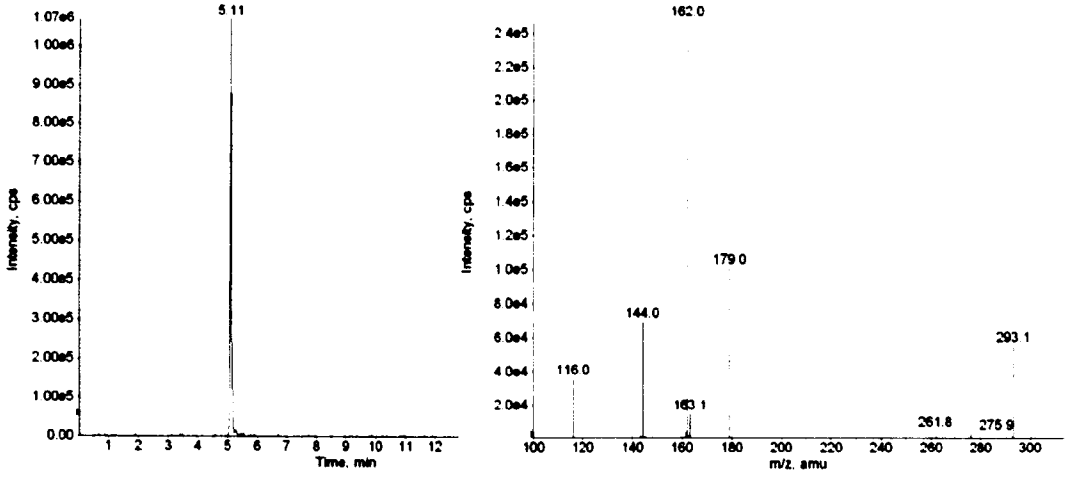



**Figure 3.13** Extracted ion chromatogram of 3M3SH-Cys-Gly (*m/z* 293) and EPI spectrum of the peak at 5.1 min from an EPI triggered PI scan of *m/z* 179.

## 3.4 Conclusions

The choice of analytical methodology to pursue is highly dependent on the level of chemical information required about the metabolites of interest as highlighted in Table 3.5. Currently, there is no one analytical technique, i.e. 'gold standard', capable of providing all the information required for analysis of apocrine sweat samples. Thus, a range of methods have been developed to provide complementary information on metabolites in apocrine sweat samples.

The LC-MS(MS) methodologies developed herein represent a significant step forward in providing identification for the full range of metabolites present in apocrine sweat samples as a whole, as well as specifically identifying and quantifying odour precursors. Although there is a large body of literature on the analysis of apocrine sweat, these are predominantly GC-MS based methods providing identities of only the volatile components present. To my knowledge there has been no global or MRM LC-MS work published to date, adding to the uniqueness of the present work. The DIMS approach did provide quick analysis times; however, the data obtained would be too complex and insufficient in providing metabolite identification, thus, will no longer be pursued.

The LC-MS(MS) methodologies have been developed using artificial sweat mixture or standard precursors. These methods will be further tested on samples that are more representative of that of apocrine sweat, which will be discussed in the following chapter.

Overall, the global MS method will provide complementary information to that of the NMR data, and with the use of a high mass accuracy instrument, empirical formula and tentative identification can be found. The MRM and precursor ion scanning-IDA-EPI approach carried out in this chapter provides informative product ion spectra which will provide a useful tool for structural confirmation. Furthermore, by monitoring for common structural moieties, the semi-targeted method is no longer restricted to the availability of standards.

**Table 3.5** Summary of the analytical conditions that have been developed.

| | Global Profiling | | Broad-profiling | Semi/Targeted |
|---|---|---|---|---|
| | NMR | DIMS | HILIC-MS for profiling polar metabolites | LC-MS/MS for amino acid conjugates |
| Chemical Information | Gives detailed structural information, particularly using 2-D NMR of isolated metabolites | Qualitative and not quantitative. Inability to distinguish between isomeric compounds | High mass accuracy (5-10 ppm) for empirical formulae generation | Characteristic fragment-ion information that is related to chemical structure |
| Chemical Bias | These methods have little chemical bias (high conc metabolites) and can be used directly on the sample | Solvent bias means it is usually more applicable to polar compounds | Solvent bias means it is usually more applicable to polar compounds | Bias to the analytes of interest i.e. odour precursors |
| Speed | 25 min | 3 min | 25 min | 18 min |
| Reproducibility | Minimal variation (<5% RSD) | Matrix and Ion suppression effects vary | Reequilibration, temperature and number of injections can all cause chromatographic variation | Reequilibration, temperature and number of injections can all cause chromatographic variation |
| Sample Size | 8 µl | 5 µl | 5 µl | 5 µl |
| Limit of Detection | Low µM concentrations | < µM concentrations | < µM concentrations | < µM concentrations |
| Advantages | non-biased detector, non-invasive, highly reproducible, relatively easy to identify metabolites | Rapid screen of metabolites, minimal sample clean up | High sensitivity, average to high chromatographic resolution, empirical formula generation | High sensitivity, structural information |
| Disadvantages | Lower sensitivity than MS | Matrix effects, identification of metabolites require MS/MS | Identification of unknown is labour intensive, limited structural information, more costly than NMR | Ion suppression effects, more costly than NMR, requires *a priory* knowledge of analytes |

# Chapter 4

## 4 Application of $^{1}H$ NMR Spectroscopy and LC-MS to an *In-Vitro* Model of Apocrine Sweat Produced from ASG5 Cell Lines

### 4.1 Introduction

Apocrine glands secrete a turbid fluid comprised of water, electrolytes, fatty acids, steroids, lipids, proteins and nitrogen metabolites such as ammonium and urea (Gower *et al.*, 1986; Zernecke *et al.*, 2010). When exposed to micro-flora populations present on the skin, the lipidic solutes, including steroids and short chain fatty acids, are transformed to malodorous compounds. Apocrine sweat is produced in low volumes, typically less than 5 μl, and secreted into the hair follicle, after which, there is a lag time of 24-48 h before further apocrine sweat can be produced. Apocrine sweat is both difficult to collect and very costly. Hence, a practical way of investigating apocrine secretions is to use a long-term proliferating ASG5 cell line (Burry *et al.*, 2008), that closely mimic the function of an apocrine gland *in situ.* The development of such an *in vitro* model has potential benefits not only in providing a suitable matrix for future method development, but potentially in assisting in the identification of transport secretary mechanisms, screening of malodorous compounds and determining effective concentrations of metabolites present.

The morphology of the ASG5 apocrine cell line are depicted in Figure 4.1, whereby, microvilli (M) and apical blebs (A) are present at the luminal membrane, as well as secretary granules (S) being present throughout the cytoplasm. The typical pinching off (mode of secretion) is also observed (Figure 4.1 B and C). This data is comparable to apocrine secretary morphology *in vivo* (Montagna *et al.*, 1953).

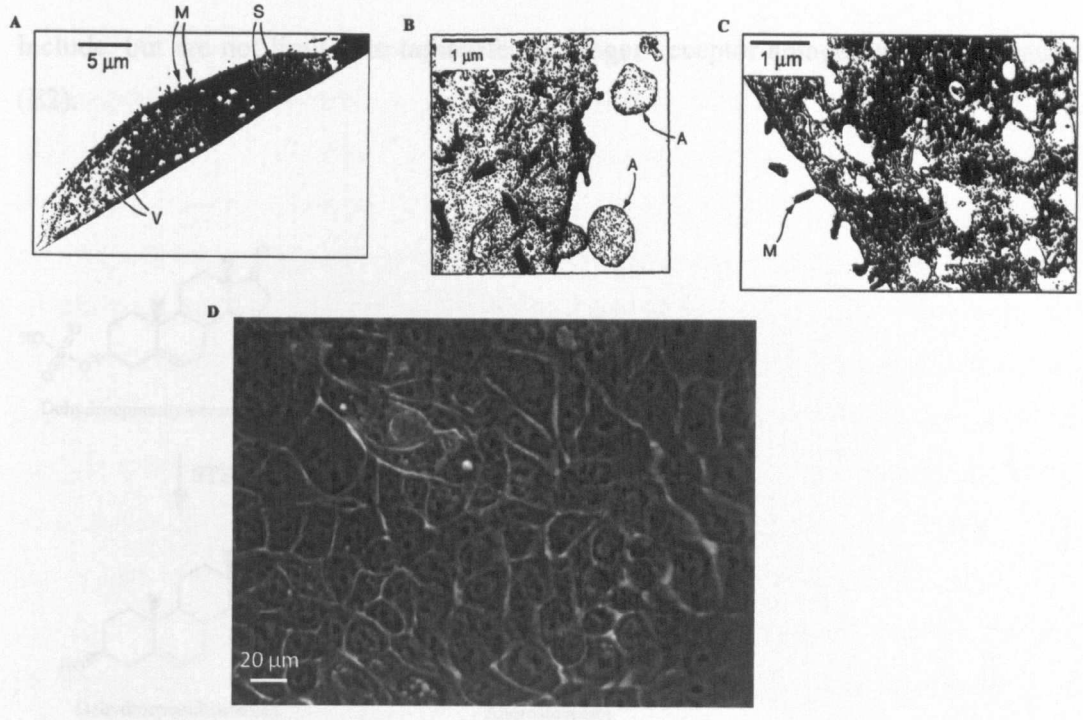



**Figure 4.1** Ultrastructure of an ASG5 apocrine cell using 200 hexagonal copper grids for transmission electron microscopy. Microvilli (M), apical blebs (A), secretary granules (S) and empty vesicles (V). Reproduced from (Burry *et al.*, 2008).

Apocrine glands do not begin to function until puberty, thus, it is thought that androgens (sex steroid hormones) play some part in the regulation of apocrine gland activity due to the presence of androgen receptors (Beier *et al.*, 2005; Labrie, 1991). This is further supported by the fact that cholesterol biosynthesis is upregulated in the presence of androgens (Heemers *et al.*, 2003), which could be used as a precursor for the production of volatile steroids (Cowley and Brooksbank, 1991; Grosser *et al.*, 2000). The ASG5 cell line possesses the genes required for androgen and estrogen synthesis, in addition to necessary β receptors required to mediate the response. Furthermore, the AGS5 cell line expresses the *ABCC11* gene, as well as the apoD, which is known to be associated with cellular secretary processes (Martin *et al.*, 2010; Spielman *et al.*, 1998; Spielman *et al.*, 1995; Zeng *et al.*, 1996b). The ASG5 cell line possesses the GG phenotype (538G→A SNP), that is associated with wet ear wax type (Yoshiura *et al.*, 2006) and increased levels of axillary odour. The ASG5 cell line also retains many of the steroidogenic features present *in vivo* secretions as depicted in Figure 4.2 (Burry *et al.*, 2008). Thus, production of malodorous compounds and odour precursors, as well as cell proliferation, would be expected to be compromised by compounds known to interfere with the androgen pathway. Such compounds

include, but are not limited to tamoxifen (estrogen receptor antagonist) or β-estradiol (E2).

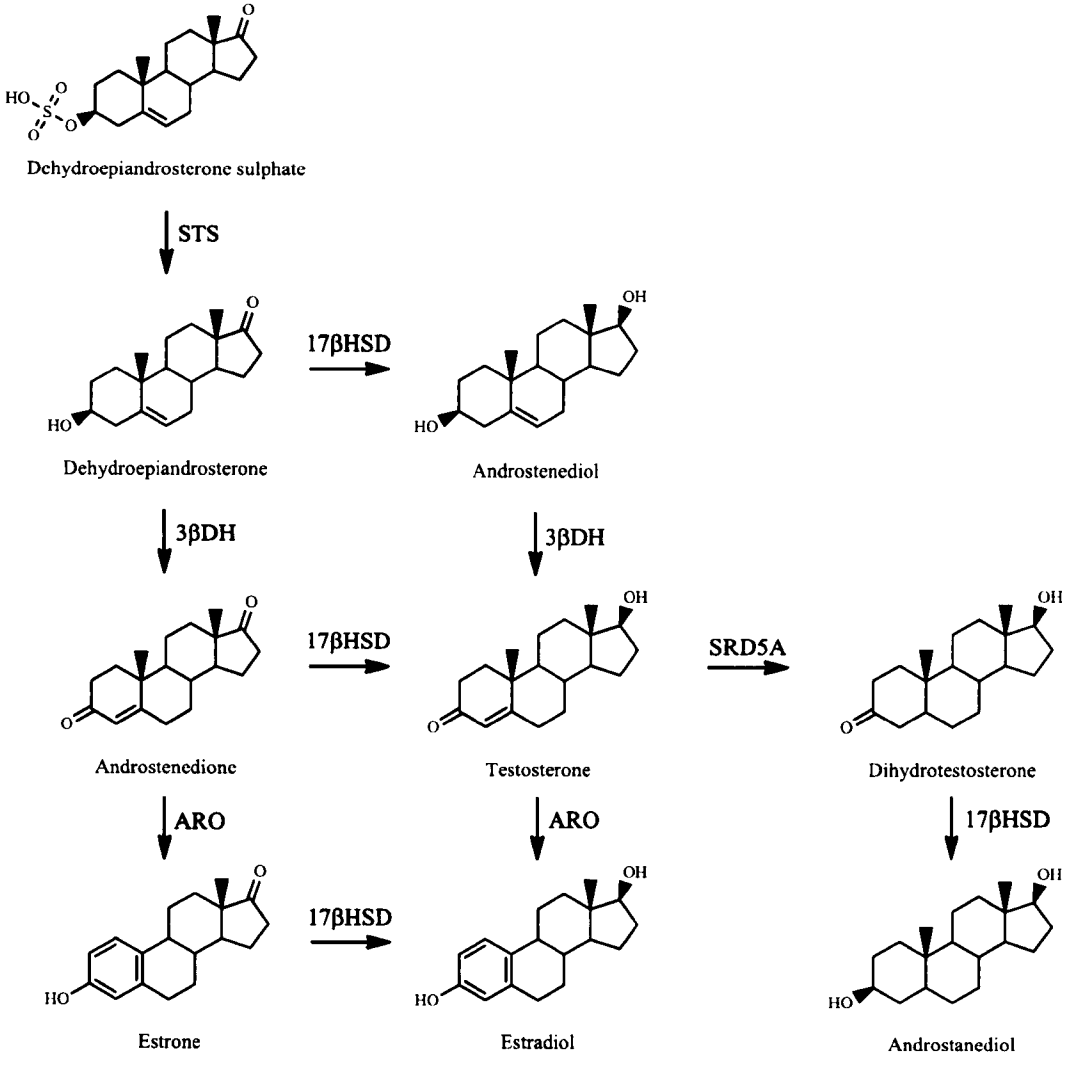



**Figure 4.2** Schematic of the steroid synthesis pathway present in ASG5 apocrine cell lines. Key: STS (steroid sulphatase), HSD (hydroxysteroid dehydrogenase), DH (dehydrogenase), ARO (aromatase) and SRD5A (5α-reductase). Reproduced with modifications (Burry *et al.*, 2008).

### 4.1.1 Metabolite Extraction from ASG5 cells

Prior to analysis with the methodologies developed in chapters two and three, the cellular metabolites have to be extracted. Ideally, the extraction method should extract

the largest number of metabolites, be nonselective, nondestructive or modify metabolites through chemical or physical means (Maharjan and Ferenci, 2003). However, there is no ideal technique to simultaneously extract all classes of metabolites, due to their high variability in the chemical and physical properties, thus, a variety of extraction methods have been developed. For example, perchloric acid is widely used to precipitate proteins and extract hydrophilic metabolites. Although advantageous for extracting amines, acidic treatment can have detrimental effects on the structural stability of metabolites (Lin *et al.*, 2007) and can directly interfere with analytical methods. Polar organic solvents (methanol, ethanol or acetonitrile) on the other hand, are typically mixed with water to extract hydrophilic metabolites, while chloroform is used to extract hydrophobic metabolites (Lin *et al.*, 2007). Given that the optimal extraction method will be essential in metabolome studies, several protocols have been developed with varying levels of success. The commonly used methods involve hot (90°C) ethanol (Tweeddale *et al.*, 1998), hot (70°C) methanol (Shryock *et al.*, 1986), cold (-40°C) methanol, perchloric acid (Shryock *et al.*, 1986), alkali (KOH) (Gonzalez *et al.*, 1997), and methanol/chloroform (Dekoning and Vandam, 1992). There have been several studies comparing the different types of extraction methods (see (Faijes *et al.*, 2007; Lin *et al.*, 2007)). Maharjan and co-workers (Maharjan and Ferenci, 2003) considered the problems associated with the different extraction protocols and concluded that the cold methanol protocol has distinct advantages over the other methodologies. Since the extraction is carried out at -40°C, stability and enzyme quenching minimise any metabolite transformations. Moreover, this method has all the advantages of hot methanol or ethanol protocols; denaturing and precipitating proteins and polysaccharides, no salts are added, easy to evaporate or concentrate extracts and minimal effect on pH.

At present, there is limited research available with regards to the use of ASG5 cell lines in metabolomic studies. The work that has been presented focuses on GC-MS as the primary analytical tool for the analysis of metabolites present in the ASG5 cell lines (Burry *et al.*, 2008). For the first time, NMR or LC-MS methodologies will be applied to analyse the metabolic composition the ASG5 apocrine cell lines. The developed analytical methodologies in both chapter two and three will have the potential to provide further insight into the apocrine cellular metabolism, as well as

identifying if male and female apocrine glands have the potential to behave differently due to the differences in circulating hormones. The use of applying multiple analytical platforms for metabolomic profiling of biological samples is gaining precedence (Hodson *et al.*, 2007; Lanza *et al.*, 2010; Lenz *et al.*, 2004; Lenz *et al.*, 2007; Williams *et al.*, 2005a), as it provides a better strategy for covering, detecting and identifying metabolites than relying on any single technique alone.

### 4.1.2 Aims

- Evaluate the analytical methodologies developed in chapters two and three by identifying whether each technique can discriminate between tamoxifen (estrogen receptor antagonist) or β-estradiol treated cell lines.

- Identify metabolites in $^{1}H$ NMR spectra which could be used as a reference for identifying metabolites from human axillary secretions.

## 4.2 Material and Methods

### 4.2.1 Sample Preparation

#### 4.2.1.1 Cell Culture

Cell culture and preparation of cell pellets was undertaken by Dr Mark Harker and co-workers at Unilever (Port Sunlight, UK). Initially, a long-term proliferating cell line derived from axillary apocrine glands (ASG5) was generated as described previously (Burry *et al.*, 2008). ASG5 cells passage number 47 or 48 were grown in 15 ml Mammary Epithelial Growth Medium (MEGM), supplemented with 10 ng/ml EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 30 μg/ml bovine pituitary extract, 50 μg/ml gentamicin and 50 ng/ml amphotericin, under incubation in a 95% air / 5% $CO_2$ humidified incubator at 37°C, the medium was changed every three days. After four or five days the cultured cells ($n$=5) were treated with tamoxifen (TAM) in ethanol (2.7 nM), β-estradiol (E2) in ethanol (10 nM), vehicle (ethanol) or left untreated for three days prior to harvest. Harvesting was performed using trypsin

0.25% solution (incubation time 8 min, 37°C). The resultant cell suspension was centrifuged (2000 rpm, 7 min) with the supernatant being discarded. The pellet was re-suspended in 1 ml fresh medium and transferred to 1.5 ml eppendorf tubes. The cells were centrifuged in a microfuge at 6000 rpm for 5 min and the supernatant discarded. The pellets were stored at -80°C.

#### 4.2.1.2 Cell Extraction

The cells were extracted with an equal volume of cold (–20°C) absolute methanol. After vortexing (30 s), the tube was transferred into a dry ice bath for 30 min and subsequently thawed in an ice bath for 10 min. Centrifugation (13,000 rpm, 10 min) was carried out and the supernatant was collected. The cell pellet was subjected to a second extraction with 50% *v/v* cold (–20°C) methanol, and the first and second extracts were combined. The pooled supernatants were evaporated to dryness with a centrifugal concentrator (~4 h) and then re-suspended in 20 µl of water. Each sample was aliquoted as follows: 5 µl aliquots into 1 mm NMR tubes with the addition of 3 µl deuterated potassium phosphate buffer (pH* 6.0, 0.1 M) containing TSP (7.6 mM), 10 µl aliquots into MS vials with the addition of 85% acetonitrile, and the remaining 5 µl was pooled together in order to create QC samples with the addition of 85% acetonitrile.

### 4.2.2 $^{1}$H NMR Spectroscopy

Development of the methodology for the analysis of human apocrine gland sweat secretions is discussed in chapter two (Section 2.3.3). In summary, spectra were acquired on a Bruker Avance 400 spectrometer, operating at 400.13 MHz $^{1}$H observation frequency equipped with a 1 mm TXI micro-volume with an internal probe temperature of 298 K and with 256 transients being collected. All spectra were manually corrected for phase and baseline distortions using TopSpin™ 2.1 (Bruker GmbH,Germany) and chemical shifts referenced to TSP standard at δ0.00 ppm.

### 4.2.3 2D NMR Spectroscopy

To aid spectral assignment, 2D $^{1}H$-$^{1}H$ COSY spectra were measured with solvent presaturation pulse sequence on each sample. Acquition and processing parameters for the COSY spectra included a relaxation decay of 1.86 s, a spectral width in F1 and F2 of 5995.2 Hz, 2 k time domain points, 128 F1 increments, 64 transients per increment and qsine apodization in F1 and F2.

### 4.2.4 HILIC-QTOF-MS

HILIC-QTOF-MS was used for a global profiling approach as previously described in chapter three (Section 3.2.4). In summary, data was acquired *m/z* 80-1000 in positive ion mode over 25 min.

### 4.2.5 Targeted LC-MS/MS

RP-HPLC-MS/MS was used to specifically target amino acid conjugates as previously described in chapter three (Section 3.2.6). This method was only conducted on the QC samples due to the limitation in sample volumes.

### 4.2.6 Quantification of Artificial Apocrine Sweat Metabolites by $^{1}$NMR Spectroscopy

Quantification was carried out as previously described in chapter two (Section 2.3.4).

### 4.2.7 NMR Spectral Data Reduction

Pre-processing of NMR spectra was carried out as previously described in chapter two (Section 2.3.5).

### 4.2.8 HILIC-QTOF-MS Data Pre-Processing

LC-MS data were prepared for multivariate data analysis using Micromass MarkerLynx Application Manager (Version 4.0, Waters, UK). MarkerLynx incorporates an ApekTrack-peak detection algorithm which allows detection and retention time alignment of the peaks eluting in each chromatogram. The data were combined into a single data matrix by aligning peaks with the same mass/retention time pair together from each data file in the dataset, along with their associated intensities. Only peaks between 1.5 min to 14 min retention times were included in the final data matrix and the intensity of each ion was normalised to the samples total signal intensity.

### 4.2.9 Multivariate Data Analysis

Umetrics SIMCA-P version 11 was used for multivariate data analysis. Two types of scaling were used, mean-centring (subtracting the calculated average of a variable from the data so that the mean for each variable is 0) and mean-centring followed by autoscaling (division of each variable by the standard deviation for that variable). The use of mean-centred but not autoscaled data in basic multivariate analyses such as PCA, often results in an emphasis on perturbation of the metabolites that are present in high concentrations, whereas autoscaled data standardise the variance of each variable to one, thus, is more sensitive to changes in the levels of minor metabolites as each variable has an equal weight. For the purpose of illustrating the results of this study, autoscaling of the data is reported.

Principal component analysis was used for initial visualization of both the $^{1}$H NMR spectra and mass spectral data. PCA is a well-established method for the interpretation of chemical data that has been thoroughly described previously (e.g. (Massart and Kaufman, 1983)). PCA reduces the dimensionality of a dataset, which has a large number of variables, while describing as much variation within the data as possible. The results of PCA are discussed in terms of component score vectors (observation coordinate along a PC) and loading vectors (direction coefficient of a PC).

Supervised analysis using principal components discriminant analysis (PC-DA) (Coolen *et al.*, 2008; Harker *et al.*, 2006; Hoogerbrugge *et al.*, 1983) was then used to maximise separation between the sample groups. PC-DA was preferred over other options such as PLS-DA because it is more suitable for multi-class clustering, such as in this study, whereas PLS-DA is more suitable for cases where there are two classes.

### 4.2.10 Compound Identification

Metabolite identification *via* HILIC-MS is based on accurately measuring pseudomolecular ions to determine their *m/z* or chemical formula in order to extract potential structures from databases. In order to calculate the most likely and chemically correct formula, rules need to be followed and adhered to in order to constrain thousands of possible candidates. A set of rules for this purpose has been published (Kind and Fiehn, 2007) and these have been used to aid compound identification from MS data in this chapter: -

- Apply heuristic restrictions for number of elements during formula generation (e.g. $^{12}C$ at 1000 Da follows 1000/12 = 83 maximum limit for a hypothetical molecule that consists exclusively of carbon)
- Nitrogen rule (odd nominal mass implies odd number of N)
- Perform isotopic pattern filter
- Perform H/C ratio check (hydrogen/carbon ratio >0.125 and <3)
- Perform NOPS ratio check (N (0-1.3), O (0-1.2), P (0-0.3), and S/C (0-0.8) ratios)
- Perform heuristic HNOPS probability check (H, N, O, P, S/C high probability ratios)

A list of freely and commercially accessible online databases are summarised in chapter five (Table 5.2). The main databases used in this chapter were biological databases such as HMDB and Lipid Maps.

## 4.3 Results and Discussion

### 4.3.1 $^1$H NMR Spectra of *In-Vitro* Model of Apocrine Sweat

Figure 4.3 shows a typical 400 MHz $^1$H NMR comparative spectra for each apocrine cell extract, acquired using only 5 µl of extract and increasing the method sensitivity by use of a 1 mm micro-volume probe. The spectrum depicted clearly illustrates the power of the miniaturized probe when there is only a limited volume of the biofluid available. The mass sensitivity per sample quantity is inversely proportional to the diameter, thus, enhancement of the probe can be approximated since S/N $\propto$ 1/(coil diameter) (Olson *et al.*, 1998; Peck *et al.*, 1995). This equated to a ~5.3-fold increase compared to conventional 5 mm NMR probes, thus, providing a similar performance to that seen on a 500 MHz spectrometer equipped with a cryo-probe (data not shown). This increase in sensitivity enabled 29 metabolites to be readily identified, with 25 being quantified. Many of the resonances have been assigned by comparison with existing literature (Harker *et al.*, 2006; Lindon *et al.*, 2000; Nicholson *et al.*, 1995) in combination with an in-house spectral database and confirmed by $^1$H-$^1$H 2D COSY. These assignments are tabulated (Table 4.1), together with typical concentrations of metabolites found in the control samples. The most abundant metabolites detected were lactic acid and several amino acids including glycine, alanine, and tyrosine, with the former being present at concentrations of 19.1 ± 10.8 mM and the latter ranging from 1.6 – 3.6 mM. Any high variability in the concentration, e.g. lactate; is likely due to overlapping peaks, e.g. $CH_2$ lipid peaks, during the integration process. In order to minimise the error, multiple resonances of the same metabolite were averaged where possible. The reported concentrations are consistent with observations made previously following the NMR spectroscopic analysis of eccrine sweat (Harker *et al.*, 2006).

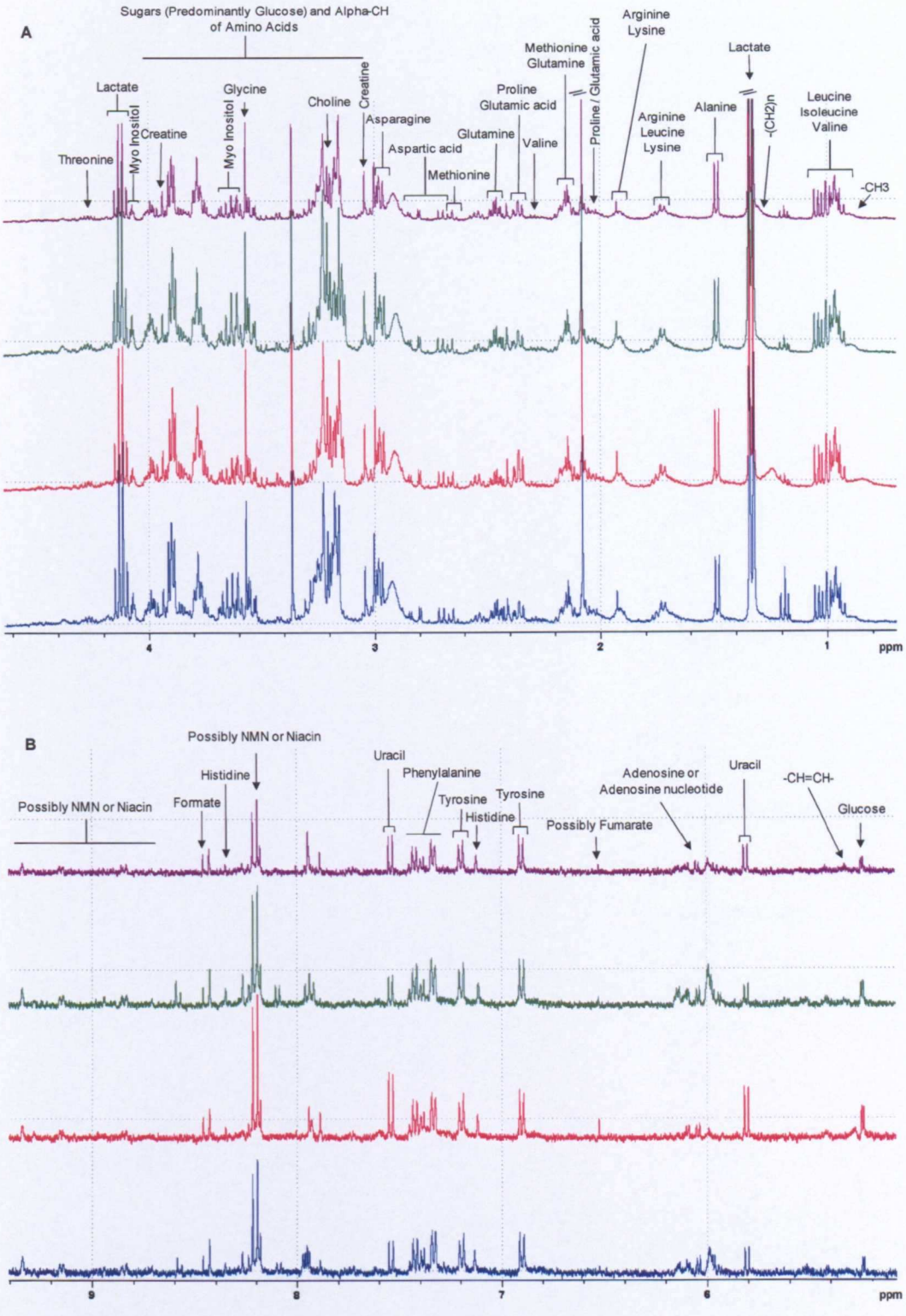



**Figure 4.3** 400-MHz 1D $^{1}$H NMR comparative spectra of an ASG5 cell extract acquired using a 1 mm TXI NMR probe, **A** δ 0.5-4.5 region and **B** δ5-9.2 region. Key, blue (TAM), red (E2), Green (Eth), and purple (Unt).

**Table 4.1** Relative concentrations of identified metabolites from AGS5 cell extract determined by NMR spectroscopy equipped with 1 mm micro-volume probe. No correction for differences in $T_1$ relaxation.

| Chemical Shift (PPM) | Assignment | Metabolite | Typical Concentration (mM) |
|---|---|---|---|
| 0.89 (m) | $CH_3$ | Lipid | - |
| 0.92 (t) | δ-$CH_3$ | Isoleucine | 2.4 ± 1.4 (0.9 - 3.8) |
| 0.99 (d) | β-$CH_3$ | | |
| 0.96 (t) | δ-$CH_3$ | Leucine | 3.6 ± 2.1 (1.3 - 5.5) |
| 1.72 (m) | $CH_2$ | | |
| 3.72 (m) | α-CH | | |
| 0.98 (d) | $CH_3$ | Valine | 1.6 ± 0.9 (0.5 - 2.4) |
| 1.03 (d) | $CH_3$ | | |
| 2.26 (m) | β-CH | | |
| 3.59 (d)[a] | α-CH | | |
| 1.18 (d) | $CH_3$ | 3-β-hydroxybutyrate | 0.2 ± 0.1 (0.04 - 0.4) |
| 2.28 (ABX)[a] | $CH_2$ | | |
| 1.30 (m) | $CH_2$ | Lipid | - |
| 1.33 (d) | $CH_3$ | Lactate | 19.1 ± 10.8 (6.6 - 28.6) |
| 4.11 (q) | CH | | |
| 1.48 (d) | $CH_3$ | Alanine | 2.6 ± 1.5 (0.9 - 4.1) |
| 1.48 (m)[a] | γ-$CH_2$ | Lysine | 4.2 ± 2.2 (1.7 - 6.2) |
| 1.73 (m) | δ-$CH_2$ | | |
| 1.90 (m) | β-$CH_2$ | | |
| 3.04 (t)[a] | ε-$CH_2$ | | |
| 1.92 (s) | $CH_3$ | Acetate | 0.6 ± 0.4 (0.2 - 0.9) |
| 2.07 (s) | $CH_3$ | Acetamide[b] | 3.6 ± 1.7 (1.8 - 5.1) |
| 2.03 (m) | γ-$CH_2$ | Proline | 1.7 ± 1 (0.5 - 2.5) |
| 2.36 (m)[a] | β-$CH_2$ | | |
| 3.35 (m) | δ-$CH_2$ | | |
| 3.43 (m) | δ-$CH_2$ | | |
| 4.15 (m)[a] | α-CH | | |

*Table 4.1 continued*

| Chemical Shift (PPM) | Assignment | Metabolite | Typical Concentration (mM) |
|---|---|---|---|
| 2.07 (m)[a] | β-$CH_2$ | Glutamate | 3.3 ± 2 (1.1 - 5) |
| 2.35 (m) | γ-$CH_2$ | | |
| 3.77 (m)[a] | α-CH | | |
| 2.15 (m)[a] | β-$CH_2$ | Glutamine | 3.4 ± 2 (1.2 - 5.1) |
| 2.46 (m) | γ-$CH_2$ | | |
| 3.77 (d)[a] | α-CH | | |
| 2.51 (AB)[a] | ½ $CH_2$ | Citrate | 1.1 ± 0.7 (0.3 - 1.8) |
| 2.65 (AB)[a] | ½ $CH_2$ | | |
| 2.68 (ABX)[a] | β-$CH_2$ | Aspartate | 0.8 ± 0.5 (0.2 - 1.4) |
| 2.82 (ABX) | β-$CH_2$ | | |
| 3.90 (ABX)[a] | α-CH | | |
| 3.04 (s) | $CH_3$ | Creatine | 1.3 ± 0.7 (0.5 - 1.9) |
| 3.93 (s) | $CH_2$ | | |
| 3.20 (s) | $N(CH_3)_3$ | Choline | 0.8 ± 0.5 (0.3 - 1.2) |
| 3.51 (m) | $NCH_2$ | | |
| 4.05 (m) | $OCH_2$ | | |
| 3.28 (t)[a] | H5 | Myo-inositol | - |
| 3.53 (dd)[a] | H1/H3 | | |
| 3.63 (dd)[a] | H4/H6 | | |
| 4.06 (t)[a] | H2 | | |
| 3.36 (s) | | Residual methanol from the extraction procedure | |
| 3.31 (ABX) | $CH_2$ | Tyrptophan[c] | - |
| 3.49 (ABX) | $CH_2$ | | |
| 4.06 (ABX) | CH | | |
| 7.21 (t) | C5H | | |
| 7.29 (t) | C6H | | |
| 7.33 (s) | C2H | | |
| 7.55 (d) | C7H | | |
| 7.74 (d) | C4H | | |

*Table 4.1 continued*

| Chemical Shift (PPM) | Assignment | Metabolite | Typical Concentration (mM) |
|---|---|---|---|
| 3.41 (m) | C-H4 | Glucose | 0.4 ± 0.2 (0.2 - 0.5) |
| 3.48 (t) | C-H3 | | |
| 3.53 (dd) | C-H2 | | |
| 3.73 (dd) | C-H6' | | |
| 3.85 (m) | C-H6 | | |
| 3.90 (dd)[a] | C-H6 | | |
| 5.24 (d) | C-H1 | | |
| 3.56 (s) | $CH_2$ | Glycine | 3.7 ± 2.3 (1.2 - 5.9) |
| 3.59 | α-CH | Threonine | 3.2 ± 1.8 (1.1 - 5) |
| 4.26 (m) | β-CH | | |
| 3.85 (dd)[a] | α-CH | Serine | 3.7 ± 2.3 (1.1 - 5.8) |
| 3.95 (dd)[a] | β-$CH_2$ | | |
| 4.00 (dd)[a] | β-$CH_2$ | | |
| 5.32 (m) | -HC=CH- | Lipid | 0.2 ± 0.2 (0.1 - 0.6) |
| 5.80 (d) | CH | Uracil[b] | 0.5 ± 0.4 (0.1 - 0.9) |
| 7.54 (d) | CH | | |
| 6.90 (d) | H3/H5 | Tyrosine | 0.6 ± 0.4 (0.2 - 1) |
| 7.19 (d) | H2/H6 | | |
| 7.33 (m) | H2/H6 | Phenylalanine | 0.7 ± 0.5 (0.2 - 1.1) |
| 7.39 (m) | H4 | | |
| 7.43 (m) | H/H5 | | |
| 8.46 (s) | CH | Formate | 0.2 ± 0.1 (0.1 - 0.3) |

[a]Partially obscured resonances

[b]Tentatively assigned

[c]Below limit of quantification

Multiplicity is indicated as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet

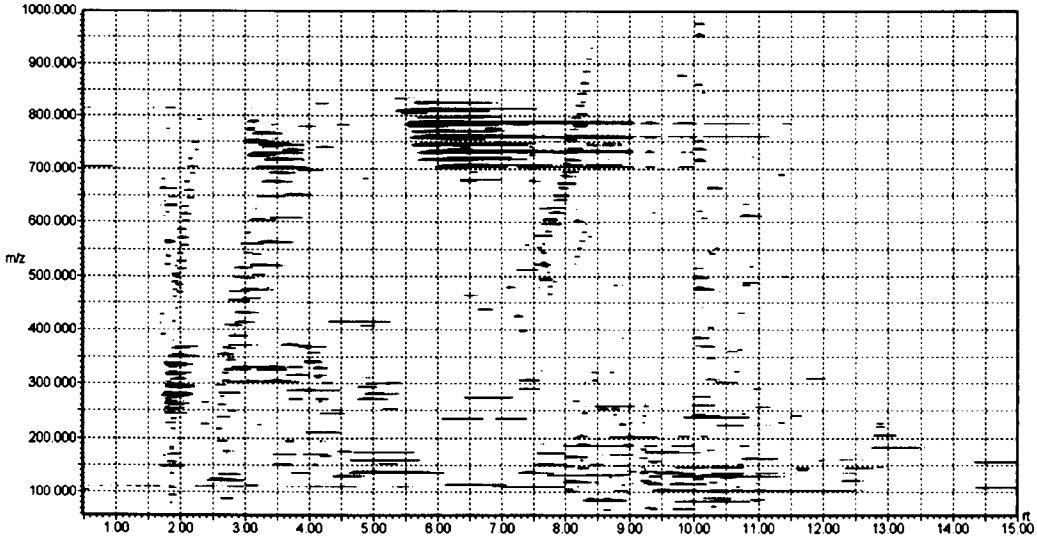
### 4.3.2 LC-Mass Spectrometry Analysis of *In-Vitro* Model of Apocrine Sweat

Figure 4.4 shows comparative 2-D LC-MS maps (horizontal axis: retention time, vertical axis: *m/z*) for each of the ASG5 cell extracts using HILIC-TOF-MS. More than 1000 features were detected in this sample during a 25 min acquisition period, highlighting both the sensitivity of the technique and the complexity of the sample. However, it should be noted that not all these features may be represented as individual compounds due to fragmentation or adducts during the ionisation process. As with the NMR data, visual inspection would be tedious, inefficient and prone to subjective error. Thus, examination of the data was carried out through the use of multivariate analysis.

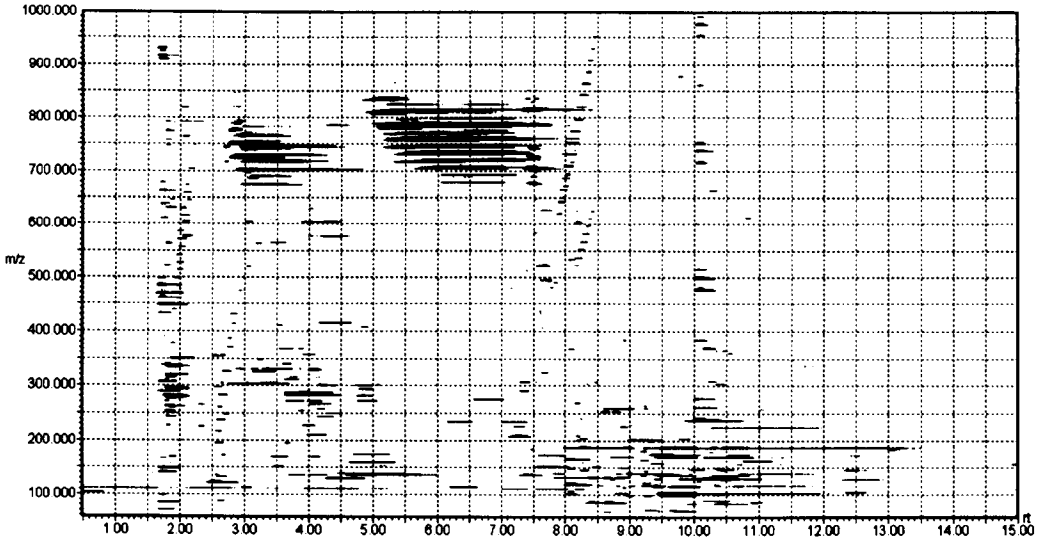
Both the global approach (HILIC-MS) and the targeted (RP-LC-MS/MS) approach proved to be unsuccessful in detecting known odour precursors in apocrine secretions, including 3M2H-Gln. However, a previous report using the ASG5 cell lines have reported the ability to detect 3M2H-Gln using GC-MS and a different extraction procedure (Burry *et al.*, 2008). Thus, it is likely that the cold methanol extraction used herein, did not extract these metabolites or that the presence was not represented in all sample classes, resulting in being diluted down in the QC samples, thereby, being below the limit of detection. Furthermore, none of the androgens in Figure 4.2 were detected. Androstanediol ($C_{19}H_{32}O_2$ *m/z* 331.2039 [M+K]) produced a possible hit with a potassium adduct, which had a retention time of 3.75 min and an associated mass error of 5.2 ppm. However, this is likely to be a false positive due to the other androgens not being detected as well not detecting the psudomolecular ion or any other adducts. Cholesterol and squalene, which have been detected previously (Burry *et al.*, 2008), were also not detected. As mentioned previously, it likely that these metabolites were not extracted with the cold methanol procedure or that they eluted off the column in the void volume. However, 275 metabolites were putatively identified such as phospholipids, glycerolipids, fatty acids and amino acids (see Appendix B). This is in line with previous reports (Burry *et al.*, 2008), and further supports that the evidence that the ASG5 cell line mimics the functions of an apocrine gland. However, false positives are always found, though could be significantly

reduced by acquiring fragmentation spectra (MS/MS or $MS^n$) and comparing it to an authentic standard or to an insilico derived fragmentation mass spectra (Wolf *et al.*, 2010).

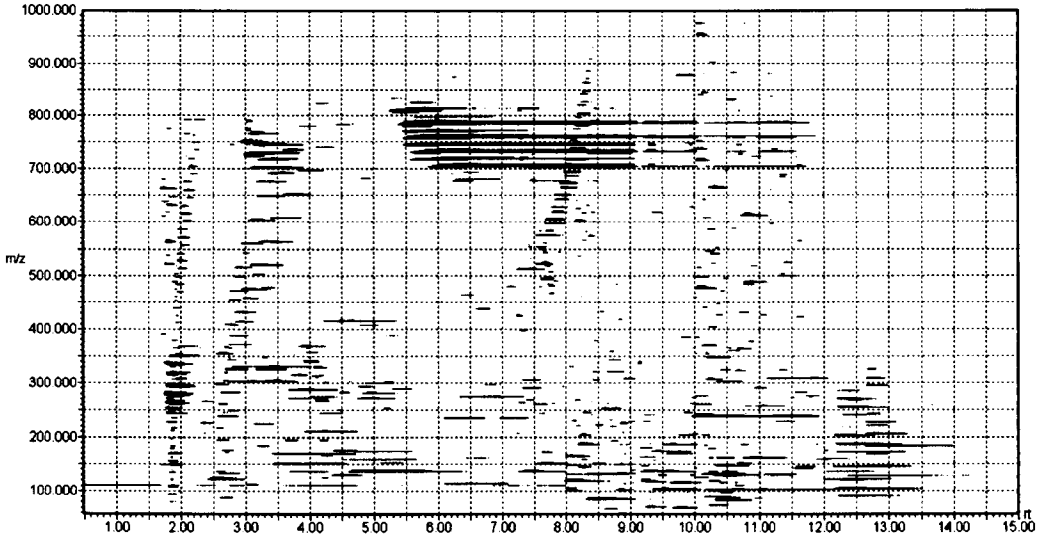
**A**




**B**



C




D

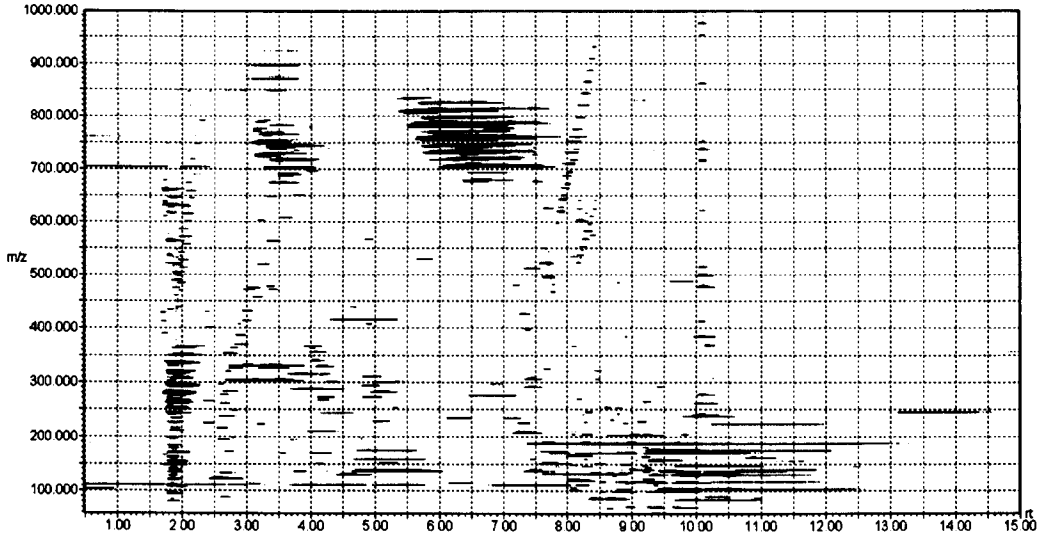



**Figure 4.4** Two dimensional mass versus retention 'map' obtained from QTOF-HILIC-MS analysis of ASG5 cell extract of **A** TAM, **B** E2, **C** Eth and **D** Unt. Typically, over 1000 features can be detected in 15 min.

### 4.3.3 Multivariate Data Analysis

Both the NMR and LC-MS data were initially analysed with PCA to obtain a global unbiased view and to find the systemic metabolic changes between each of the treatments. UV scaling was used for all the models presented in this section, as compared to mean centred data, all metabolites become equally important. PCA scores plots of the $^{1}$H NMR spectral data and HILIC-MS spectra are depicted in Figure 4.5 A and B, which explains 54.2% (PC1 vs PC2 vs PC3) and 54.8% (PC1 vs PC2) of the total variation, respectively.

PCA of the NMR data showed separation of the treatment groups split across three PCs, and therefore supervised clustering was performed *via* PC-DA to ease visualisation and interpretation of the data. PC-DA model employs *a priori* knowledge relating to group information in order to construct the model. Due to the limited sample size, PC-DA models were initially constructed on the average response to the different cell treatments. The original data were then projected onto the model in order to determine the predictability of the model. The clustering of samples shown in Figure 4.6 is consistent with that of the LC-MS data, with the samples clustering according to the different treatments; however, ethanol and tamoxifen treated were clustered together across all discriminant axes, regardless of the analytical method. Thus, TAM can be considered as having no additional effect to that of the vehicle or any effect incurred by the TAM is masked by the effect of the vehicle. The first discriminant axis is mainly responsible for explaining the difference between treatments with the E2 inhibitor. In a similar way, the second discriminant axis is important for explaining the difference between untreated and TAM and ethanol treated cells. It can be seen in Figure 4.5 and Figure 4.6 that the E2 treated and control groups can be easily distinguished using both PCA and PC-DA, thus, demonstrating a clear influence that hormones have on the metabolic composition of apocrine sweat *in vitro*, as well as highlighting the complementary nature of both analytical methodologies.

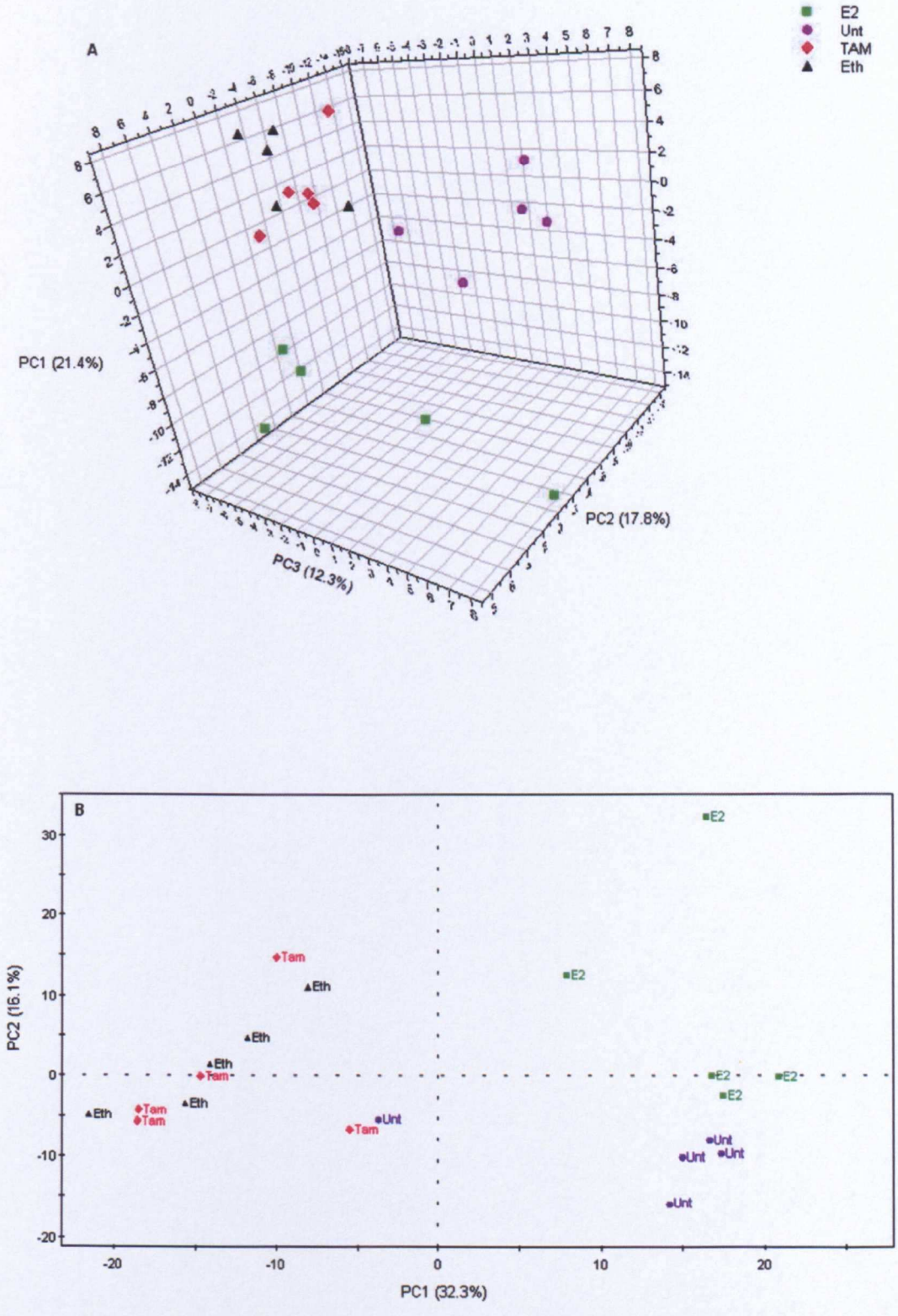



**Figure 4.5** PCA scores plot (UV scaled) obtained from **A** $^{1}$H NMR spectra and **B** HILIC MS data from the analysis of ASG5 apocrine cell lines treated with tamoxifen, β-estradiol and ethanol ($n$=5).

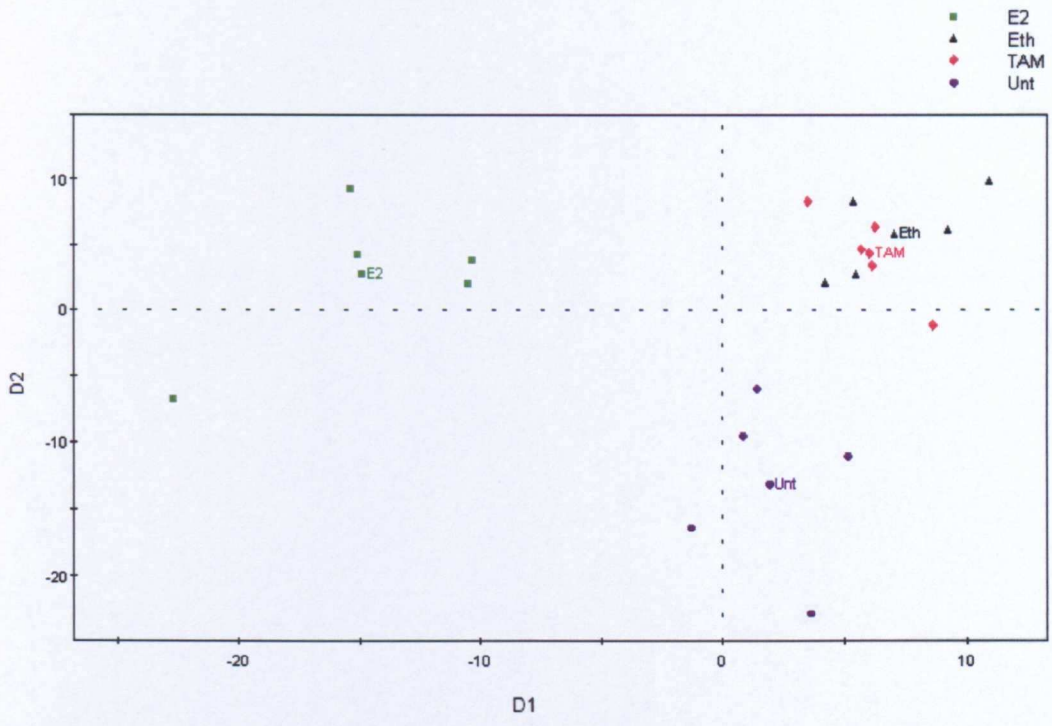



**Figure 4.6** PC-DA score plot (UV-scaled) obtained from $^{1}$H NMR spectra from the analysis of ASG5 apocrine cell lines treated with tamoxifen, β-estradiol and ethanol ($n$=5).

Determining the regions of interest between the different treatments is extremely difficult as there is an abundance of information from both methodologies. The NMR data showed a clear distinction between E2 and TAM, ethanol, and untreated groups, while the difference between untreated and TAM and ethanol were more subtle. In the case of $^{1}$H NMR the metabolites responsible for clustering of the E2 group include glucose, acetate, and β-hydroxybutyrate whilst the TAM and ethanol treated samples are characterised by lactate, myo-inositol and unknown metabolites that are putatively identified as nucleotides or nucleotide derivatives. Untreated cells are grouped by other amino acids such as valine, leucine and isoleucine as well as lipids peaks and formate. Table 4.2 summarises the main findings, where changes were supported by the PC-DA generated loading plot of the NMR data. The perturbations in metabolite profiles highlighted here show that the cells react differently to different levels of circulating hormones.

**Table 4.2** Key changes in the endogenous metabolites responsible for defining the hormonal treatment observed using PC-DA. The mean normalised bucketed values are reported.

| | | $\bar{x} \pm SD$ | | | |
|---|---|---|---|---|---|
| **Tentative Assignment** | **Buckets** | **Untreated** | **β-Estradiol** | **Ethanol** | **Tamoxifen** |
| Lactate | 4.14, 4.1, 1.38, 1.34, 1.3 | 4.60 ± 0.42 | 3.56 ± 0.82 | 5.26 ± 0.75 | 4.68 ± 0.21 |
| Trp/Myo Inositol | 4.06 | 0.70 ± 0.05 | 0.61 ± 0.14 | 0.97 ± 0.11 | 0.92 ± 0.08 |
| Unkown | 4.02 | 0.69 ± 0.08 | 0.65 ± 0.05 | 0.88 ± 0.04 | 0.8 ± 0.11 |
| Phe/Ser | 3.98 | 1.05 ± 0.14 | 0.97 ± 0.16 | 1.51 ± 0.17 | 1.39 ± 0.11 |
| Creatine/A.A possibly Tyr | 3.94 | 1.23 ± 0.39 | 1.18 ± 0.21 | 1.06 ± 0.09 | 1.01 ± 0.07 |
| A.A possibly Asp | 3.9 | 3.36 ± 0.33 | 3.49 ± 0.32 | 2.99 ± 0.23 | 3.31 ± 0.25 |
| Sugar possibly Glucose | 3.86, 3.82 | 0.89 ± 0.11 | 1.30 ± 0.49 | 1.06 ± 0.19 | 1.00 ± 0.09 |
| Unkown possibly A. A | 3.78 | 3.12 ± 0.14 | 3.14 ± 0.19 | 2.46 ± 0.21 | 2.76 ± 0.15 |
| Glucose | 3.74, 3.7, 3.68, 3.5, 3.46, 3.42, 3.38 | 0.65 ± 0.13 | 1.13 ± 0.38 | 0.71 ± 0.07 | 0.68 ± 0.07 |
| Myo inositol | 3.62, 3.54 | 1.19 ± 0.09 | 1.10 ± 0.10 | 1.62 ± 0.20 | 1.56 ± 0.11 |
| Proline | 3.34, 2.02 | 0.90 ± 0.23 | 0.83 ± 0.21 | 0.66 ± 0.09 | 0.69 ± 0.03 |
| Myo inositol/A. A possibly Pro | 3.3 | 2.01 ± 1.13 | 2.01 ± 0.89 | 1.37 ± 0.61 | 1.22 ± 0.19 |
| A.A possibly Tyr | 3.18 | 4.13 ± 1.35 | 3.67 ± 0.67 | 3.78 ± 0.55 | 4.6 ± 0.74 |
| A.A possibly Phe | 3.14 | 1.79 ± 1.02 | 1.75 ± 1.03 | 2.41 ± 1.30 | 2.10 ± 0.77 |
| Creatine/A.A possibly Lys | 3.02 | 1.93 ± 0.70 | 1.85 ± 0.63 | 1.27 ± 0.38 | 1.37 ± 0.29 |
| A.A probably Asn | 2.98 | 1.75 ± 0.21 | 1.71 ± 0.14 | 1.57 ± 0.23 | 1.64 ± 0.15 |
| Lipid | 2.94, 2.9 | 1.93 ± 0.57 | 1.89 ± 0.55 | 1.81 ± 0.47 | 1.96 ± 0.24 |
| Unknown possibly Methylamine | 2.54 | 0.53 ± 0.05 | 0.55 ± 0.03 | 0.37 ± 0.06 | 0.47 ± 0.03 |
| Glutamine | 2.46, 2.18, 2.14 | 1.31 ± 0.06 | 1.06 ± 0.07 | 0.95 ± 0.06 | 1.02 ± 0.05 |
| Glutamic acid | 2.38, 2.34 | 0.70 ± 0.07 | 0.83 ± 0.09 | 0.55 ± 0.08 | 0.65 ± 0.05 |
| Unknown possibly acetamide | 2.06 | 3.14 ± 0.71 | 3.46 ± 0.58 | 2.86 ± 0.10 | 2.86 ± 0.31 |
| Lipid $(CH_2)_n$ | 1.26, 1.22 | 0.33 ± 0.07 | 1.00 ± 0.43 | 0.33 ± 0.07 | 0.32 ± 0.05 |
| β-hydroxybutyrate | 1.18 | 0.27 ± 0.13 | 1.21 ± 1.11 | 0.37 ± 0.11 | 0.38 ± 0.18 |
| Leu/Ile/val | 1.02, 0.98, 0.94, 0.9 | 1.29 ± 0.09 | 1.13 ± 0.11 | 1.13 ± 0.07 | 1.13 ± 0.03 |
| lipid $(CH_3)$ | 0.86, 0.82 | 0.09 ± 0.03 | 0.40 ± 0.16 | 0.11 ± 0.03 | 0.11 ± 0.02 |

TAM has a complex pharmacology as it is a selective estrogen receptor modulator which can prevent estrogen from stimulating growth as well as possessing agonistic effects such as the induction of calcium signalling (Zhang *et al.*, 2000) and influencing bone and lipid metabolism (Love *et al.*, 1992; Riggs and Hartmann, 2003). TAM has been shown to increase intracellular triglycerides (Hozumi *et al.*, 1998) and disrupts fatty acid metabolism (Lelliott *et al.*, 2005). Furthermore, TAM can affect the lipoprotein concentrations by reducing levels LDL-cholesterol due to increasing LDL receptor activity (Bruning *et al.*, 1988; Powles *et al.*, 1989; Walsh *et al.*, 1991). However, with the data presented it is difficult to state whether they agree with the current literature as cholesterol compounds and their derivatives were not detected. Lactate dehydrogenase, which catalyzes the conversion of pyruvate into

lactate, has been shown to increase with E2 (Thomas *et al.*, 1989). However, the data presented contradicts these findings, although, it does agree with the fact that TAM has no effect on lactate production. TAM ($C_{26}H_{29}NO$; *m/z* 372.2327 [M+H]) is known to be metabolised into *N*-desmethyltamoxifen ($C_{25}H_{27}NO$; *m/z* 358.2170 [M+H]) and 4-hydroxytamoxifen ($C_{26}H_{29}NO_2$; *m/z* 388.2276 [M+H]), where oxidation of these primary metabolites results in formation of 4-hydroxy-*N*-desmethyltamoxifen (endoxifen; $C_{25}H_{27}NO_2$; *m/z* 374.2119 [M+H]) by hepatic cytochrome P450s (Desta *et al.*, 2004; Hoskins *et al.*, 2009). These metabolites along with E2 ($C_{18}H_{24}O_2$; *m/z* 273.1854 [M+H]) were not identified in the samples prior to MVA. In comparison to the NMR data analysis, TAM and E2 were considered to be below the limit of detection. It is important to remove any drug metabolites to avoid miss-interpretation of the data, as the models produced would predominately describe the variation between the different drugs and their corresponding metabolites.

The principle ions contributing to the PCA grouping from HILIC-MS data were, *m/z* 447.3474, 289.1790, 461.3638, 312.3637, 368.4240, 312.3637, 284.3296, 256.2993 and 228.2674 which were shown to be only present in the E2 group. The untreated group were grouped due to the lipid content (e.g. glycerophosphocholines) whilst those at *m/z* 247.0581, 405.0090 152.0561 and 229.1538 were more prevalent in the TAM and ethanol treated samples. Furthermore, the phospholipids content is generally lower in concentration with TAM treatment when compared against the E2 treated cells, while nucleotide metabolites were shown to be elevated in the TAM treated cells. Molecular mass, postulated elemental composition and chromatographic retention time data for the ions relating to the differences are given in Table 4.3. However, as with tentative assignments for the ions responsible for distinguishing between the different treatments, these metabolites are as yet unidentified despite searches of available databases using the accurate masses and atomic compositions. Further investigations, such as metabolite isolation *via* fraction collection which would then be subjected to further NMR and MS analysis, would be required in an attempt to characterise and identify these compounds. Moreover, a continuing problem with the confirmation of the identity of many compounds is the lack of authenticated standards to provide unequivocal structures, thus, calculating the correct empirical formula is essential (Kind and Fiehn, 2007). Many of the changes observed are also unlikely to be directly relevant to apocrine sweat. There will be many

changes caused by the addition of ethanol and other disruptions caused by hormone interactions which will produce a rippling effect across intracellular metabolism. Nonetheless, the analytical methodologies used can reveal many of these global changes. However, it is more difficult to attribute these changes to specific pathways, but not impossible since many of the steroid hormone effects are characterised.

**Table 4.3** Key changes in endogenous metabolites after addition of either E2, TAM, ethanol, with respect to the untreated control observed from PCA analysis with HILIC-MS data acquired in positive ion mode.

| Ret. Time (min) | *m/z* | Tentative Formula | *m/z* Error (ppm) | Tentative Assignment | $\bar{x} \pm$ SD (Peak area) Untreated | E2 | Ethanol | TAM |
|---|---|---|---|---|---|---|---|---|
| *Principle ions from E2* | | | | | | | | |
| 1.72 | 483.3455[b] | $C_{26}H_{43}NO_6$ | 3.31 | Glycocholic acid | 0 ± 0 | 2.94 ± 0.99 | 0 ± 0 | 0 ± 0 |
| 1.72 | 447.3474[b] | $C_{23}H_{43}NO_6$ | 8.05 | Hexadecanedioic acid mono-L-carnitine ester | 0.02 ± 0.05 | 204.15 ± 38.58 | 0 ± 0 | 0.01 ± 0.01 |
| 1.73 | 433.3304 | $C_{27}H_{44}O_4$ | 4.15 | 24-Hydroxycalcitriol | 0 ± 0 | 7.66 ± 1.64 | 0 ± 0 | 0 ± 0 |
| 1.73 | 167.0338[a] | $C_6H_8O_4$ | 10.78 | 3-Methylglutaconic acid/3-Hexenedioic acid | 1.39 ± 0.41 | 2.60 ± 0.39 | 0.80 ± 0.16 | 1.07 ± 0.24 |
| 1.73 | 307.1892[f] | $C_8H_{14}O_2$ | 2.28 | 2-Octenoic acid | 0 ± 0 | 12.37 ± 1.75 | 0 ± 0 | 0 ± 0.01 |
| 1.73 | 307.1892[b] | $C_{13}H_{23}NO_6$ | 7.49 | 3-Methylglutarylcarnitine | 0 ± 0 | 12.37 ± 1.75 | 0 ± 0 | 0 ± 0.01 |
| 1.73 | 289.1797 | $C_{18}H_{24}O_3$ | 2.42 | 16,17-Epiestriol/17-Epiestriol/2-Hydroxyestradiol | 0 ± 0.01 | 27.6 ± 5.45 | 0 ± 0 | 0 ± 0 |
| 1.73 | 461.3638[d] | $C_{28}H_{44}O_3$ | 1.52 | Ercalcitriol | 0 ± 0 | 29.44 ± 6.17 | 0 ± 0 | 0 ± 0 |
| 1.74 | 945.6660[f] | $C_{24}H_{47}NO_7$ | 6.03 | Galactosylsphingosine | 0 ± 0 | 0.95 ± 0.94 | 0 ± 0 | 0 ± 0 |
| 3.29 | 326.3772 | $C_{22}H_{47}N$ | 4.60 | - | 57.90 ± 34.04 | 202.32 ± 292.04 | 154.64 ± 113.55 | 114.49 ± 155.67 |
| 3.41 | 332.3306 | $C_{18}H_{41}N_3O_2$ | 8.73 | - | 11.50 ± 4.65 | 32.41 ± 43.72 | 22.50 ± 15.43 | 18.62 ± 21.09 |
| 3.47 | 304.2982 | $C_{16}H_{37}N_3O_2$ | 5.92 | - | 40.04 ± 17.63 | 122.47 ± 167.07 | 85.80 ± 55.31 | 68.67 ± 79.31 |
| 3.68 | 368.424 | $C_{25}H_{53}N$ | 4.34 | - | 0.01 ± 0.02 | 10.36 ± 1.41 | 0 ± 0.01 | 0 ± 0 |
| 3.7 | 340.3945 | $C_{23}H_{49}N$ | 0.59 | - | 0 ± 0 | 4.66 ± 0.68 | 0 ± 0 | 0 ± 0 |
| 3.76 | 312.3637 | $C_{21}H_{45}N$ | 2.24 | - | 0.01 ± 0.02 | 14.96 ± 1.73 | 0 ± 0 | 0 ± 0 |
| 3.85 | 284.3293 | $C_{19}H_{41}N$ | 8.44 | - | 0.14 ± 0.30 | 830.27 ± 115.47 | 0.22 ± 0.14 | 0.23 ± 0.22 |
| 3.95 | 256.2993 | $C_{17}H_{37}N$ | 4.29 | - | 0.01 ± 0.01 | 2.64 ± 2.49 | 0 ± 0 | 0.01 ± 0.01 |
| 4.04 | 228.2674 | $C_{15}H_{33}N$ | 7.45 | - | 0.05 ± 0.06 | 10.82 ± 2.80 | 0.04 ± 0.07 | 0.04 ± 0.06 |
| 4.25 | 258.2784[b] | $C_{16}H_{32}O$ | 5.03 | Palmitaldehyde | 0 ± 0 | 3.25 ± 3.11 | 0.06 ± 0.11 | 0.05 ± 0.08 |
| 4.34 | 300.3259[b] | $C_{19}H_{41}NO$ | 2.33 | Pristanal | 0 ± 0 | 10.69 ± 1.53 | 0 ± 0 | 0 ± 0 |
| 5.49 | 834.6022 | $C_{48}H_{84}NO_8P$ | 0.12 | PC | 6.13 ± 5.26 | 15.89 ± 3.69 | 1.27 ± 1.49 | 1.98 ± 0.85 |
| 5.56 | 806.5686 | $C_{46}H_{80}NO_8P$ | 1.74 | PC | 11.22 ± 2.69 | 15.74 ± 1.89 | 5.93 ± 2.77 | 7.07 ± 2.22 |
| 6.85 | 776.5832[d] | $C_{41}H_{78}NO_8P$ | 2.70 | PE | 10.46 ± 3.53 | 16.17 ± 3.36 | 1.36 ± 1.53 | 1.50 ± 2.23 |

*Table 4.3 continued*

| Ret. Time (min) | *m/z* | Tentative Formula | *m/z* Error (ppm) | Tentative Assignment | $\bar{x}$ ± SD (Peak area) Untreated | E2 | Ethanol | TAM |
|---|---|---|---|---|---|---|---|---|
| *Principle ions from Untreated* | | | | | | | | |
| 1.81 | 282.2789 | $C_{18}H_{35}NO$ | 2.83 | Oleamide | 145.01 ± 44.74 | 60.24 ± 11.27 | 90.1 ± 22.97 | 85.31 ± 36.36 |
| 1.87 | 280.2621 | $C_{18}H_{33}NO$ | 6.78 | - | 101.54 ± 36.81 | 44.00 ± 15.02 | 58.46 ± 13.11 | 57.41 ± 14.75 |
| 3.03 | 776.5591 | $C_{45}H_{78}NO_7P$ | 0.39 | PE | 6.78 ± 2.48 | 7.30 ± 4.81 | 1.62 ± 1.33 | 1.10 ± 1.51 |
| 3.3 | 674.5131 | $C_{37}H_{72}NO_7P$ | 0.89 | PE | 17.45 ± 6.29 | 17.02 ± 8.38 | 8.46 ± 1.06 | 9.83 ± 3.92 |
| 3.3 | 742.5393 | $C_{41}H_{76}NO_8P$ | 0.80 | PE or PC | 17.79 ± 7.37 | 5.23 ± 4.18 | 1.87 ± 2.28 | 1.17 ± 1.61 |
| 3.37 | 744.5562 | $C_{41}H_{78}NO_8P$ | 2.55 | PE or PC | 90.54 ± 50.48 | 40.30 ± 16.46 | 28.00 ± 6.02 | 28.94 ± 4.77 |
| 3.4 | 716.5237 | $C_{39}H_{74}NO_8P$ | 2.79 | PE | 57.11 ± 17.18 | 26.49 ± 10.36 | 13.73 ± 3.17 | 14.75 ± 2.44 |
| 3.51 | 690.5083 | $C_{37}H_{72}NO_8P$ | 1.30 | PE | 16.19 ± 11.11 | 5.25 ± 3.90 | 2.00 ± 2.78 | 0.26 ± 0.58 |
| 4.46 | 130.1579 | $C_8H_{19}N$ | 13.06 | - | 45.48 ± 15.35 | 28.96 ± 16.49 | 28.23 ± 6.01 | 18.35 ± 7.77 |
| 5.79 | 784.5883 | $C_{44}H_{82}NO_8P$ | 3.44 | PC | 143.58 ± 17.01 | 121.29 ± 19.49 | 64.04 ± 39.66 | 78.61 ± 27.46 |
| 5.88 | 786.6024 | $C_{44}H_{84}NO_8P$ | 1.40 | PC | 873.87 ± 310.62 | 793.55 ± 350.45 | 570.02 ± 67.74 | 612.78 ± 50.16 |
| 5.89 | 756.5544 | $C_{42}H_{78}NO_8P$ | 0.13 | PC/PE | 72.36 ± 10.6 | 42.21 ± 5.58 | 41.89 ± 5.83 | 42.25 ± 7.68 |
| 5.97 | 758.5714 | $C_{42}H_{80}NO_8P$ | 1.85 | PC/PE/PE-NMe | 936.35 ± 88.15 | 768.21 ± 93.32 | 480.43 ± 154.36 | 563.43 ± 73.98 |
| 6.09 | 760.5859 | $C_{42}H_{82}NO_8P$ | 0.39 | PC | 454.41 ± 492.96 | 548.11 ± 327.08 | 565.63 ± 41.83 | 389.76 ± 266.78 |
| 6.18 | 732.5549 | $C_{40}H_{78}NO_8P$ | 0.82 | PE or PC | 752.84 ± 127.35 | 405.25 ± 207.14 | 457.57 ± 55.35 | 415.8 ± 124.28 |
| 6.51 | 706.5389 | $C_{38}H_{76}NO_8P$ | 0.42 | PE or PC | 163.77 ± 66.44 | 47.19 ± 36.98 | 50.24 ± 46.82 | 41.95 ± 34.73 |
| 8.02 | 775.4798[g] | $C_{21}H_{36}O_5$ | 4.77 | Cortol | 5.34 ± 1.57 | 3.82 ± 1.95 | 1.94 ± 0.32 | 2.35 ± 0.86 |
| 8.09 | 819.5059[e] | $C_{42}H_{79}O_{10}P$ | 8.42 | PG | 4.09 ± 1.23 | 3.23 ± 1.48 | 1.83 ± 0.20 | 2.12 ± 0.51 |
| 8.82 | 104.1064[b] | $C_5H_{10}O$ | 10.57 | Iso-Valeraldehyde | 3.98 ± 1.72 | 3.05 ± 1.65 | 0.56 ± 0.39 | 0.83 ± 0.56 |
| 9 | 141.1132 | $C_6H_{12}N_4$ | 5.67 | - | 2.20 ± 0.85 | 1.63 ± 0.76 | 0.51 ± 0.06 | 0.36 ± 0.21 |
| 9.28 | 139.0494 | $C_6H_6N_2O_2$ | 5.10 | Nicotinamide N-oxide | 30.14 ± 15.05 | 15.32 ± 9.57 | 3.58 ± 2.04 | 6.12 ± 3.66 |
| 9.6 | 137.0707 | $C_7H_8N_2O$ | 5.84 | N-Methylnicotinamide | 83.89 ± 14.83 | 64.18 ± 22.85 | 38.00 ± 7.05 | 46.33 ± 17.65 |
| 10.22 | 132.0764 | $C_4H_9N_3O_2$ | 6.81 | Creatine | 130.89 ± 17.9 | 95.21 ± 40.32 | 101.74 ± 15.85 | 116.02 ± 22.66 |
| *Principle ions from TAM/Ethanol* | | | | | | | | |
| 2.54 | 123.0549 | $C_6H_6N_2O$ | 3.16 | Niacinamide | 83.73 ± 40.06 | 73.46 ± 22.97 | 143.85 ± 24.57 | 147.74 ± 39.04 |
| 2.68 | 133.0855 | $C_6H_{12}O_3$ | 3.16 | Hydroxyisocaproic acid/5-Hydroxyhexanoic acid | 4.52 ± 1.96 | 2.86 ± 1.85 | 7.35 ± 1.23 | 7.82 ± 1.45 |

*Table 4.3continued*

| Ret. Time (min) | *m/z* | Tentative Formula | *m/z* Error (ppm) | Tentative Assignment | $\bar{x}$ ± SD (Peak area) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Untreated | E2 | Ethanol | TAM |
| 2.69 | 177.1119[d] | $C_8H_{16}O_4$ | 4.52 | 4-Hydroxycyclohexylcarboxylic acid | 2.04 ± 0.81 | 1.07 ± 0.69 | 3.77 ± 0.60 | 3.99 ± 0.79 |
| 3.76 | 331.2052[c] | $C_{19}H_{32}O_2$ | 3.92 | Androstanediol | 0 ± 0 | 0.58 ± 1.01 | 9.96 ± 2.84 | 5.23 ± 5.05 |
| 4.91 | 137.0452 | $C_5H_4N_4O$ | 2.84 | Hypoxanthine | 694.13 ± 213.43 | 747.66 ± 214.98 | 826.09 ± 110.12 | 912.67 ± 117.18 |
| 4.91 | 137.0452 | $C_4H_8O_5$ | 1.46 | Erythronic acid/Threonic acid | 694.13 ± 213.43 | 747.66 ± 214.98 | 826.09 ± 110.12 | 912.67 ± 117.18 |
| 6.4 | 114.066 | $C_4H_7N_3O$ | 6.13 | Creatinine | 5.12 ± 7.02 | 5.08 ± 5.37 | 18.61 ± 5.68 | 21.24 ± 4.72 |
| 7.44 | 137.0450 | $C_4H_8O_5$ | 4.01 | Threonic acid | 32.16 ± 16.46 | 20.39 ± 9.41 | 85.26 ± 29.29 | 65.17 ± 10.52 |
| 7.61 | 480.3438 | $C_{24}H_{50}NO_6P$ | 3.33 | LysoPC(P-16:0) | 0.15 ± 0.11 | 0.30 ± 0.09 | 1.35 ± 0.73 | 1.06 ± 0.29 |
| 8.08 | 120.0803 | $C_8H_9N$ | 8.33 | - | 162.00 ± 65.75 | 124.41 ± 47.82 | 220.71 ± 36.44 | 234.7 ± 39.99 |
| 8.08 | 166.0858 | $C_9H_{11}NO_2$ | 6.02 | L-Phenylalanine | 64.86 ± 25.32 | 50.01 ± 20.49 | 87.26 ± 13.93 | 83.53 ± 17.39 |
| 8.87 | 104.0528[h] | $C_2H_6S$ | 5.77 | Dimethylsulfide | 2.29 ± 3.57 | 2.56 ± 1.75 | 7.40 ± 1.63 | 7.53 ± 2.16 |
| 8.87 | 150.058 | $C_5H_{11}NO_2S$ | 5.99 | Methionine | 6.38 ± 4.37 | 4.92 ± 2.1 | 12.71 ± 3.03 | 13.25 ± 3.27 |
| 8.87 | 133.0313 | $C_5H_8O_2S$ | 7.51 | - | 11.43 ± 8.2 | 8.57 ± 3.58 | 23.36 ± 6.13 | 24.76 ± 6.87 |
| 9.56 | 104.1065 | $C_5H_{13}NO$ | 9.60 | Choline | 412.51 ± 118.73 | 323.33 ± 88.53 | 362.83 ± 15.67 | 427.40 ± 111.21 |
| 9.76 | 116.0704 | $C_5H_9NO_2$ | 6.89 | L-Proline | 51.79 ± 39.02 | 36.99 ± 18.55 | 89.81 ± 22.14 | 94.93 ± 25.63 |
| 9.88 | 247.0581[g] | $C_4H_8O_3$ | 1.21 | 4-Hydroxybutyric acid/3-Hydroxybutyric acid | 0.09 ± 0.17 | 0.03 ± 0.05 | 1.30 ± 0.37 | 1.07 ± 0.54 |
| 10.01 | 206.048 | $C_7H_{11}NO_4S$ | 3.40 | - | 0.26 ± 0.47 | 0.09 ± 0.17 | 1.26 ± 0.35 | 1.40 ± 0.57 |
| 10.09 | 477.2047 | $C_{27}H_{11}H_5O$[c] | 4.98 | - | 100.97 ± 68.23 | 79.98 ± 41.4 | 179.53 ± 34.62 | 194.84 ± 47.48 |
| 10.09 | 239.1050 | $C_{14}H_{16}O_2$[a] | 2.5 | 10E,12E-tetradecadiene-4,6-diynoic acid | 625.82 ± 173.44 | 551.78 ± 208.66 | 767.59 ± 121.32 | 786.49 ± 89.47 |
| 10.17 | 204.0869[a] | $C_7H_{11}N_5O$ | 3.91 | 6-methyltetrahydropterin | 0.63 ± 1.25 | 0.09 ± 0.20 | 3.67 ± 0.94 | 4.19 ± 0.52 |
| 10.17 | 608.0911 | $C_{17}H_{27}N_3O_{17}P_2$ | 2.79 | Uridine diphosphate-N-acetylgalactosamine | 0.43 ± 0.89 | 0.02 ± 0.02 | 2.78 ± 0.76 | 3.29 ± 0.32 |
| 10.26 | 136.062 | $C_5H_5N_5$ | 2.20 | Adenine | 0.28 ± 0.63 | 0 ± 0 | 2.75 ± 0.63 | 2.06 ± 0.77 |
| 10.27 | 405.009 | $C_9H_{14}N_2O_{12}P_2$ | 2.47 | Uridine 5'-diphosphate | 0.36 ± 0.8 | 0.01 ± 0.02 | 5.40 ± 1.05 | 4.22 ± 0.65 |
| 10.29 | 120.0656 | $C_4H_9NO_3$ | 4.16 | Homo-serine/Allothreonine | 1.13 ± 2.53 | 0.49 ± 1.09 | 4.86 ± 0.56 | 4.8 ± 0.87 |
| 10.43 | 134.0454 | $C_4H_7NO_4$ | 4.55 | Aspartate | 0.67 ± 1.49 | 0.43 ± 0.95 | 3.52 ± 0.87 | 3.63 ± 2.09 |
| 10.44 | 130.0498 | $C_5H_7NO_3$ | 4.61 | N-Acryloylglycine/Pyroglutamic acid | 61.17 ± 73.74 | 33.1 ± 26.85 | 130.1 ± 38.3 | 134.64 ± 29.43 |
| 10.46 | 97.0283 | $C_5H_4O_2$ | 7.21 | - | 0.80 ± 1.11 | 0.26 ± 0.35 | 5.37 ± 0.66 | 4.23 ± 1.12 |
| 10.68 | 112.0501 | $C_4H_5N_3O$ | 3.92 | Cytosine | 0.85 ± 1.66 | 0.13 ± 0.28 | 3.11 ± 0.76 | 3.43 ± 1.2 |

*Table 4.3 continued*

| Ret. Time (min) | *m/z* | Tentative Formula | *m/z* Error (ppm) | Tentative Assignment | $\bar{x}$ ± SD (Peak area) Untreated | E2 | Ethanol | TAM |
|---|---|---|---|---|---|---|---|---|
| 10.68 | 324.0596 | $C_9H_{14}N_3O_8P$ | 0.31 | Cytidine 2'-phosphate/Cytidine monophosphate | 1.29 ± 2.68 | 0.33 ± 0.54 | 5.35 ± 1.25 | 4.97 ± 1.67 |
| 10.72 | 152.0561 | $C_5H_5N_5O$ | 7.23 | Guanine/8-Hydroxyadenine/2-Hydroxyadenine | 0.77 ± 1.71 | 0.07 ± 0.07 | 10.26 ± 2.22 | 7.52 ± 3.13 |
| 10.82 | 613.1587 | $C_{20}H_{32}N_6O_{12}S_2$ | 1.79 | Oxidized glutathione | 19.23 ± 41.41 | 10.34 ± 19.1 | 58.25 ± 24.05 | 76.31 ± 21.33 |
| 10.85 | 489.1153 | $C_{14}H_{26}N_4O_{11}P_2$ | 1.81 | Citicoline | 6.19 ± 12.7 | 1.49 ± 3.04 | 28.89 ± 4.34 | 27.82 ± 8.52 |
| 10.89 | 162.1122 | $C_7H_{15}NO_3$ | 4.93 | Carnitine | 55.23 ± 31.08 | 27.53 ± 11.37 | 92.65 ± 13.43 | 90.31 ± 15.86 |
| 11.35 | 229.1538 | $C_{11}H_{20}N_2O_3$ | 6.10 | L-leucyl-L-proline/L-isoleucyl-L-proline | 0.41 ± 0.86 | 0.07 ± 0.11 | 5.38 ± 1.62 | 4.26 ± 0.98 |
| 11.68 | 146.1176 | $C_7H_{15}NO_2$ | 3.42 | 3-Dehydroxycarnitine | 2.17 ± 3.16 | 1.39 ± 1.21 | 8.97 ± 1.89 | 8.73 ± 1.15 |
| 12.8 | 206.0555[i] | $C_5H_6N_2O_3$ | 6.31 | 5-Hydroxymethyluracil | 4.29 ± 9.59 | 2.04 ± 4.57 | 19.38 ± 4.89 | 21.8 ± 5.68 |

Adduct - [a]Na, [b]$NH_3$ adduct, [c]K adduct, [d]CH3OH+H, [e]M+2Na-H, [f]2M+Na, [g]2M+K, [h]M+ACN+H, [i]M+ACN+Na

PE-Nme - Glycerophosphoethanolamines

PC - Glycerophosphocholines

PE - Glycerophosphoethanolamines

## 4.4 Conclusions

A $^{1}$H NMR and HILIC-MS based metabolomics approach was used to investigate the effect of adding hormonal drugs to an ASG5 apocrine cell lines. Both analytical methods produced high quality data in which untreated and the E2 treated cells could clearly be differentiated from the TAM and ethanol treated cells when coupled with MVA techniques. Furthermore, it was observed that TAM and ethanol could not be differentiated, thus, concluded that TAM produced no additional effect to that of the ethanol-treated cells. The differences in the metabolic profile between the different hormonal treatments observed both by $^{1}$H NMR spectroscopy and HILIC–MS were based on different sets of markers. Thus, this illustrates the complementary nature of the two analytical methodologies and demonstrates the potential value of applying both techniques to analyse biological samples.

Definitive identification of metabolites is a very challenging aspect of metabolomics for both NMR spectroscopy and LC-MS. Identification of metabolites detected by NMR was aided by the large body of information already available on human biological fluids. Thus, the information provided herein will provide a good base for the identification of apocrine sweat *in vivo*. In comparison, the available data for metabolite identification by LC-MS is less extensive and the identity of most of the metabolites contributing to the different treatments is currently unknown. However, a number of putative identifications (either metabolite ID or elemental composition) were achieved by relying on the mass accuracy of the QTOF system.

Limited conclusions could be drawn on the effect of hormonal drugs on the ASG5 cells; nevertheless, this is vastly more than is currently available in the literature. Currently, the ASG5 cells are not well characterised, thus, it is unknown whether TAM or E2 (as well as any other chemical mediators) will effect cell proliferation or have an effect on the androgen pathway. TAM would not be expected to interfere with androgen pathway directly since it inhibits the estrogen receptor, which is down stream of androgen synthesis. However, it is unsure whether the inhibition of the receptor may invoke a feedback response. Nonetheless, there is clear difference in the

cellular metabolism between the different hormone treatments which suggest that apocrine cells from males and females might behave differently due to the different levels of circulating hormones.

# Chapter 5

## 5 Metabolomic Investigations of Human Axillary Secretions

### 5.1 Introduction

The human axilla region is populated with two classes of sweat glands: the eccrine glands, which produce a watery secretion in response to heat, and the apocrine glands, which produce micro-droplets of a viscous secretion in response to emotional stress. The characteristic axillary odour (commonly referred to as "body odour") is generated when bacteria, predominately of the *Corynebacteria* and *Staphylococci* genera (Leyden *et al.*, 1981; Shehadeh and Kiligman, 1963), on the skin surface interact with apocrine secretions (Shelley *et al.*, 1953). The main contributors to axillary odour are unsaturated or hydroxylated branched fatty acids, (E)-3-methyl-2-hexenoic acid (3M2H) and 3-hydroxy-3-methyl-hexanoic acid (HMHA), with the former being the key component (Zeng *et al.*, 1991); sulfanylalkanols, particularly 3-methyl-3-sulfanylhexan-1-ol (3M3SH) (Gautschi *et al.*, 2007; Hasegawa *et al.*, 2004; Natsch *et al.*, 2004; Troccaz *et al.*, 2004); and the odoriferous steroids, 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol (Bird and Gower, 1981; Claus and Alsing, 1976). The precursor of the odorant acids have been shown to be secreted as glutamine conjugates, that are then cleaved specifically by a bacterial Zn-dependent $N_{\alpha}$-acyl-glutamine-aminoacylase (N-AGA) from *Corynebacteria* Ax20 (Natsch *et al.*, 2006; Natsch *et al.*, 2003). Sulfanylalkanols have been shown to be secreted as either a cysteine-(S) or cysteine-glycine-(S) conjugate, which are then cleaved by the sequential action of a bacterial dipeptidase and a cysteine β-lyase (Emter and Natsch, 2008; Starkenmann *et al.*, 2005). There is evidence that odoriferous steroids are formed from nonodoriferous steroid precursors, conjugated to a sulphate or glucuronic acid (Froebe *et al.*, 1990), as a result of bacterial metabolism (Decreau *et al.*, 2003; Gower *et al.*, 1997; Labows *et al.*, 1979).

These latest findings confirm the importance of bacterial activity and sweat odour precursors for the generation of body odours. It is commonly known that gender, ethnicity, emotional, physiological, and environmental factors may influence individual odours (Akutsu *et al.*, 2006; Dixon *et al.*, 2007; Penn *et al.*, 2007; Troccaz *et al.*, 2009). Furthermore, several studies have concluded that individuals have a distinct odour type, which may be used for mate selection (Wedekind and Furi, 1997; Wedekind *et al.*, 1995) as well as individual or kin recognition (Weisfeld *et al.*, 2003). Thus it is suggested that an individual's odour profile is partly determined by their major histocompatibility complex (also known as human leucocyte antigen region) (Roberts *et al.*, 2005), however, the underlying mechanism is currently unknown (Natsch *et al.*, 2010). Furthermore, MHC-dependent odour discrimination and mating preference have been presented in mice (Yamazaki *et al.*, 2000; Yamazaki *et al.*, 1976), reptiles (Olsson *et al.*, 2003), birds (Freeman-Gallant *et al.*, 2003) and fish (Reusch *et al.*, 2001), where MHC-dissimilarity may help to maximise the frequency of MHC-heterozygotes in their offspring, providing increasing resistance to disease and prevent inbreeding (Penn and Potts, 1999).

To date, the literature has limited information on the importance of skin bacteria or sweat composition in generating the characteristic axillary odour from an individual. Furthermore, most studies only consider the volatile composition or odour perception without considering their origin (i.e. left or right side) or do not specify whether samples come from one axilla or are pooled together from one individual or several donors. Side related differences have seldom been assessed in humans, and what has been reported is often contradictory. Bird and Gower found that high level of androstenone correlated with the handedness, except for one individual (Bird and Gower, 1982). Ferdenzi and co-workers reported that the left and right axillary are perceptually equivalent when sampling the general population, with only a significant difference in the left axilla in left-handed people (Ferdenzi *et al.*, 2009). However, they stated that this difference is likely to be attributed to the perception of female raters who did not use hormone-based contraception and who were likely to be in the fertile phase of their cycle (Ferdenzi *et al.*, 2009). This is consistent with the idea that there is increased awareness of stimuli between mating and fertilisation issues at the time of ovulation (Lundstrom *et al.*, 2006).

Axillary secretions and odours are of both a biological and commercial importance. Studies have shown that odours contain chemical cues that can affect an individual's mood (Jacob *et al.*, 2002; Preti *et al.*, 2003), brain activity (Jacob *et al.*, 2001; Savic *et al.*, 2001), or even alter length and timing of the human menstrual cycle (McClintock, 1978). In addition, the nature and biogenesis of these odorants that contribute to ones 'volatile metabolome', is a focus of a multibillion-pound consumer cosmetic industry, which seeks new products to counteract either odour production or perception.

In summary, the literature on apocrine sweat composition is biased towards the volatile components, thus, this chapter will use the methodologies developed in chapter two and three to provide further identification of the non-volatile components, through the use of in-house metabolite libraries and external databases. Particular emphasis will be on the odour precursors as these are considered key metabolites for odour production. Furthermore, pattern recognition techniques will be used to analyse the spectral data obtained from six volunteers, with each underarm collected separately, across five days in order to determine the inter-day or inter-individual variation.

## 5.2 Aims

- To identify metabolites present in human apocrine sweat, with emphasis on odour precursors, using NMR spectroscopy, HILIC-MS (QTOF) and LC-MS/MS (QTRAP) methodologies developed in chapters two and three.

- UPLC-Orbitrap-MS method initially developed by Dr Catherine Ortori will also be applied to the analysis of human apocrine sweat.

- To assess inter-subject variability in the measured profiles of odour precursors in apocrine sweat.

- To determine whether there are any intra-individual differences in the odour precursors measured in apocrine sweat from the left and right arm.

## 5.3 Materials and Methods

### 5.3.1 Chemicals

MS grade caffeine, sodium dodecyl sulfate, and sodium taurocholate were purchased from Sigma Aldrich (Poole, UK). Mass spec grade Ultramark 1621 and MRFA tetrapeptide was purchased from ABCR GmbH & Co. KG (Karlsruhe, Germany) and Research Plus Inc (Barnegat, NJ) respectively.

### 5.3.2 LC-MS Column

A Zorbax RRHD Eclipse Plus $C_{18}$ column (1.8 µm, 2.1 x 150 mm) was purchased from Agilent Technologies (Cheshire, UK). The column was equilibrated with mobile phase prior to use and washed as per manufacture recommendations.

### 5.3.3 Collection of Odourless Axillary Secretions

The collection of human apocrine secretions was carried out by Dr Mark Harker (Unilever, Port Sunlight) as follows: The axillae of six male volunteers who had a controlled wash in their underarms on the morning of day one with subsequent collections on morning of days two to five from both underarms, which were collected separately (right and left). After each collection their underarms were washed again. Thus, each collection point represents a 24 h time point between samples during the course of the week-long test. Both underarms were sampled using a Teflon cup and stick. The Teflon cup was placed on the skin in the underarm whilst panelists lay down with their arm raised. 1.5 ml water was applied to the buffer cup and the water was stirred with the Teflon stick for 60 s. This water was then collected using a pipette and stored on ice. This process was repeated and the two fractions were pooled. This process was then repeated for the other underarm. The two samples for each panelist were treated separately. The samples were spun at 14 000xg for 5 min to remove dead skin and bacteria. Samples were maintained at 4°C after collection until storage when they were kept at -80°C.

### 5.3.4 Sample Preparation

All samples were evaporated to dryness with a centrifugal evaporator (25°C, 2 h) and then re-suspended in 20 µl water. Each sample was aliquoted as follows: 5 µl aliquots into 1 mm NMR tubes with the addition of 3 µl deuterated phosphate buffer (pH* 6.0, 0.1 M) containing TSP (7.6 mM), 10 µl aliquots into MS vials with the addition of 85% acetonitrile, and the remaining 5 µl was pooled together in order to create QC samples with the addition of 85% acetonitrile for LC-MS analysis.

### 5.3.5 $^{1}$H NMR Spectroscopy for the Global Profiling of Axillary Secretions

Development of the methodology for the analysis of human apocrine gland sweat secretions is discussed in chapter two (Section 2.3.3). In summary, spectra were acquired on a Bruker Avance 400 spectrometer, operating at 400.13 MHz $^{1}$H observation frequency equipped with a 1 mm TXI micro-volume probe with an internal probe temperature of 298 K and with 256 transients being collected. All spectra were manually corrected for phase and baseline distortions using TopSpin™ 2.1 (Bruker GmbH,Germany) and chemical shifts referenced to TSP standard at δ0.00 ppm.

### 5.3.6 2D NMR Spectroscopy

To aid spectral assignment, 2D $^{1}$H-$^{1}$H COSY spectra were measured with solvent presaturation pulse sequence on each sample. Acquisition and processing parameters for the COSY spectra included a relaxation decay of 1.86 s, a spectra width in F1 and F2 of 5995.2 Hz, 2 k time domain points, 128 F1 increments, 64 transients per increment and qsine apodization in F1 and F2.

### 5.3.7 HILIC-QTOF-MS for the Global Profiling of Axillary Secretions

HILIC-QTOF-MS was used for a global profiling approach as previously described in chapter three (Section 3.2.4). In summary, data was acquired *m/z* 80-1000 in positive ion mode over 25 min.

### 5.3.8 UPLC-Orbitrap-MS for the Global Profiling of Axillary Secretions

In addition to using the HILIC-QTOF-MS developed specifically for the project, a benchtop Orbitrap mass spectrometer (Thermo Scientific Exactive), was used as another approach for global profiling. The orbitrap mass analyser has a higher resolution than that of most time-of-flight instruments (~10 000) and approaches that of FTICR (~100 000) to ensure high mass accuracy and to enable confident discrimination between co-eluting, isobaric compounds in complex samples. Such applications require mass accuracy of less than 5 ppm. Furthermore, the Exactive is capable of higher scan speeds and polarity switching without sacrificing mass accuracy. Thus, positive and negative spectra can be obtained in a single run.

All experiments were conducted on an Accela U-HPLC system equipped with quaternary pumps coupled to an Exactive orbitrap mass spectrometer (all from Thermo Scientific, Hemel Hempstead, UK), controlled by Xcalibur 2.1 software. The instrument was equipped with an ESI probe and was operated in positive-negative polarity switching mode. The spray voltage was set to 3 kV or -3 kV for positive or negative ion mode respectively. The sheath gas, auxiliary and sweep gas flows were set to 70, 40, and 2 respectively (arbitrary units) and the capillary temperature was set to 350°C. The instrument was operated in full scan mode from *m/z* 100-1000 and from *m/z* 50-200. This was in order to maintain mass accuracy at the high *m/z* end. It is a known limitation of the instrument where the last mass $\leq$ 20 x first mass. The mass spectrometer was calibrated according to the manufacturer's guidelines prior to use with either caffeine, MRFA tetrapeptide (Met-Arg-Phe-Ala), and Ultramark 1621 or sodium dodecyl sulfate, sodium taurocholate, and Ultramark 1621 in an acenonitrile:methanol:water solution containing 1% acetic acid for positive and negative mode respectively.

A 5 µl aliquot of apocrine sweat mixture was injected onto a Zorbax RRHD Eclipse Plus $C_{18}$ column (1.8 µm particle size, 2.1 x 150 mm) operated at 40°C, with all the eluent from the LC column being directly transferred into the ion source of the MS without post column splitting. The eluent solvents were [A] water with 0.1% formic acid and [B] acetonitrile and 0.1% formic acid. The gradient duration was 16 min and was based on an in-house method developed by Dr Catharine Ortori and is summarised in Table 5.1 below.

**Table 5.1** LC-MS/MS conditions for the analysis of apocrine sweat secretions. Mobile phase [A] water + 0.1% formic acid and [B] acetonitrile + 0.1% formic acid.

| Time (min) | Parameter [B] | Flow rate µl/min |
|---|---|---|
| 0 | 10 | 300 |
| 4 | 22 | 300 |
| 7 | 99 | 300 |
| 12 | 99 | 450 |
| 13 | 10 | 600 |
| 16 | 10 | 450 |

### 5.3.9 Conjugate Profiling using RP-HPLC-MS/MS

RP-HPLC-MS was used as a survey scanning method to detect amino acid conjugates without making prior assumption of identity as previously described in chapter three (Section 3.2.6). The precursor ion survey scanning method was split into two analytical replicates instead of three due to the limited sample volume. The Gln (*m/z* 147) and Cys (*m/z* 105) containing conjugates were monitored together within the same acquisition, while the Cys-Gly conjugate (*m/z* 179) were monitored separately. Thus, it must be noted that EPI spectra would only be available for the most intense ion, i.e. if Gln and a Cys conjugate co-eluted, EPI spectra will only be produced for the most concentrated metabolite.

### 5.3.10 Targeted Profiling using RP-HPLC-MS/MS

RP-HPLC-MS/MS was used to specifically target amino acid conjugates as previously described in chapter three (Section 3.2.6).

### 5.3.11 Data Analysis

Methodology for the pre-processing and subsequent data-analysis of the NMR, HILIC-MS and Q-TRAP data were carried out as discussed previously in chapter two (Section 2.3.5), chapter three (Section 3.2.8) and chapter four (Section 4.2.8). For the Exactive work, the raw data was analysed using Sieve version 1.0 (Thermo Scientific, UK); this application manager integrates peaks in the UPLC-MS data by using ChromAlign$^{TM}$ peak detection.

The ion intensities for each peak detected are then exported to Microsoft Excel 2007 to be normalised, within each sample, to the sum of the peak intensities of that sample. The resulting normalised peak intensities are then exported in to SIMCA P and analysed as discussed in chapter four (Section 4.2.9).

### 5.3.12 Compound Identification by LC-MS

Metabolite identification *via* LC-MS is based on two computational methods: matching MS/MS fragment patterns to spectral libraries or accurately measuring pseudomolecular ion to determine their *m/z* or chemical formula in order to extract potential structures from databases. In order to calculate the most likely and chemically correct formula, rules need to be followed and adhered to in order to constrain thousands of possible candidates. This work flow is discussed in chapter four (Section 4.2.10) and a recent review (Kind and Fiehn, 2007).

There are currently a range of freely and commercially accessible online databases, as summarised in Table 5.2. These databases can be searched by name, mass or structure and some have additional information, like MS fragmentation or metabolic pathways, which may provide further assistance with metabolite identification. The main databases used in this study were the biological databases such as HMDB or Lipid Maps, as these are specific to metabolites that have been reported in human biological samples. However, synthesized authentic standards are necessary for ultimate confirmation of an unknown metabolite identity. The confirmation of a metabolite's identity is not trivial and can be considered the most challenging and time consuming

process in metabolomics. Hence, the level of evidence in supporting a proposed metabolite identity is indicated by a one to three star rating for ease of interpretation in this chapter; one star, putative identification, two stars has common fragmentations ions to that of what would be expected, and three stars confirmed with a standard.

**Table 5.2** Some commonly used metabolomic databases used for metabolite identification.

| Name | Content |
|---|---|
| **Compound specific databases** | |
| PubChem | Chemical compounds and structures, links to Entrez databases, bioactivity data from high throughput screening programs |
| HMDB | A comprehensive database >7900 human metabolites obtained from NMR, GC-MS and MS/MS |
| Lipid Maps | Structure and annotations of >10,000 biological lipids in human and other mammals |
| KNApSAcK | A cross-species metabolite database of >28,500 compounds mostly from plants and microorganisms |
| MMD | A knowledgebase of 42,687 endogenous and exogenous metabolites |
| Drugbank | Drug, drug targets, physicochemical, clinical, and pharmacological properties, links to other databases, 3D and chemical structure |
| ChemSpider | ChemSpider is an aggregated database of organic molecules containing more than 20 million compounds from many different providers. At present the database contains information from such diverse sources as a marine natural products database, ACD-Labs chemical databases, the EPAs DSSTox databases and from a series of chemical vendors |
| **Reference databases** | |
| NIST 08 | A commerical library of >220,000 EI mass spectra from >190,000 pure chemical compounds |
| GMD | Mass spectra and retention indices of known plant metabolites analysed by GC-MS |
| METLIN | A database of LC-MS, LC-MS/MS, and LC-FTMS mass spectra of matabolites from human and microbial species. It contains >25,000 structures, including more than 8000 di and tripeptides. |
| MassBank | A high resolution MS/MS spectral database for standard chemical substances. More than 30,000 spectra from >1900 different compounds are available |
| MS2T | A MS/MS spectral tag library of phytochemical compounds |
| MMCD | A NMR and LC-MS spectral library of metabolite standards. It contains approximately >20,000 metabolite entries and experimental spectral data on >600 compounds |
| Fiehn GC-MS Database | This library contains data on 713 compounds (name, structure, CAS ID, other links) for which GC/MS data (spectra and retention indices) have been collected by the Fiehn laboratory |
| **Pathway specific databases** | |
| KEGG | A composite database consisting of collections of pathway maps, genes, organisms, enzymes and ligands (metabolites, drugs and other small molecules) |
| BioCyc | A collection of 1004 pathway/genome databases of organisms with completely or partially sequenced genomes |
| EcoCyc | A scientific database of Escherichia coli K-12 MG1655 |
| MetaCyc | A database of nonredundant, experimentally elucidated metabolic pathways on >1,600 pathways from >2000 different organisms |
| HumanCyc | A human metabolic pathway and human genome database on 28,783 genes, their products and the metabolic reactions and pathways they catalyze |
| Reactome | A database of human metabolic pathways and biological processess involving small metabolites, biomolecules and their reactions/interactions. Reactome has data and pathway diagrams for >2700 proteins, 2800 reactions and 860 pathways for humans |

## 5.4 Results and Discussion

### 5.4.1 Identification of Small Molecule Components of Human Apocrine Sweat

#### 5.4.1.1 Global Analysis using $^1$H NMR Spectroscopy

To my knowledge, this is the first report of applying NMR spectroscopy to the global profiling of human apocrine sweat. The results obtained in the present study confirm that NMR based metabolomics can be successfully applied to the metabolic profiling of apocrine gland sweat. A typical 400 MHz $^1$H NMR spectrum of human apocrine sweat acquired using a 1 mm micro-volume probe is depicted in Figure 5.1. The increased sensitivity obtained from using a micro-volume probe has allowed 25 metabolites to be identified. Many resonances have been assigned by comparison with existing literature (Harker *et al.*, 2006; Lindon *et al.*, 2000; Nicholson *et al.*, 1995) in combination with an in-house spectral database and are tabulated (Table 5.3), together with concentrations of metabolites found in a typical sample. The major classes of metabolites identified included TCA cycle intermediates (e.g. citric acid, formic acid), ketone bodies (3-hydroxybutyrate), energy metabolites (creatinine, lactate), amino acids and nucleotide derivatives. Lactic acid was found to be the most abundant metabolite, which is consistent with observations made previously following spectroscopic analysis of human eccrine sweat (Harker *et al.*, 2006). A number of unassignable compounds or tenatively assignable compounds were also observed. In particular, the chemical shifts downfield (aromatic region) are presumed to be nucleotides or nucleotide derivatives. Furthermore, from the 2D $^1$H-$^1$H COSY spectra, it was shown that the doublets observed at 6.41 ppm are coupled with doublets at 7.32 ppm, indicating an AX spin system.

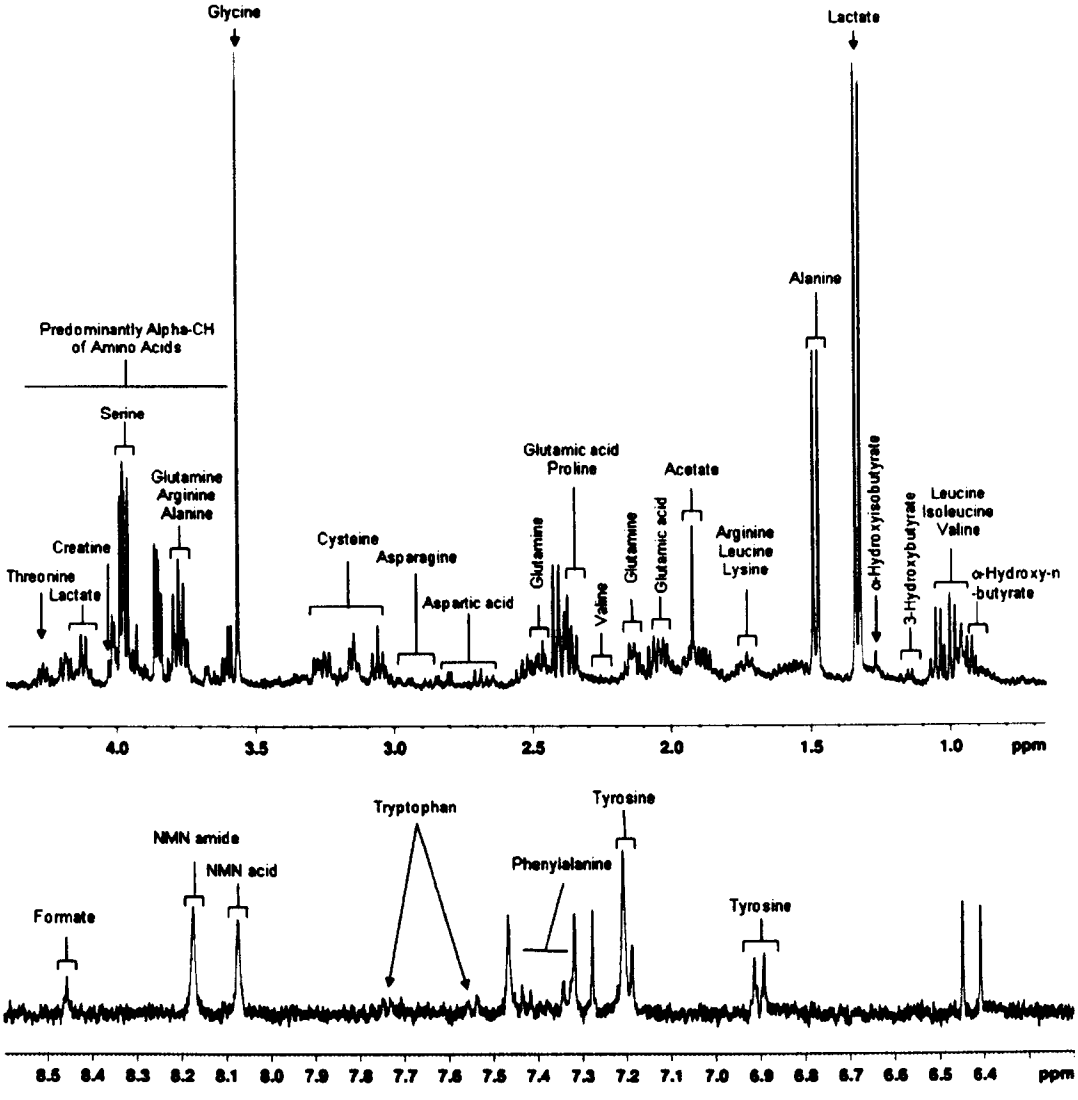



**Figure 5.1** Typical 400 MHz $^{1}$H NMR spectrum of human apocrine sweat obtained using 1 mm micro-volume probe. Some metabolites are tentatively assigned. Spectra were acquired using a standard noesy preset pulse sequence with 256 scans.

**Table 5.3** Peaks assigned to metabolites in apocrine sweat and experimentally determined concentrations. No corrections for difference in $T_1$ relaxation have been made.

| Chemical Shift | Assignment | Metabolite | Typical Concentration (mM) |
|---|---|---|---|
| 0.90 (t)[a] | $CH_3$ | α–hydroxy-n-butyrate[b] | - |
| 0.92 (t) | δ-$CH_3$ | Isoleucine | 1.9 ± 1 (0.9 - 3.8) |
| 0.99 (d) | β-$CH_3$ | | |
| 0.96 (t) | δ-$CH_3$ | Leucine | 1.2 ± 0.6 (0.7 - 2.1) |
| 1.72 (m) | $CH_2$ | | |
| 3.72 (m) | α-CH | | |
| 0.98 (d) | $CH_3$ | Valine | 0.9 ± 0.3 (0.5 - 1.4) |
| 1.03 (d) | $CH_3$ | | |
| 2.26 (m) | β-CH | | |
| 3.59 (d) | α-CH | | |
| 1.14 (d) | $CH_3$ | Isobutyrate[b] | - |
| 1.18 (d) | $CH_3$ | 3-β-hydroxybutyrate[b] | 0.2 ± 0.1 (0.1 - 0.5) |
| 2.28 (ABX) | $CH_2$ | | |
| 1.26 (s) | $CH_3$ | α-hydroxyisobutyrate[b] | 1.8 ± 1 (0.9 - 3.4) |
| 1.33 (d) | $CH_3$ | Lactate | 13.2 ± 13.8 (2.4 - 44.8) |
| 4.11 (q) | CH | | |
| 1.48 (d) | $CH_3$ | Alanine | 2.4 ± 1.1 (1.2 - 4.2) |
| 1.48 (m) | γ-$CH_2$ | Lysine | - |
| 1.73 (m) | δ-$CH_2$ | | |
| 1.90 (m) | β-$CH_2$ | | |
| 3.04 (t) | ε-$CH_2$ | | |
| 1.56 (m) | γ-$CH_2$ | Citrullene[b] | - |
| 1.86 (m) | β-$CH_2$ | | |
| 3.13 (t) | α-$CH_2$ | | |
| 3.74 (t) | α-$CH_2$ | | |
| 1.92 (s) | $CH_3$ | Acetate | 1.9 ± 0.8 (0.9 - 3.1) |
| 2.03 (m) | γ-$CH_2$ | Proline | - |
| 2.36 (m)[a] | β-$CH_2$ | | |
| 3.35 (m) | δ-$CH_2$ | | |
| 3.43 (m) | δ-$CH_2$ | | |
| 4.15 (m)[a] | α-CH | | |

*Table 5.3 continued*

| Chemical Shift | Assignment | Metabolite | Typical Concentration (mM) |
|---|---|---|---|
| 2.07 (m)[a] | β-$CH_2$ | Glutamate | 6.8 ± 3.7 (3 - 12.8) |
| 2.35 (m) | γ-$CH_2$ | | |
| 2.15 (m)[a] | β-$CH_2$ | Glutamine | 2.2 ± 1.4 (0.9 - 4.1) |
| 2.46 (m) | γ-$CH_2$ | | |
| 2.51 (AB)[a] | ½ $CH_2$ | Citrate | - |
| 2.65 (AB)[a] | ½ $CH_2$ | | |
| 2.68 (ABX) | β-$CH_2$ | Aspartate | 0.2 ± 0.1 (0.1 - 0.4) |
| 2.82 (ABX) | β-$CH_2$ | | |
| 3.90 (ABX) | α-CH | | |
| 3.04 (s) | $CH_3$ | Creatine | 0.2 ± 0.1 (0.1 - 0.5) |
| 4.02 (s) | $CH_2$ | | |
| 3.31 (ABX) | $CH_2$ | Tyrptophan | 0.5 ± 0.4 (0.1 - 1) |
| 3.49 (ABX) | $CH_2$ | | |
| 4.06 (ABX) | CH | | |
| 7.21 (t) | C5H | | |
| 7.29 (t) | C6H | | |
| 7.33 (s) | C2H | | |
| 7.55 (d) | C7H | | |
| 7.74 (d) | C4H | | |
| 3.56 (s) | $CH_2$ | Glycine | 3.2 ± 1.5 (1.5 - 5.9) |
| 3.59 | α-CH | Threonine | 1.7 ± 0.9 (0.7 - 3.1) |
| 4.26 (m) | β-CH | | |
| 3.85 (dd) | α-CH | Serine | 3.7 ± 1.8 (1.7 - 6.4) |
| 3.95 (dd) | β-$CH_2$ | | |
| 4.00 (dd) | β-$CH_2$ | | |
| 6.90 (d) | H3/H5 | Tyrosine | 0.4 ± 0.2 (0.2 - 0.7) |
| 7.19 (d) | H2/H6 | | |
| 7.33 (m) | H2/H6 | Phenylalanine | 0.3 ± 0.1 (0.2 - 0.5) |
| 7.39 (m) | H4 | | |
| 7.43 (m) | H/H5 | | |
| 8.46 (s) | CH | Formate | 0.6 ± 0.4 (0.2 - 1.4) |

[a]Partially obscured resonances, [b]Tentatively assigned
Multiplicity is indicated as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet

### 5.4.1.2 Global Analysis using High Mass Accuracy LC-MS (HILIC and $C_{18}$)

HILIC methodology was developed to identify and characterise the aqueous fraction of apocrine sweat secretions, as discussed in chapter 3. Figure 5.2 shows a 2D LC-MS map (horizontal axis: retention time, vertical axis: *m/z*) for a typical QC sample acquired in positive ESI mode. A total of 580 peaks were detected with an average cumulative variation of 103% across all QC samples. This variation is considered to be large; indicating the method was not performing as expected. Only 37 metabolites had a CV of less than 25%. Altering the processing parameters, such as minimum peak intensity, to that of a higher value, e.g. 600 counts, the number of extracted peaks dropped to 190 metabolites with an average CV of 91.5%. This error also incorporates any error or inconsistencies from peak integration during the pre-processing stage. Therefore, it is considered that there was a sensitivity detection issue with the instrument which would cause this high CV error. Nonetheless, valuable information can still be obtained from this data set in regards to the presence of amino acid conjugates.

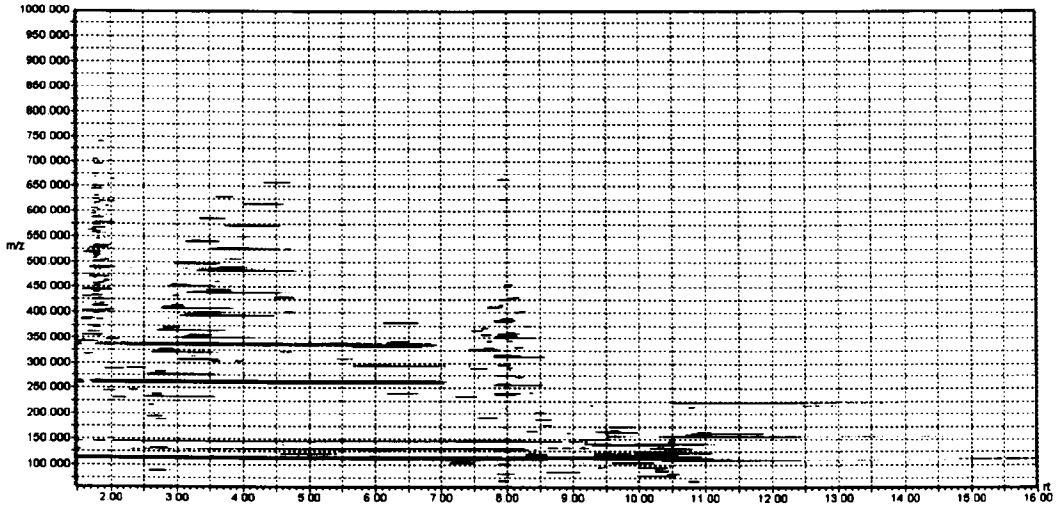



**Figure 5.2** Two dimensional mass versus retention 'map' (*m/z* 80-1000) obtained from QTOF-HILIC-MS analysis of a human apocrine sweat sample. In this typical chromatogram, ~580 ions can be detected in 15 min.

An 'in-house' metabolomic method, previously developed in our group, using a RP-UPLC $C_{18}$ column coupled to an Orbitrap-MS was used for analysis of semi-polar/non-polar fraction. A typical total ion chromatogram obtained from a representative QC apocrine sweat sample is depicted in Figure 5.3 for both positive and negative ESI mode. The initial pre-processing of the UPLC-MS data using Sieve, extracted 1359 peaks with an average CV of 36% and 988 peaks with an average CV of 23%, across all QC samples in positive and negative mode respectively. Using the information gathered from the literature about the composition of human apocrine sweat (see Appendix A), putative identification of the odour precursors are summarised Table 5.4, while putative identification of known metabolites previously reported in apocrine sweat are summarised in Table 5.5.

Identification of the amino acid conjugates was based on using the accuracy of the instrument in order to extract the individual peaks from the TIC. In total, nine amino acid conjugates were shown to be detected with both the HILIC-QTOF and UPLC-Orbitrap methodologies, with 3M2H-Gln and HMHA-Gln being the most abundant conjugates detected. N-α-9-hydroxy- non-anoyl- L-glutamine, N-α-3-hydroxy-3-methyloct anoyl- L-glutamine and N-α-3-hydroxy-4-methyloct anoyl- L-glutamine (where each metabolite has a corresponding *m/z* 303.1927) all produce one chromatographic peak at 7.12 min, thus, no further identification is possible. 3M3SH-Cys-Gly was detected with the HILIC methodology, however, was marginally above the limit of detection. S-[1-(2-hydroxyethyl)-butyl]-L-cysteinylglycine (*m/z* 279.1379) produced two chromatographic peaks at 6.07 min and 8.53 min. The mass error at 6.07 min is 20.42 ppm, thus, is likely the $Na^+$ adduct of 3M2H-Gln, while the peak at 8.53 min is marginally above the limit of detection in the HILIC method. Furthermore, S-[1-(2-hydroxyethyl)-butyl]-L-cysteinylglycine produced several chromatographic peaks (ranging from 1.39-12.89 min) from the UPLC $C_{18}$ method in negative mode only. Again, all the representative peaks were only marginally above the limit of detection, with the most intense peak eluting at 11.29 min, thus, no retention time can be reliably obtained.

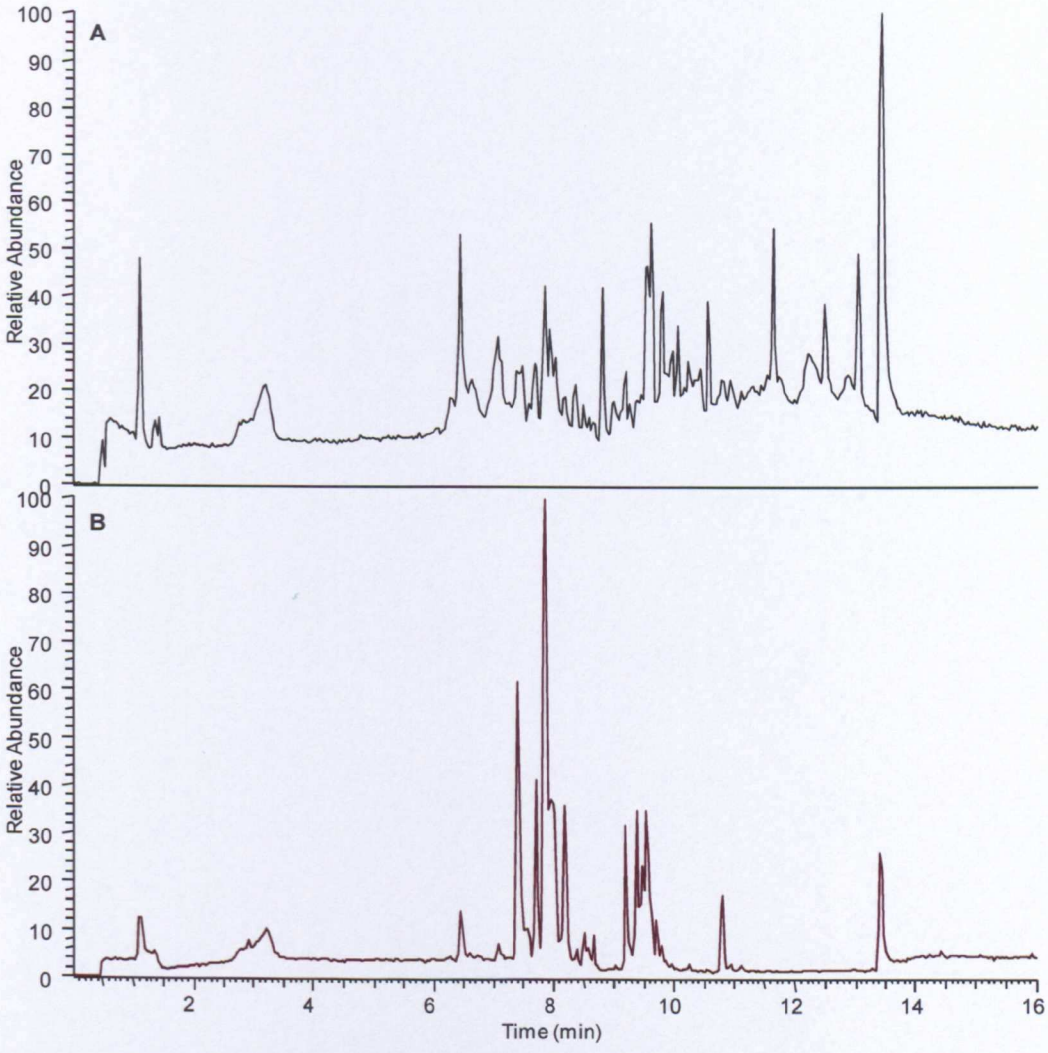



**Figure 5.3** UPLC-MS total ion chromatogram acquired in **A** positive mode **B** negative mode, of a representative human apocrine sweat sample.

**Table 5.4** Summary of the HILIC and UPLC retention times and HR mass spectra of the principle odour precursors in both positive and negative ion mode

| Tentative ID | Formula | HILIC-QTOF-MS | | | UPLC-Orbitrap-MS | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Positive mode | | Retention Time (min) | Positive mode | | Negative mode | | Retention Time (min) |
| | | Detected *m/z* | Delta (ppm) | | Detected *m/z* | Delta (ppm) | Detected *m/z* | Delta (ppm) | |
| **Gln Conjugates** | | | | | | | | | |
| N-α-3-hydroxy-3-methylhexanoyl- L-glutamine | $C_{12}H_{22}N_2O_5$ | 275.1612[b] | 1.81 | 8.02 | 297.1424[a] | 1.32 | 273.1452 | -1.32 | 3.16 |
| N-α-3-methylhex-2-enoyl-L-glutamine | $C_{12}H_{20}N_2O_4$ | 279.1322[a] | 0.34 | 6.07 | 279.1319[a] | 1.32 | 255.1346 | -1.76 | 6.45 |
| N-α-4-methyl-3-oct-enoyl- L-glutamine | $C_{14}H_{24}N_2O_4$ | 285.1821 | 2.45 | 4.71 | 307.1632[a] | 1.04 | 283.1661 | -1.00 | 7.13 |
| N-α-4-ethyl-hept-anoyl- L-glutamine | $C_{14}H_{25}N_2O_4$ | 287.2002 | 10.79 | 8.08 | - | - | 285.1814 | -2.16 | 7.21 |
| N-α-3-hydroxy- 3-methylhept-anoyl- L-glutamine | $C_{13}H_{24}N_2O_5$ | 289.1757[b] | -2.07 | 7.58 | 311.1579[a] | 0.40 | 287.1610 | -1.03 | 6.25 |
| N-α-3-hydroxy-oct-anoyl- L-glutamine | $C_{13}H_{24}N_2O_5$ | 289.1757[b] | -2.07 | 7.58 | 311.1579[a] | 0.40 | 287.1610 | -1.03 | 6.25 |
| N-α-8-hydroxy-oct-anoyl- L-glutamine | $C_{13}H_{24}N_2O_5$ | 289.1757[b] | -2.07 | 7.58 | 311.1579[a] | 0.40 | 287.1610 | -1.03 | 6.25 |
| N-α-3-hydroxy-4-methylhept anoyl- L-glutamine | $C_{13}H_{24}N_2O_5$ | 289.1757[b] | -2.07 | 7.58 | 311.1579[a] | 0.40 | 287.1610 | -1.03 | 6.25 |
| N-α-4-ethyl-oct-anoyl- L-glutamine | $C_{15}H_{28}N_2O_4$ | 301.2129 | 0.66 | 4.33 | 323.1949[a] | 2.25 | 299.1974 | -0.91 | 7.49 |
| N-α-9-hydroxy- non-anoyl- L-glutamine | $C_{14}H_{26}N_2O_5$ | 303.1927 | 2.31 | 7.12 | 325.1736[a] | 0.68 | 301.1765 | -1.37 | 6.77 |
| N-α-3-hydroxy-3-methyloct anoyl- L-glutamine | $C_{14}H_{26}N_2O_5$ | 303.2023 | 2.31 | 7.12 | 325.1736[a] | 0.68 | 301.1765 | -1.37 | 6.77 |
| N-α-3-hydroxy-4-methyloct anoyl- L-glutamine | $C_{14}H_{26}N_2O_5$ | 303.2023 | 2.31 | 7.12 | 325.1736 | 0.68 | 301.1765 | -1.37 | 6.77 |
| **Cys-Gly Conjugates** | | | | | | | | | |
| S-[1-(2-hydroxyethyl)-butyl]-L-cysteinylglycine | $C_{11}H_{22}N_2O_4S$ | 279.1368 | 8.53 | -3.94 | - | - | - | - | - |
| S-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cysteinylglycine | $C_{12}H_{24}N_2O_4S$ | 293.1534 | 8.45 | -0.34 | - | - | - | - | - |

[a]$Na^+$ adduct

[b]$Na^+$ detected but less dominant than pseudomolecular ion

**Table 5.5** Tentative identities of known compounds previously reported in apocrine sweat.

| | | Positive mode | | | Negative mode | | |
|---|---|---|---|---|---|---|---|
| **Compound Name** | **Formula** | **Detected *m/z*** | **Delta (ppm)** | **Actual RT** | **Detected *m/z*** | **Delta (ppm)** | **Actual RT** |
| α-Pinene | $C_{10}H_{16}$ | 137.1327 | 1.78 | 7.16 | - | - | - |
| (4-Hydroxyphenyl)acetic acid | $C_8H_8O_3$ | 153.0548 | 1.33 | 8.23 | - | - | - |
| Geranial | $C_{10}H_{16}O$ | 153.1274 | 0.32 | 7.40 | - | - | - |
| Geraniol | $C_{10}H_{18}O$ | 155.1433 | 1.93 | 7.16 | - | - | - |
| Isopropylacetophenone | $C_{11}H_{14}O$ | 163.1120 | 1.58 | 7.94 | - | - | - |
| 2-Phenylethyl acetate | $C_{10}H_{12}O_2$ | 165.0912 | 1.18 | 9.17 | - | - | - |
| Eugenol | $C_{10}H_{12}O_2$ | 165.0912 | 1.18 | 9.17 | - | - | - |
| Isoeugenol | $C_{10}H_{12}O_2$ | 165.0912 | 1.18 | 9.17 | - | - | - |
| Jasmone | $C_{11}H_{16}O$ | 165.1276 | 1.13 | 7.35 | - | - | - |
| (Z)-4-Methylnon-3-enoic acid | $C_{10}H_{18}O_2$ | 171.1381 | 0.79 | 10.37 | - | - | - |
| (E)-4-Methylnon-3-enoic acid | $C_{10}H_{18}O_2$ | 171.1381 | 0.79 | 10.37 | - | - | - |
| 9-Decenoic acid | $C_{10}H_{18}O_2$ | 171.1381 | 0.79 | 10.37 | - | - | - |
| Octanedioic acid (suberic acid) | $C_8H_{14}O_4$ | 175.0964 | -0.72 | 9.88 | - | - | - |
| E-Cinnamyl acetate | $C_{11}H_{12}O_2$ | 177.0913 | 1.79 | 8.04 | - | - | - |
| 10-Undecenoic acid | $C_{11}H_{20}O_2$ | 185.1539 | 1.34 | 7.35 | - | - | - |
| 2-Hexyl 2-pentenoate | $C_{11}H_{20}O_2$ | 185.1539 | 1.34 | 7.35 | - | - | - |
| 1-Dodecene | $C_{12}H_{24}$ | 186.2219 | 1.27 | 8.59 | - | - | - |
| Citronellol acetate | $C_{12}H_{22}O_2$ | 199.1695 | 0.97 | 8.82 | - | - | - |
| Farnesene | $C_{15}H_{24}$ | 205.1954 | 1.59 | 12.26 | - | - | - |
| Pentyl salicylate | $C_{12}H_{16}O_3$ | 209.1175 | 1.15 | 8.23 | - | - | - |
| Methyl trans-jasmonate | $C_{12}H_{18}O_3$ | 211.1331 | 0.95 | 6.78 | - | - | - |
| Cyclotetradecane | $C_{14}H_{28}$ | 214.2532 | 1.37 | 7.65 | - | - | - |
| 1-Tetradecene | $C_{14}H_{28}$ | 214.2532 | 1.37 | 7.65 | - | - | - |
| N,N-Dimethyl-1-dodecylamine | $C_{14}H_{31}N$ | 214.2532 | 1.37 | 7.65 | - | - | - |
| 1-Hexenyl salicylate | $C_{13}H_{16}O_3$ | 221.1172 | -0.01 | 9.07 | - | - | - |
| Tetradecanal | $C_{14}H_{28}O$ | 230.2481 | 1.16 | 7.69 | - | - | - |
| 2-Tetradecanone | $C_{14}H_{28}O$ | 230.2481 | 1.16 | 7.69 | - | - | - |
| 2-Phenylundecane | $C_{17}H_{28}$ | 233.2269 | 2.17 | 12.22 | - | - | - |
| 3-Phenylundecane | $C_{17}H_{28}$ | 233.2269 | 2.17 | 12.22 | - | - | - |
| 4-Phenylundecane | $C_{17}H_{28}$ | 233.2269 | 2.17 | 12.22 | - | - | - |
| 5-Phenylundecane | $C_{17}H_{28}$ | 233.2269 | 2.17 | 12.22 | - | - | - |
| 6-Phenylundecane | $C_{17}H_{28}$ | 233.2269 | 2.17 | 12.22 | - | - | - |
| 2-Pentadecanone | $C_{15}H_{30}O$ | 244.2639 | 1.61 | 7.65 | 225.2216 | -3.30 | 9.99 |
| 9-Hexadecenoic acid | $C_{16}H_{30}O_2$ | 255.2321 | 1.08 | 7.97 | 253.2168 | -1.94 | 10.25 |
| a Hexadecadienol | $C_{16}H_{30}O$ | 256.2639 | 1.54 | 7.62 | - | - | - |
| Hexadecanal | $C_{16}H_{32}O$ | 258.2795 | 1.55 | 7.59 | - | - | - |
| 2-Hexadecanone | $C_{16}H_{32}O$ | 258.2795 | 1.55 | 7.59 | - | - | - |
| 2-Phenyldodecane | $C_{18}H_{30}$ | 264.2688 | 0.73 | 12.22 | - | - | - |
| 5-Phenyldodecane | $C_{18}H_{30}$ | 264.2688 | 0.73 | 12.22 | - | - | - |
| 6-Phenyldodecane | $C_{18}H_{30}$ | 264.2688 | 0.73 | 12.22 | - | - | - |
| 9-Heptadecenoic acid | $C_{17}H_{32}O_2$ | 269.2480 | 1.67 | 8.13 | 267.2325 | -1.53 | 10.68 |
| 7-Hexadecenoic acid methyl ester | $C_{17}H_{32}O_2$ | 269.2480 | 1.67 | 8.13 | 267.2325 | -1.53 | 10.68 |
| 9-Hexadecenoic acid methyl ester | $C_{17}H_{32}O_2$ | 269.2480 | 1.67 | 8.13 | 267.2325 | -1.53 | 10.68 |
| Androsta-4, 16-dien-3-one | $C_{19}H_{26}O$ | 271.2060 | 1.18 | 7.85 | - | - | - |
| Androsta-5, 16-dien-3-one | $C_{19}H_{26}O$ | 271.2060 | 1.18 | 7.85 | - | - | - |

*Table 5.5 continued*

| | | Positive mode | | | Negative mode | | |
|---|---|---|---|---|---|---|---|
| **Compound Name** | **Formula** | **Detected *m/z*** | **Delta (ppm)** | **Actual RT** | **Detected *m/z*** | **Delta (ppm)** | **Actual RT** |
| 5a-Androst-16-en-3-one | $C_{19}H_{28}O$ | 273.2216 | 1.08 | 7.40 | - | - | - |
| Androsta-4, 16-dien-3α-ol | $C_{19}H_{28}O$ | 273.2216 | 1.08 | 7.40 | - | - | - |
| 5α-Androsta-5, 16-dien-3β-ol | $C_{19}H_{28}O$ | 273.2216 | 1.08 | 7.40 | - | - | - |
| Androsta-5, 16-dien-3a-ol | $C_{19}H_{28}O$ | 273.2216 | 1.08 | 7.40 | - | - | - |
| Hexadecanoic acid | $C_{16}H_{32}O_2$ | 274.2744 | 1.31 | 7.46 | 255.2324 | -2.14 | 10.94 |
| Methyl 9-methyltetradecanoate | $C_{16}H_{32}O_2$ | 274.2744 | 1.31 | 7.46 | 255.2324 | -2.14 | 10.94 |
| Pentadecanoic acid methyl ester | $C_{16}H_{32}O_2$ | 274.2744 | 1.31 | 7.46 | 255.2324 | -2.14 | 10.94 |
| Ethyl tetradecanoate | $C_{16}H_{32}O_2$ | 274.2744 | 1.31 | 7.46 | 255.2324 | -2.14 | 10.94 |
| Oleic acid | $C_{18}H_{34}O_2$ | 283.2634 | 0.96 | 8.33 | 281.2482 | -1.55 | 11.10 |
| 1-Nonadecene | $C_{19}H_{38}$ | 284.3315 | 1.18 | 8.46 | - | - | - |
| Heptadecanoic acid | $C_{17}H_{34}O_2$ | 288.2901 | 1.26 | 7.65 | 269.2482 | -1.50 | 11.29 |
| 2-Methylhexadecanoic acid | $C_{17}H_{34}O_2$ | 288.2901 | 1.26 | 7.65 | 269.2482 | -1.50 | 11.29 |
| Hexadecanoic acid methyl ester | $C_{17}H_{34}O_2$ | 288.2901 | 1.26 | 7.65 | 269.2482 | -1.50 | 11.29 |
| Ethyl pentadecanoate | $C_{17}H_{34}O_2$ | 288.2901 | 1.26 | 7.65 | 269.2482 | -1.50 | 11.29 |
| 5a-Dihydrotestosterone | $C_{19}H_{30}O_2$ | 291.2319 | 0.26 | 9.56 | - | - | - |
| Dodecyl benzoate | $C_{19}H_{30}O_2$ | 291.2319 | 0.26 | 9.56 | - | - | - |
| Cyclopentanetridecanoic acid methyl ester | $C_{19}H_{36}O_2$ | 297.2791 | 0.93 | 8.56 | 295.2636 | -2.28 | 11.69 |
| Octadecanoic acid | $C_{18}H_{36}O_2$ | 302.3059 | 1.73 | 7.82 | 283.2639 | -1.41 | 12.18 |
| Heptadecanoic acid methylester | $C_{18}H_{36}O_2$ | 302.3059 | 1.73 | 7.82 | 283.2639 | -1.41 | 12.18 |
| Ethyl hexadecanoate | $C_{18}H_{36}O_2$ | 302.3059 | 1.73 | 7.82 | 283.2639 | -1.41 | 12.18 |
| Dodecyl hexanoate | $C_{18}H_{36}O_2$ | 302.3059 | 1.73 | 7.82 | 283.2639 | -1.41 | 12.18 |
| Decyl octanoate | $C_{18}H_{36}O_2$ | 302.3059 | 1.73 | 7.82 | 283.2639 | -1.41 | 12.18 |
| Tridecyl benzoate | $C_{20}H_{32}O_2$ | 305.2480 | 1.57 | 7.51 | - | - | - |
| Isooctanedioldibutyrate | $C_{16}H_{30}O_4$ | 309.2035 | -0.55 | 9.81 | 285.2068 | -1.21 | 7.09 |
| Hexadecanoic acid isopropyl ester | $C_{19}H_{38}O_2$ | 316.3215 | 1.57 | 8.01 | - | - | - |
| Ethyl heptadecanoate | $C_{19}H_{38}O_2$ | 316.3215 | 1.57 | 8.01 | - | - | - |
| Propanedioic acid dimethyl ester | $C_5H_8O_4$ | - | - | - | 114.0087 | 1.92 | 8.97 |
| Methyl N,N-diethylthiocarbamate | $C_6H_{13}NOS$ | - | - | - | 146.0642 | -2.15 | 6.44 |
| 10-Methyltridecanoic acid | $C_{14}H_{28}O_2$ | - | - | - | 227.2011 | -2.66 | 10.03 |
| Tetradecanoic acid | $C_{14}H_{28}O_2$ | - | - | - | 227.2011 | -2.66 | 10.03 |
| Dodecanoic acid | $C_{14}H_{28}O_2$ | - | - | - | 227.2011 | -2.66 | 10.03 |
| Tridecanoic acid methyl ester | $C_{14}H_{28}O_2$ | - | - | - | 227.2011 | -2.66 | 10.03 |
| 9-Pentadecenoic acid | $C_{15}H_{28}O_2$ | - | - | - | 239.2011 | -2.33 | 9.86 |
| Pentadecanoic acid | $C_{15}H_{30}O_2$ | - | - | - | 241.2168 | -2.29 | 10.45 |
| n -Methyltetradecanoic acid | $C_{15}H_{30}O_2$ | - | - | - | 241.2168 | -2.29 | 10.45 |
| Tetradecanoic acid methyl ester | $C_{15}H_{30}O_2$ | - | - | - | 241.2168 | -2.29 | 10.45 |
| Isopropyl dodecanoate | $C_{15}H_{30}O_2$ | - | - | - | 241.2168 | -2.29 | 10.45 |
| 2-Ethylhexyl salicylate | $C_{15}H_{22}O_3$ | - | - | - | 249.1492 | -1.80 | 9.35 |
| Dodecyl octanoate | $C_{20}H_{40}O_2$ | - | - | - | 311.2955 | -0.17 | 9.38 |
| 17-Oxo-5-androsten-3a-yl sulfate | $C_{19}H_{28}O_5S$ | - | - | - | 367.1580 | -1.30 | 7.86 |
| 17-Oxo-5a-androstan-3a-yl sulfate | $C_{19}H_{30}O_5S$ | - | - | - | 369.1736 | -1.53 | 7.38 |

The associated error with respect to the tentative identities reported in Table 5.5 are generally less than 2 ppm, however, would need to be confirmed by authenticated standards. Moreover, the metabolites that were detected in both positive and negative mode report different retention times, thus, it is likely that another unknown metabolite has the same *m/z* ratio. No further identification can be made from the data collected.

The high mass accuracy (<5 ppm) of the Exactive instrument was exploited in order to use the accurate masses of parent ions for databases searches (see Section 5.3.12) in order to find tentative identities to the known unknown[2] metabolites. The result of these searches are summarised in Appendix C, in which 473 metabolites were putatively identified. This generally works well when the composition of biological fluid is well characterised (i.e. plasma). However, when analysing biological fluids where the composition is poorly known (i.e. apocrine sweat), highly variable or limited information is only available. As a result, only ~21% of the total extracted data from Sieve produced any potential matches. Furthermore, each of the searched *m/z* often produced several possible metabolites hits. This can be reduced by including restriction on the elemental composition i.e. C, H, O, N, S and P, hydrogen/carbon ratios, heuristic chemical structure and bonding rules and isotopic abundances, as discussed in Tobias Kind and Oliver Fiehn (Kind and Fiehn, 2007). However, the volume of unknown unknowns[3] highlight the fact that LC-MS metabolite databases are currently lagging behind NMR and GC-MS, which is a view echoed by others (Williams *et al.*, 2005b). One approach in providing identities of these unknown unknowns is to use hyphenated techniques e.g. LC-NMR-MS.

#### 5.4.1.3 Semi-Targeted analysis using LC-MS Survey Scanning

As mentioned previously, precursor ion survey scan coupled with EPI spectra has the potential to identify common structural moieties, such as amino acid conjugates. This

[2] Known unknown corresponds to metabolites that are known, but have not been formally identified in a biological mixture.

[3] Unknown unknown corresponds to metabolites that are truly novel and have never been identified before.

is achieved by monitoring for a specific fragment; Gln (*m/z* 147), Cys (*m/z* 105), and Cys-Gly (*m/z* 179). Using precursor ion survey scan coupled with full product ion spectra, a number of known and unknown amino acid conjugates were detected. In order to provide tentative assignments to the unknowns, where standards were not available, a simple set of rules were followed. Firstly, the amino acid moiety is known, thus, the FA fragment can be calculated as the remainder of the *m/z* ratio. Secondly, from the literature (Appendix A) it is also known that the FA fragment contains no more than four oxygen elements, with the majority of reported structures containing two or three, and generally no nitrogen elements, thereby, eliminating a number of possible empirical formula. Finally, the chromatographic retention gives additional information on the polarity of the molecule. Since RP-LC is employed, it would be expected that shorter acyl chains would elute faster than longer acyl chains due to fewer interactions with stationary phase. Moreover, the increase in unsaturation results in reduced retention due to the *cis* double bonds reducing the overall surface area that can interact with the stationary phase.

*Gln conjugates*

HMHA-Gln and 3M2H-Gln eluted at 5.2, and 6.5 min respectively. These amino acid conjugates were identified on the basis of retention time and EPI spectra compared to reference standards, as illustrated in Figure 5.16 and Figure 5.17. Furthermore, a number of analytes were tentatively identified as Gln conjugates. Standards were not available to confirm identification for these unknowns. However, it is known that the Gln conjugates fragments into *m/z* 147, which can further fragment into *m/z* 130. Thus, this information can provide additional confidence that the detected unknowns are actually an amino acid conjugate and not another unknown metabolite that also produces a fragment of *m/z* 147. Table 5.6 summarises the main finding, with the evidence to support the identities given below.

**Table 5.6** Summary of the potential odour precursors identified in human apocrine sweat by LC-MS/MS using PI scanning screening approach.

| *m/z* | FA Fragment (*m/z*) | Retention Time (min) | Tentative Formula | Tentative Identity | Confidence |
|---|---|---|---|---|---|
| *Gln Conjugates* | | | | | |
| 275 | 129 | 5.2 | $C_7H_{13}O_2$ | HMHA-Gln | *** |
| 257 | 111 | 6.5 | $C_7H_{11}O$ | 3M2H-Gln | *** |
| 289 | 142 | 6.2 | $C_8H_{15}O_2$ | N-α-3-hydroxy-4-methylhept anoyl- L-glutamine / N-α-3-hydroxy- 3-methylhept-anoyl- L-glutamine | ** |
| 289 | 142 | 6.5 | $C_8H_{15}O_2$ | N-α-3-hydroxy-4-methylhept anoyl- L-glutamine / N-α-3-hydroxy- 3-methylhept-anoyl- L-glutamine | ** |
| 289 | 142 | 8.5 | $C_8H_{15}O_2$ | N-α-3-hydroxy-oct-anoyl- L-glutamine / N-α-8-hydroxy-oct-anoyl- L-glutamine | ** |
| 303 | 156 | 7.1 | $C_9H_{17}O_2$ | N-α-3-hydroxy-3-methyloct anoyl- L-glutamine / N-α-3-hydroxy-4-methyloct anoyl- L-glutamine | ** |
| 285 | 138 | 7.5 | $C_9H_{15}O$ | N-α-4-methyl-3-oct-enoyl- L-glutamine | ** |
| 301 | 154 | 8.1 | $C_{10}H_{19}O$ | N-α-4-ethyl-oct-anoyl- L-glutamine | ** |
| 271 | 124 | 6.9 | $C_8H_{13}O$ | 7-Octenoic acid | * |
| 317 | 170 | 7.7 | $C_{10}H_{19}O_2$ | 3-Hydroxydecanoic acid / azelaic acid | * |
| 299 | 152 | 7.9 | $C_{10}H_{17}O$ / $C_{10}H_{17}O_2$ | (Z/E)-4-Methylnon-3-enoic acid / 9-Decenoic acid | * |
| 313 | 166 | 8.2 | $C_{11}H_{19}O$ / $C_{10}H_{15}O_2$ | 10-Undecenoic acid | * |
| 315 | 168 | 8.3 | $C_{11}H_{20}O$ / $C_{10}H_{16}O_2$ | 2-Methyldecanoic acid / 4-Ethylnonanoic acid | * |
| 329 | 182 | 8.7 | $C_{12}H_{23}O$ / $C_{11}H_{19}O_2$ | 8-Methylundecanoic acid / 4-Ethyldecanoic acid / | * |

*Table 5.6 continued*

| *m/z* | FA Fragment (*m/z*) | Retention Time (min) | Tentative Formula | Tentative Identity | Confidence |
|---|---|---|---|---|---|
| 357 | 210 | 9.07 | $C_{14}H_{27}O$ / $C_{13}H_{23}O_2$ | 10-Methyltridecanoic acid / Tetradecanoic acid | * |
| 311 | 165 | 1.4 | $C_{10}H_{13}O_2$ | Uknown | |
| 206 | 59 | 4.1 | $C_3H_8O$ | Uknown | |
| *Cys or Cys-Gly Conjugates* | | | | | |
| 208 | 87 | 3.2 | $C_5H_{11}O$ | 2M3H-Cys | *** |
| 293 | 115 | 5.05 | $C_7H_{15}O$ | 3-Methyl-3-sulfanylhexan-1-ol | *** |
| 265 | 86 | 1.65 | $C_5H_{11}O$ | 2-Methyl-3-sulfanylbutan-1-ol / 3-Sulfanylpentan-1-ol | ** |
| 279 | 100 | 3.6 | $C_6H_{13}O$ | 3-Sulfanylhexan-1-ol | ** |
| 313 | 134 | 5.13 | $C_9H_{11}O$ | Uknown thiol | |
| 307 | 128 | 5.37 | $C_8H_{17}O$ | Uknown thiol | |
| 309 | 130 | 6.8 | $C_8H_{19}O$ | Uknown thiol | |
| 310 | 131 | 6.9 | $C_8H_{21}O$ | Uknown thiol | |
| 365 | 186 | 6.6 | $C_{12}H_{17}O$ | Uknown thiol | |
| 358 | 179 | 6.8 | $C_{12}H_{20}O$ | Uknown thiol | |
| 375 | 196 | 7.6 | $C_{13}H_{25}O$ | Uknown thiol | |
| 387 | 265 | 8.09 | $C_{18}H_{33}O$ | Uknown thiol | |
| 295 | 116 | 9.5 | $C_7H_{17}O$ | Uknown thiol | |

There are four potential matches where the pseudomolecular ion corresponds to an amino acid conjugate that has been reported in the literature (see Appendix A). Firstly, unknown *m/z* 289, which has three chromatographic peaks at retention times of 6.2, 6.5, and 8.5 min (Figure 5.18). The FA fragment is expected to have an *m/z* 142 which is likely to have the formula of $C_8H_{15}O_2$. There are four ions of *m/z* 289 that have been reported in the literature; N-α-3-hydroxy-4-methylhept anoyl- L-glutamine, N-α-3-hydroxy- 3-methylhept-anoyl- L-glutamine, N-α-3-hydroxy-oct-anoyl- L-glutamine, and N-α-8-hydroxy-oct-anoyl- L-glutamine (Natsch *et al.*, 2006). The former two conjugates would be considered the most likely candidates for the retention time of 6.2 and 6.5 min with N-α-3-hydroxy-4-methylhept anoyl- L-glutamine being slightly more retained. Where, N-α-3-hydroxy-oct-anoyl- L-glutamine would be the most likely candidate for the peak that elutes at 8.5 min.

Secondly, unknown *m/z* 303 has a retention time of 7.1 min (Figure 5.19). The FA fragment is expected to have an *m/z* 156 which is likely to have the formula of $C_9H_{17}O_2$. There are four ions of *m/z* 303 that have been reported in the literature; N-α-3-hydroxy-3-methyloct anoyl- L-glutamine, N-α-3-hydroxy-4-methyloct anoyl- L-glutamine, N-alpha-7-carboxy-hept-anoyl- L-glutamine, and N-α-9-hydroxy- non-anoyl- L-glutamine (Natsch *et al.*, 2006). Out of these reported metabolites, it would be expected the former two would be most likely candidate as the later two would be considered to be more polar. On closer inspection of the extracted precursor ion chromatogram, there are two closely eluting peaks which further suggest that N-α-3-hydroxy-3-methyloct anoyl- L-glutamine and N-α-3-hydroxy-4-methyloct anoyl- L-glutamine, with the later eluting later, as the most probable candidate.

Thirdly, unknown *m/z* 285 has a retention time of 7.5 min (Figure 5.20). The FA fragment is expected to have an *m/z* 138 which is likely to have the formula of $C_9H_{15}O$. There is only one reported match in the literature, thus, is tentatively assigned as N-α-4-methyl-3-oct-enoyl- L-glutamine, with the FA being (E)-4-Methyloct-3-enoic acid (Natsch *et al.*, 2006).

Finally, unknown *m/z* 301 eluted at 8.1 min (Figure 5.21). The FA fragment is expected to have an *m/z* 154 which is likely to have the formula of $C_{10}H_{19}O$. There is

one reported match in the literature, thus, is tentatively assigned as N-α-4-ethyl-oct-anoyl- L-glutamine, with the FA being 4-ethyloctanoic acid (Natsch *et al.*, 2006).

There were also a number of possible conjugates that have no potential match within the reported literature. However, the calculated FA fragments in most cases do match VFA that has been detected in apocrine sweat from the literature (see Appendix A). These are all summarised in below.

- Unknown *m/z* 271 has a retention time of 6.9 min (Figure 5.22). The FA fragment is expected to have an *m/z* 124 which is likely to have the formula of $C_8H_{13}O$.
- Unknown *m/z* 317 has a retention time of 7.7 min (Figure 5.23). The FA fragment is expected to have an *m/z* 170 which is likely to have the formula of $C_{10}H_{19}O_2$.
- Unknown *m/z* 299 has a retention time of 7.9 min (Figure 5.24). The FA fragment is expected to have an *m/z* 152 which is likely to have the formula of $C_{10}H_{17}O$ or $C_9H_{13}O_2$.
- Unknown *m/z* 313 has a retention time of 8.2 min (Figure 5.25). The FA fragment is expected to have an *m/z* 166 which is likely to have the formula of $C_{10}H_{15}O_2$ or $C_{11}H_{19}O$.
- Unknown *m/z* 329 has a retention time of 8.7 min (Figure 5.26). The FA fragment is expected to have an *m/z* 182 which is likely to have the formula of $C_{12}H_{23}O$ or $C_{11}H_{19}O_2$
- Unknown *m/z* 357 has a retention time of 9.07 min (Figure 5.27). The FA fragment is expected to have an *m/z* 210 which is likely to have the formula of $C_{14}H_{27}O$ or $C_{13}H_{23}O_2$.

Unknown *m/z* 311 has a retention time of 1.40 min (Figure 5.28). The FA fragment is expected to have an *m/z* 165 which is likely to have the formula of $C_{10}H_{13}O_2$. However, with this size fragment you would expect the analyte to elute a lot later. Also, there is no *m/z* 130 peak present. Thus, it is likely that this metabolite is a false positive.

Unknown *m/z* 206 has a retention time of 4.1 min (Figure 5.29). The FA fragment is expected to have an *m/z* 59 which is likely to have the formula of $C_3H_8O$. Again there is no peak at *m/z* 130, thus, expected to be a false positive.

*Cys or Cys-Gly conjugates*

2M3H-Cys and 3M3SH-Cys-Gly eluted at 3.2 and 5.08 min respectively. These amino acid conjugates were identified on the basis of retention time and EPI spectra compared to reference standard, as illustrated in Figure 5.30 and Figure 5.31. Furthermore, a number of analytes were tentatively identified as Cys-Gly conjugates. Standards were not available to confirm identification for these unknowns, thus, tentative assignment was based upon characteristic product ions observed in the EPI spectra of the 3M3SH-Cys-Gly conjugate. It is known from the 3M3SH-Cys-Gly standard that the fragmentation pattern produces common product ions of *m/z* 179, 162, and 144 which are characteristic fragments of the Cys-Gly residue. Thus, it is reasonable to suggest that any other Cys-Gly conjugate would contain the same product ions. Table 5.6 summarises the main finding, with the evidence to support the identities given below.

There are two potential matches where an unknown pseudomolecular ion corresponds to an amino acid conjugate previously reported in the literature (see Appendix A). Firstly, an unknown *m/z* 265 eluted at 1.65 min (Figure 5.32). The FA fragment is expected to have an *m/z* 86 which is likely to have the formula of $C_5H_{11}O$. If this fragment is a thiol then it could be tentatively assigned to 3-sulfanylpentan-1-ol or 2-methyl-3-sulfanylbutan-1-ol. Secondly, an unknown *m/z* 279 has a retention time of 3.3 min (Figure 5.33). The FA fragment is expected to have an *m/z* 100 which is likely to have the formula of $C_6H_{13}O$. This thiol containing compound could be tentatively assigned to 3-sulfanylhexan-1-ol. Another addition of $CH_2$ group would result in 3M3SH-Cys-Gly conjugate which elutes at 5.08 min (Figure 5.31). The addition of each $CH_2$ group would result in a stronger retention due to the metabolite becoming less polar. This chromatographic behaviour is consistent with other metabolites that have been reported on RP systems, for example; homoserine lactones had a predictable increase in retention with increasing acyl chain length (Ortori *et al.*, 2007).

There were also a number of possible conjugates that have no potential match within the reported literature. However, the assumption would be that the non-amino acid fragment would presumably be a thiol.

- Unknown *m/z* 313 has a retention time of 5.13 min (Figure 5.34). The FA fragment is expected to have an *m/z* 134 which is likely to have the formula of $C_9H_{11}O$. The increase in retention due to increasing the carbon length is offset by the presence of double bonds, which reduce the retention time compared to their corresponding linear isomers, due to the overall surface area being reduced.

- Unknown *m/z* 307, 309, and 310 has a retention time of 5.37, 6.8, and 6.9 min respectively. As depicted in Figure 5.35, Figure 5.36 and Figure 5.37, these unknown metabolites have fragments higher than the pseudomolecular ion. This could be a result of being multiple charged, where that resultant fragments also fragments into common product ions being monitored. The resolution of this work is not sufficient to be able to distinguish multiple charged species by using isotope distribution of $C^{12}/C^{13}$. However, for a doubly charged species you would expect the isotope cluster to be half an amu apart (Hoffmann and Stroobant, 2001). The FA fragment is expected to have an *m/z* ~128 which is likely to have a formula of $C_8H_{16}O$.

- Unknown *m/z* 365 has a retention time of 6.6 min (Figure 5.38). The FA fragment is expected to have an *m/z* 186 which is likely to have the formula of $C_{12}H_{27}O$.

- Unknown *m/z* 358 has a retention time of 6.8 min (Figure 5.39). The FA fragment is expected to have an *m/z* 186 which is likely to have the formula of $C_{12}H_{20}O$.

- Unknown *m/z* 375 has a retention time of 7.6 min (Figure 5.40). The FA fragment is expected to have an *m/z* 196 which is likely to have the formula of $C_{13}H_{25}O$.

- Unknown *m/z* 295 has a retention time of 9.5 min (Figure 5.41). The FA fragment is expected to have an *m/z* 196 which is likely to have the formula of $C_7H_{17}O$.

#### 5.4.1.4 RP-LC-MS/MS Multiple Reaction Monitoring for Targeted Analysis of Odour Precursors in Human Apocrine Sweat Secretions

Targeted MRM analysis requires prior knowledge of the metabolites of interest. Ideally, the CE and DP need to be optimised for each metabolite in order to obtain maximum sensitivity for the transition which is being monitored. However, due to the limited availability of standards, this could not be optimised for all the amino acid conjugates that have been reported in the literature (see Appendix A). Since all the reported odour precursors have a common structure (amino acid moiety), it is reasonable to suggest that these reported amino acid conjugates would fragment in a similar way, thereby, leaving the amino acid residue to be monitored. In comparison to precursor ion survey scanning, this approach is considered to be more sensitive, as the spectrometer is no longer scanning, since Q1 and Q3 are fixed, thus, allowing the spectrometer to monitor the precursor and fragment ions over longer times (Hoffmann and Stroobant, 2001).

As previously stated, 14 mass transitions were monitored for the analysis of odour precursors present in human apocrine sweat samples. This was based on predictions of the MRM transition, for all the reported amino acid conjugates in literature, to which standards were not available. A representative total ion chromatogram is depicted in Figure 5.4. A number of analytes were tentatively identified as amino acid conjugates as summarised in Table 5.7. As mentioned previously, standards were not available to confirm identification for these unknowns.

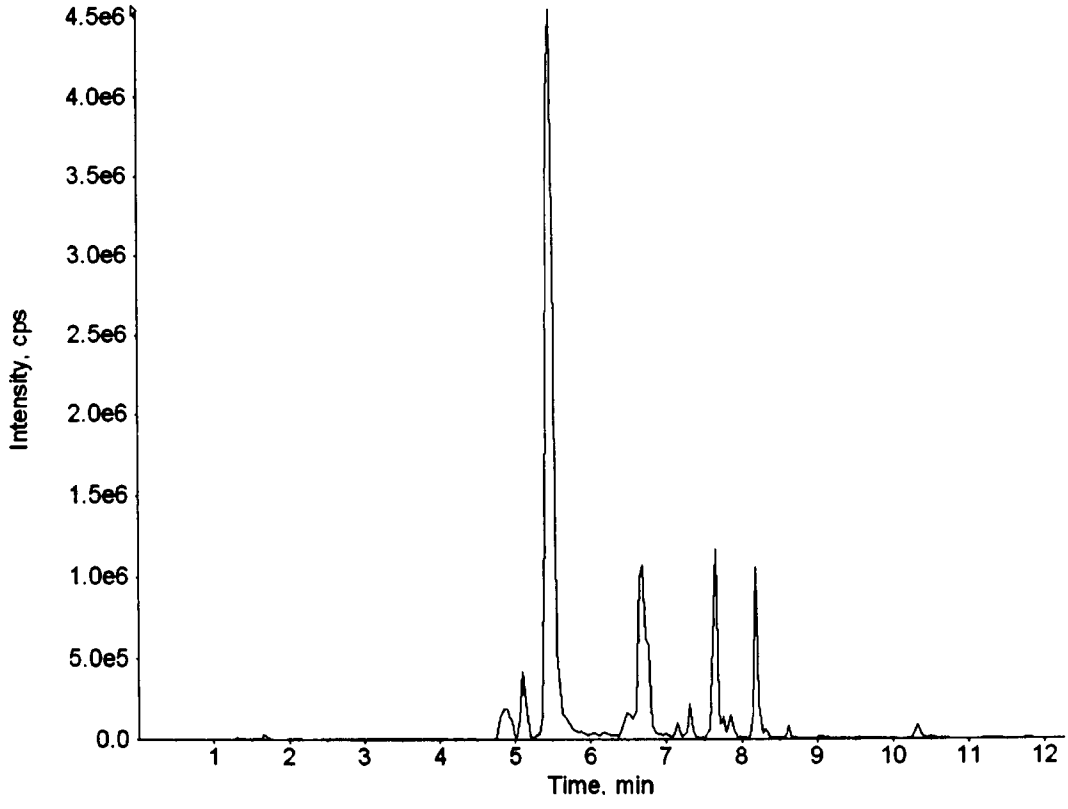



**Figure 5.4** HPLC-MS/MS total ion chromatogram of a representative human apocrine sweat sample in MRM mode.

**Table 5.7** Summary of the potential odour precursors identified in human apocrine sweat by LC-MS/MS using MRM.

| *m/z* | % Peak Area | Retention Time (min) | Tentative Identity | Confidence |
|---|---|---|---|---|
| ***Gln conjugates*** | | | | |
| 257/147 | 11.40 | 6.5 | N-α-3-methylhex-2-enoyl-L-glutamine | *** |
| 275/147 | 72.08 | 5.2 | N-α-3-hydroxy-3-methylhexanoyl- L-glutamine | *** |
| 289/147 | 5.39 | 6.5 | N-α-3-hydroxy-4-methylhept anoyl- L-glutamine | ** |
| 303/147 | 1.28 | 7.3 | N-α-3-hydroxy-3-methyloct anoyl- L-glutamine | ** |
| 285/147 | 1.97 | 7.5 | N-α-4-methyl-3-oct-enoyl- L-glutamine | ** |
| 301/147 | 0.52 | 8.2 | N-α-4-ethyl-oct-anoyl- L-glutamine | ** |
| 287/147 | 0.30 | 7.9 | N-α-4-ethyl-hept-anoyl- L-glutamine | ** |
| 259/147 | 0.36 | 6.6 | N-α-3-methyl-2-oxopent-anoyl- L-glutamine | |
| **Cys or Cys-Gly conjugates** | | | | |
| 208/105 | 0.37 | 3.2 | S-[1-(2-hydroxy-1-methylethyl)-ethyl]-L-cysteine | *** |
| 293/179 | 6.21 | 5.1 | S-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cysteinylglycine | *** |
| 279/179 | 0.03 | 4.7 | S-[1-(2-hydroxyethyl)-butyl]-L-cysteinylglycine | ** |
| 236/105 | 0.10 | 5.4 | S-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cystein | |

2M3H-Cys, 3M3SH-Cys-Gly, HMHA-Gln and 3M2H-Gln have retention times of 3.2, 5.0, 5.2, and 6.5 min respectively. These conjugates were identified on the basis of retention time and EPI spectra compared to reference standards, as noted above. While screening for the known amino acid conjugates from the literature in MRM mode, potentially, three additional conjugates were also detected that were not detected/present in the PI scanning mode. Firstly, the transition *m/z* 287/147 resulted in a peak that eluted at 7.9 min (Figure 5.5). This has been tentatively assigned to N-α-4-ethyl-hept-anoyl- L-glutamine which was also detected with the UPLC-Orbitrap-MS (Table 5.4). Secondly, the transition *m/z* 259/147 resulted in a peak that eluted at 6.6 min (Figure 5.6 A). This has been tentatively assigned to N-α-3-methyl-2-oxopent-anoyl- L-glutamine. Finally, the transition *m/z* 236/105 resulted in a peak that eluted at 5.4 min (Figure 5.6 B). This has been tentatively assigned to N-α-3-methyl-2-oxopent-anoyl- L-glutamine. EPI spectra were not obtained for the latter two metabolites, due to the low concentration, thus, no further evidence can suggest if these metabolite are amino acid conjugates. Evidence for the tentative identification of *m/z* 289, 303, 285, 301 and 279 were discussed above. Furthermore, it is also evident that the two most intense peaks are HMHA-Gln and 3M2H-Gln conjugates.

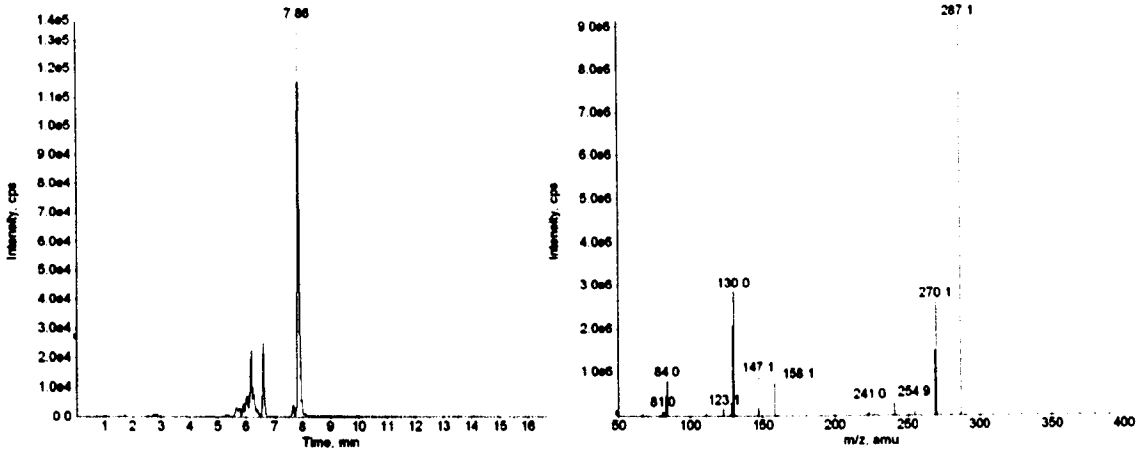



**Figure 5.5** Extracted ion chromatogram from the MRM transition *m/z* 287/147 and EPI spectrum of the peak at 7.6 min.

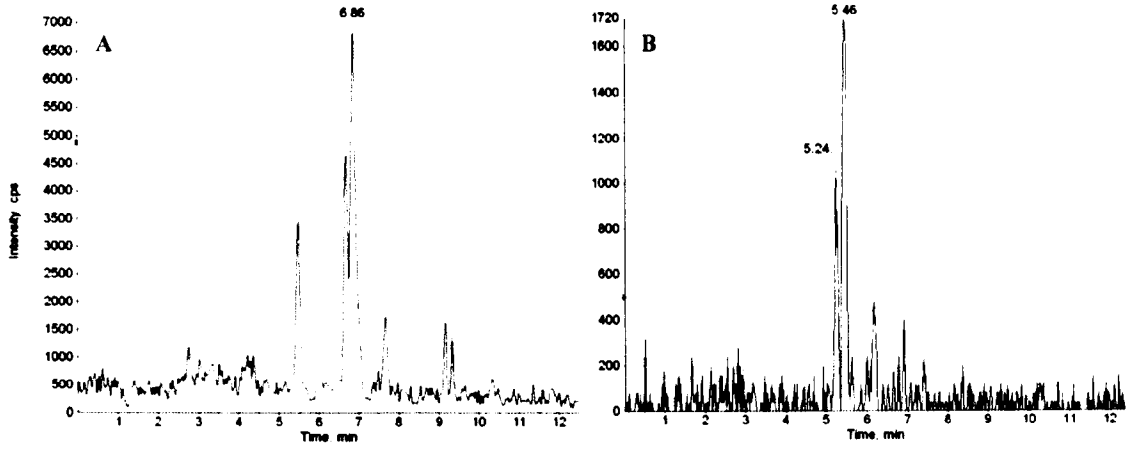



**Figure 5.6** Extracted ion chromatogram from the MRM transition **A** *m/z* 259/147 and **B** *m/z* 236/105. No EPI spectra were obtained for these metabolites.

*Summary of identification of small molecules compounds of human apocrine sweat.*

To my knowledge, this is the first time NMR spectroscopy has been applied to human axillary secretions in which 25 metabolites were readily identified and quantified. Furthermore, identification of metabolites detected by NMR was aided by the large body of information already available on human biological fluids. While the detection of biomarkers was facile in regards to the LC-MS methodologies, the process of identification however is more demanding, due in part to the limited database available. Both global methodologies were able to detect 9 amino acid conjugates which were confirmed by a targeted MS/MS approach. The precursor ion survey scanning approach has successfully identified four amino acid conjugates present the human apocrine sweat secretions, for which standards were available and tentatively identified 26 other amino acid conjugates. Further identification of these unknown metabolites is not possible with the data presented here; all that can be hypothesized is that these are likely to be a Gln, Cys, or Cys-Gly conjugate. There is no evidence in the literature to support the presence or identity of these analytes. Moreover, this method is not selective towards the detection of amino acid conjugates alone due to prevalence of other endogenous metabolites which could produce similar fragments, thereby highlighting the limitations of this methodology.

### 5.4.2 Study of Temporal, Inter- and Intra-Individual Differences in Human Axillary Secretions

#### 5.4.2.1 Inter- and Intra-Individual Differences

Visual comparison of this type of data, from both NMR spectroscopy and LC-MS data-sets could be performed, however, such analysis would be tedious, inefficient and prone to subjective error. Thus, multivariate analysis such as PCA (unsupervised) and PC-DA (supervised) were applied to all the NMR and global LC-MS (QTOF and Exactive) data-sets obtained from the apocrine sweat samples. Both mean centred and UV scaling lead to a similar degree of separation between the six classes, thus, only UV scaled models will be shown as a representative example. The PCA model (UV scaled) obtained from the NMR data resulted in $R^2X$ (cum) of 0.354 using two PCs, where PC1 and 2 explained 21.3% and 14.1% of the total variation, respectively, as depicted in Figure 5.7. The HILIC-MS PCA model (UV scaled) resulted in $R^2X$ (cum) of 0.275 using two PCs, where PC1 and 2 explained 17.8% and 9.6% of the total variation, respectively, as depicted in Figure 5.8. PCA model (UV scaled) obtained from the Exactive data in positive mode resulted in $R^2X$ (cum) of 0.467 using two PCs, where PC1 and 2 explained 29.3% and 17.4% of the total variation, respectively. The negative mode PCA model (UV scaled) resulted in $R^2X$ (cum) of 0.554 using two PCs, where PC1 and 2 explained 35.6% and 19.7% of the total variation, respectively, as depicted in Figure 5.9.

These global methodologies provided complementary information to one another as inter-person variation is clearly evident across all PCA models. As would be expected, PC-DA further improved the separation between each individual (data not shown). It is also evident that the clustering between the intra-individual variations in the PCA model obtained from the negative electrospray data are more closely defined compared to that of the NMR or MS positive mode data. This is likely to be due to the main differences between groups being metabolites that are only observed in negative mode.

The ten amino acid conjugates that were identified or tentatively identified confidently (indicated with two or more stars in Table 5.7) also provided complementary evidence to the global methodologies. Figure 5.10 illustrates the inter-volunteer variation across day one, which is a representative example across all days. The inter-person variation of the amino acid conjugates is consistent with other biological fluids, due to variation in genetic profiles as well as environmental life styles. However, it is evident that HMHA-Gln conjugate (*m/z* 275) is the most dominant peak, which is consistent across all volunteers, while 2M3H-Cys conjugate (*m/z* 208) is predominantly weak. 3M2H-Gln conjugate (*m/z* 257) remains relatively consistent across all samples, with slightly more produced for volunteer two. 3M3SH-Cys-Gly conjugate (*m/z* 293) was the most variable between each volunteer, with a higher concentration produced in volunteer two compared to the least produced in volunteer five.

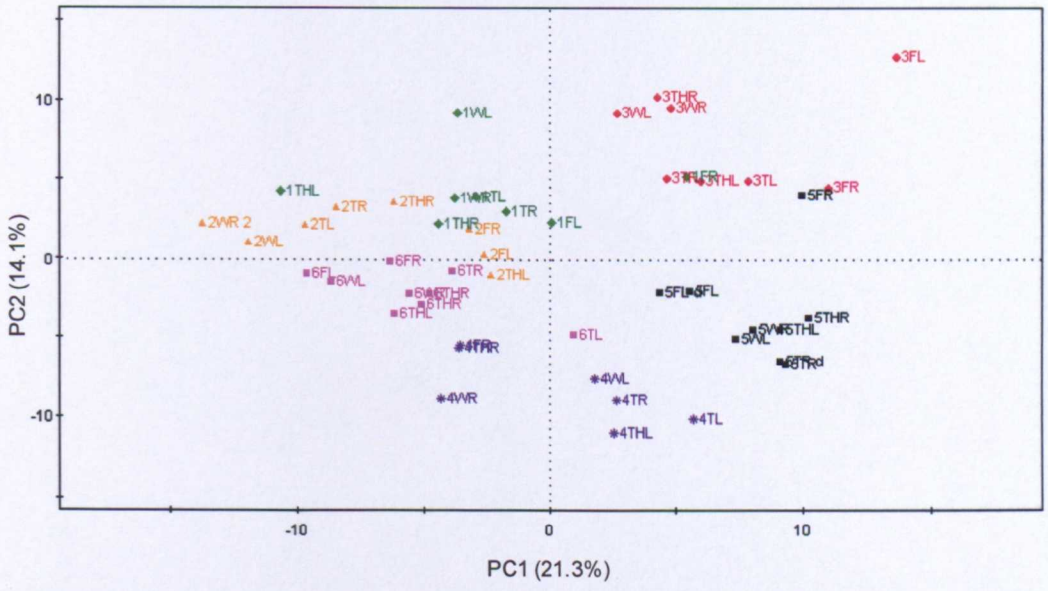



**Figure 5.7** PCA score plot (UV scaled) of human apocrine sweat samples analysed by NMR spectroscopy of six healthy male volunteers (left and right axillae measured separately) across four days. Key:- Coloured by subject, T – day 1, W – day 2, TH – day 3, F – day 4 and L or R indicate the side which was sampled.

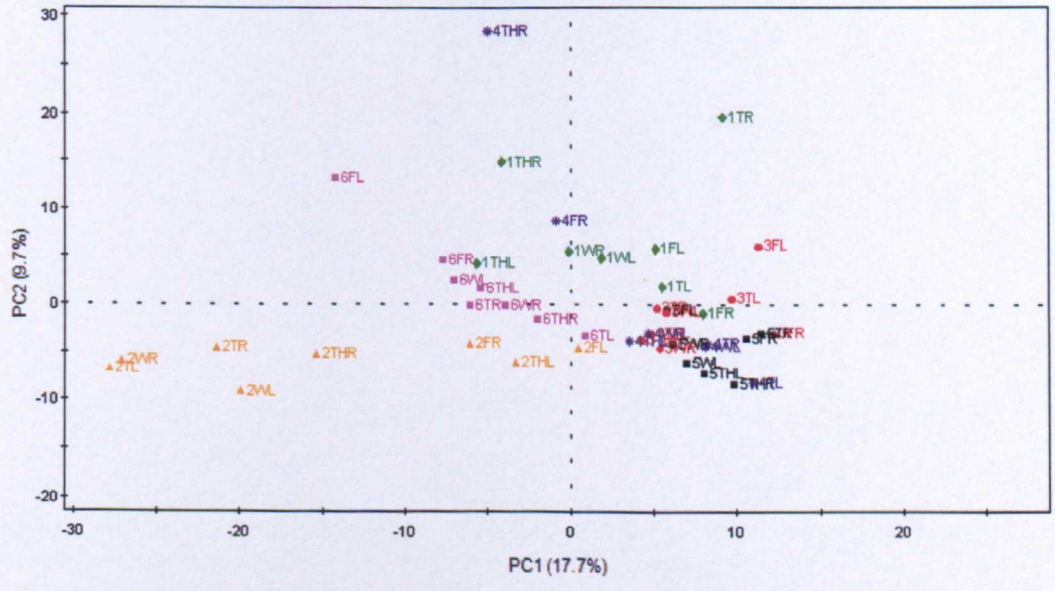
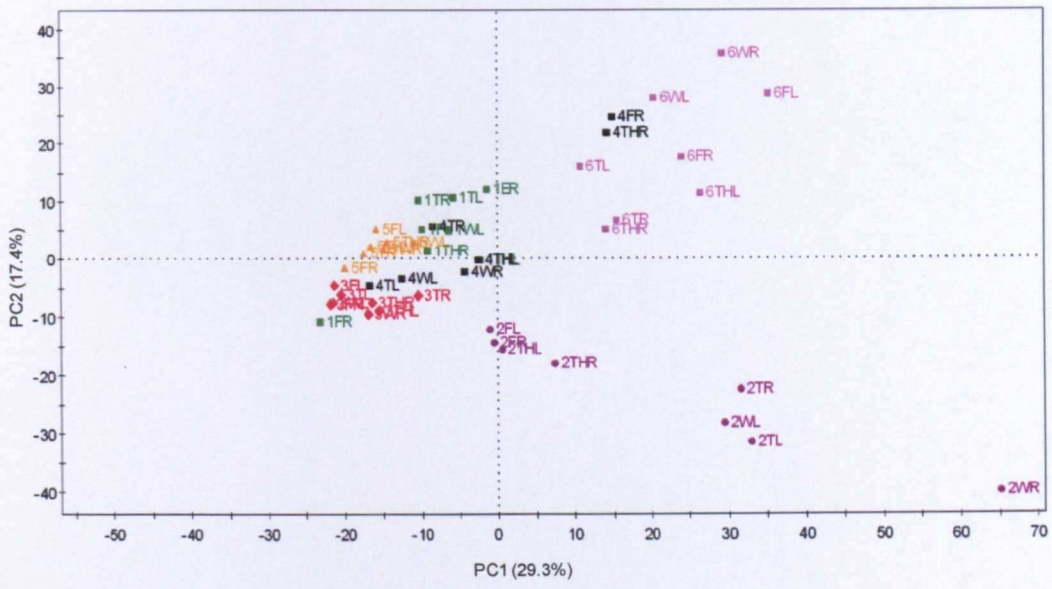



**Figure 5.8** PCA score plot (UV scaled) of human apocrine sweat samples analysed by HILIC-MS of six healthy male volunteers (left and right axillae measured separately) across four days. Key:- Coloured by subject, T – day 1, W – day 2, TH – day 3, F – day 4 and L or R indicate the side which was sampled.

A




B

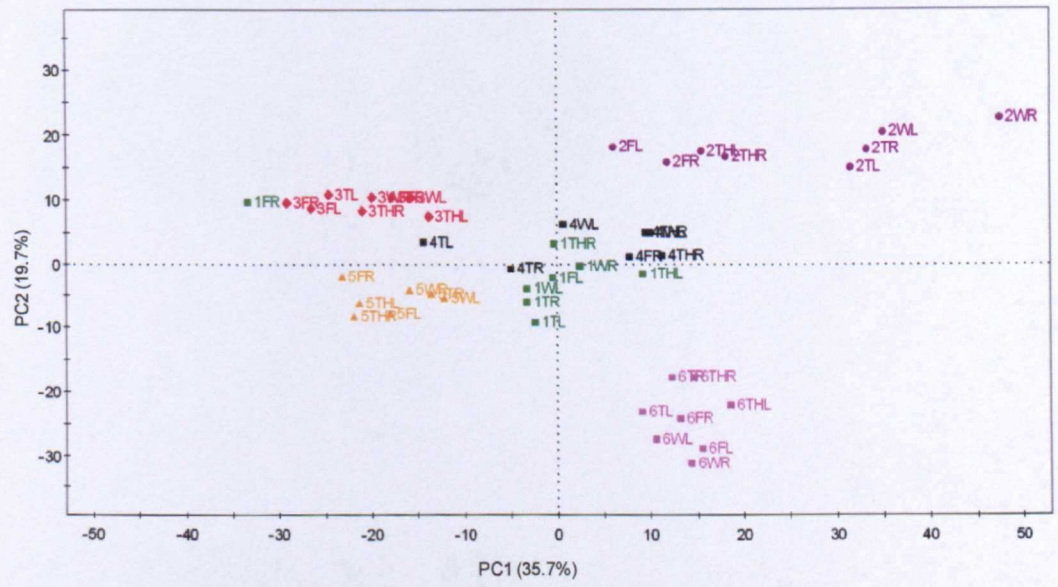



**Figure 5.9** PCA score plot (UV scaled) of human apocrine sweat samples analysed by UPLC-MS of six healthy male volunteers (left and right axillae measured separately) across four days. **A** Positive mode data, **B** negative mode data. Key:- Coloured by subject, T – day 1, W – day 2, TH – day 3, F – day 4 and L or R indicate the side which was sampled.

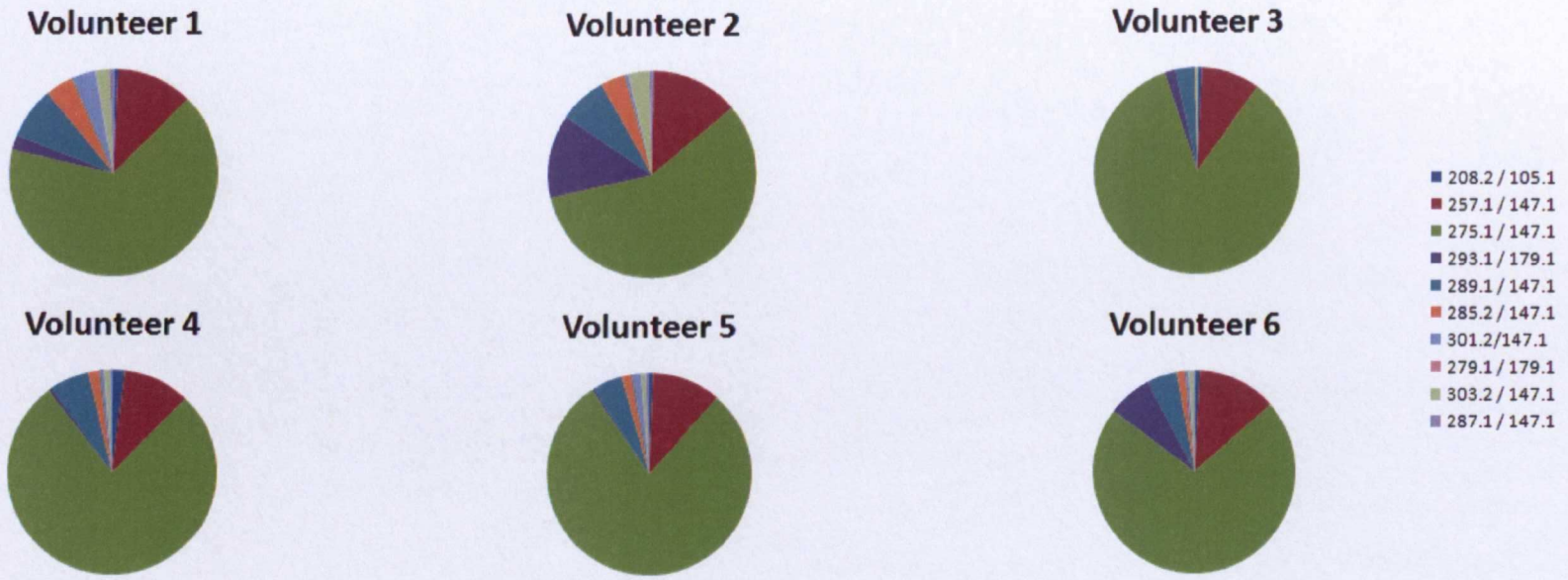


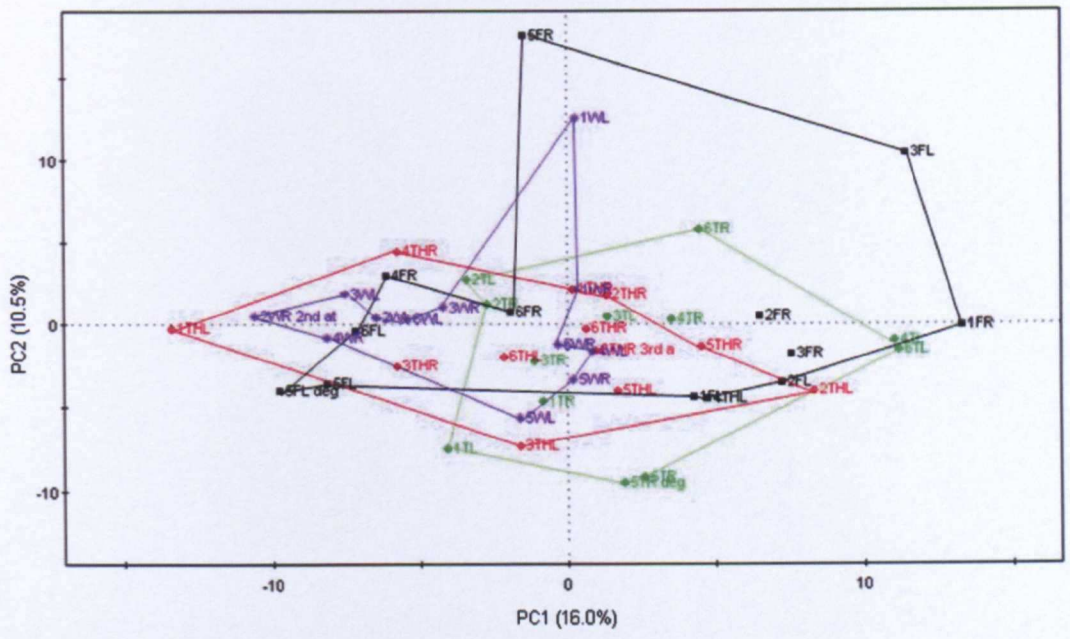

**Figure 5.10** Summarises the intra-subject variation across day one using the data on amino acid conjugated obtained by targeted LC-MS/MS analysis of study samples.

### 5.4.2.2 Inter-Day Differences

To assess whether there is any inter-day variation from sample collection or to identify whether the sweat produced is constant across the four days; the data was mean centred per individual. This effectively minimises the dominant inter-person variation from the model, so only intra-person variation or time trends will influence the separation in the model. Both mean centring per person and UV scaling per person lead to a similar degree of separation between the four classes for all analytical methodologies, thus, only UV scaled models from NMR and LC-MS will be shown as a representative example. The PCA models from the first two PCs account for 26.6% and 44.8% of the total variation, obtained from the NMR and Exactive data respectively. PCA of the apocrine sweat profiles did not display intrinsic clustering related to sample collection as depicted in Figure 5.11.

The ten amino acid conjugates (indicated with two or more stars in Table 5.7) were selected in order to identify the degree of inter-day variation across all volunteers. As depicted in Figure 5.12, there is little difference between the 24 h time points of sample collection. This is consistent with the above finding from both the global NMR and MS data as well as the work published by Leydon and co-workers (Leyden *et al.*, 1981). However, intra-volunteer difference across each day was observed for all amino acid conjugates. A representative example is shown in Figure 5.13, highlighting a gross inter-day effect for all amino acid conjugates. This gross effect was observed for all volunteers, however, the actual inter-day levels varied between each volunteer. Thus, the observed change is attributed to a concentration effect arising from how the sample was collected or how much sweat was secreted rather than inter-day effect, as the relative proportions remain consistent across all metabolites.

A




B

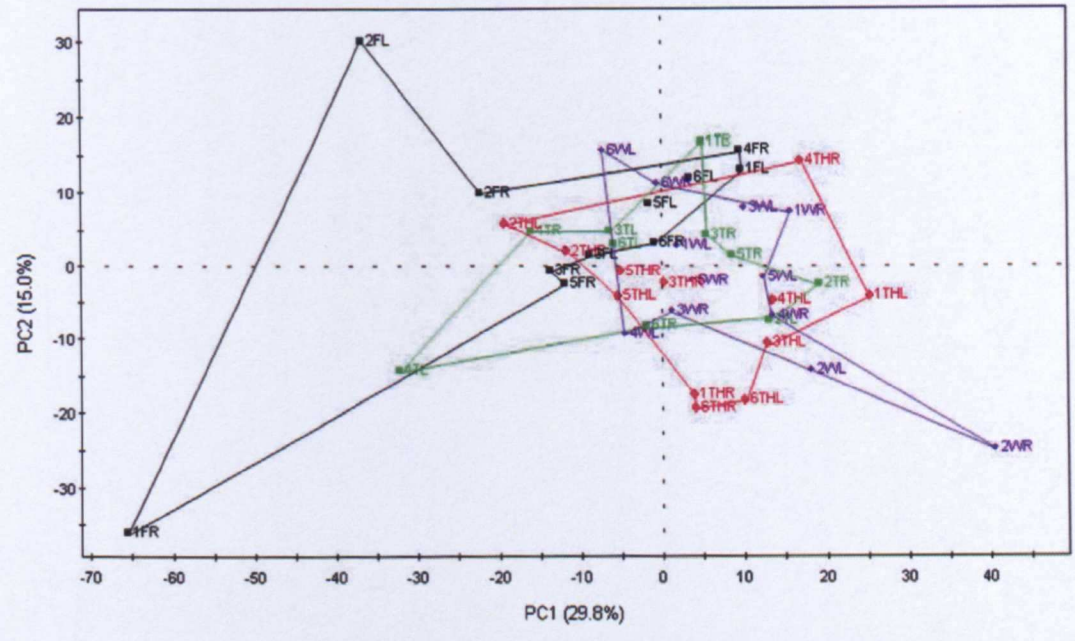



**Figure 5.11** PCA scores plot (UV scaled per person) of human apocrine sweat samples analysed by **A** NMR spectroscopy **B** UPLC-MS negative mode, of six healthy male volunteers (left and right axillae measured separately) across four days. Coloured to show day to day differences, green – day 1, purple – day 2, red – day 3 and black day 4.

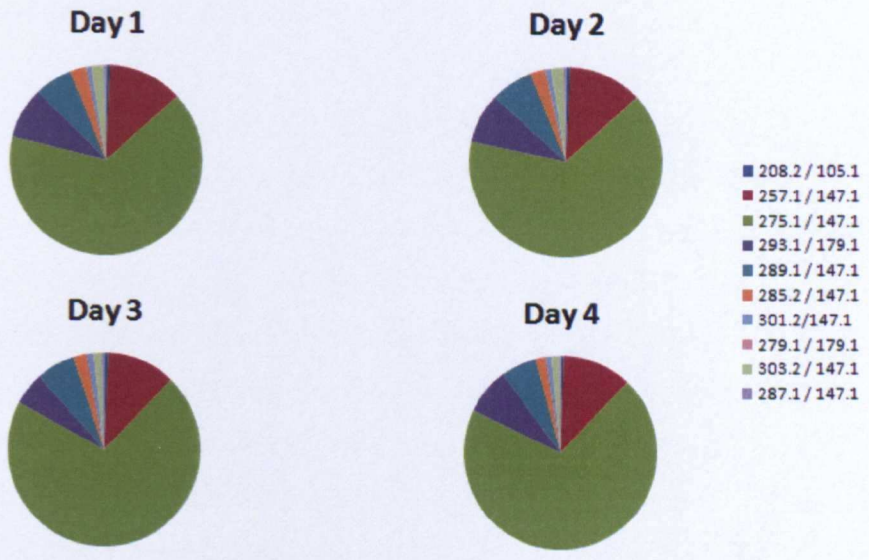



**Figure 5.12** Pie charts illustrating the average inter-day variation of the targeted amino acid conjugates (LC-MS/MS), with each day representing a 24 h time point.

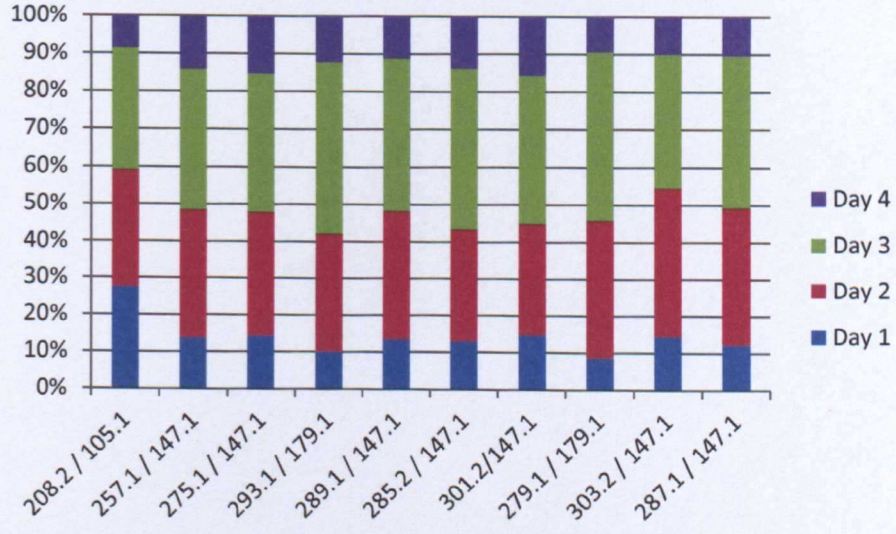
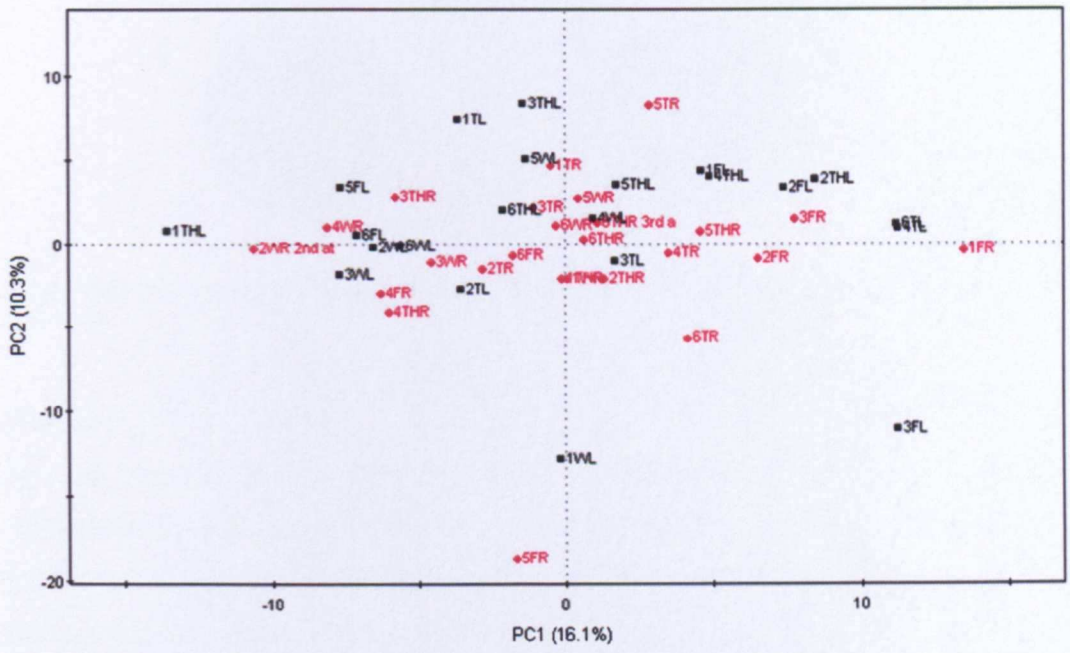



**Figure 5.13** Summarises the intra-day variation of each amino acid conjugate, with each day representing a 24 h time point, obtained from volunteer one.

### 5.4.2.3 Left and Right Arm Differences

To assess whether there are any side related differences between the left and right axilla, the data was mean centred per person as discussed previously (Section 5.4.2.2). Both mean centring per person and UV scaled per person lead to a similar degree of separation between the two classes for all analytical methodologies, thus, only UV scaled models from NMR and LC-MS will be shown as a representative example. The PCA models from the first two PCs account for 26.6% and 44.8% of the total variation obtained from the NMR and Exactive data respectively. PCA of the apocrine sweat profiles did not display any side related differences as depicted in Figure 5.14. Furthermore, the data obtained from the targeted analysis was consistent with the above finding from both the global NMR and LC-MS data, as depicted in Figure 5.15. All identified amino acid conjugates (indicated with two or more stars in Table 5.7) were secreted in equal quantities in both the left and right arm across each day.

A




B

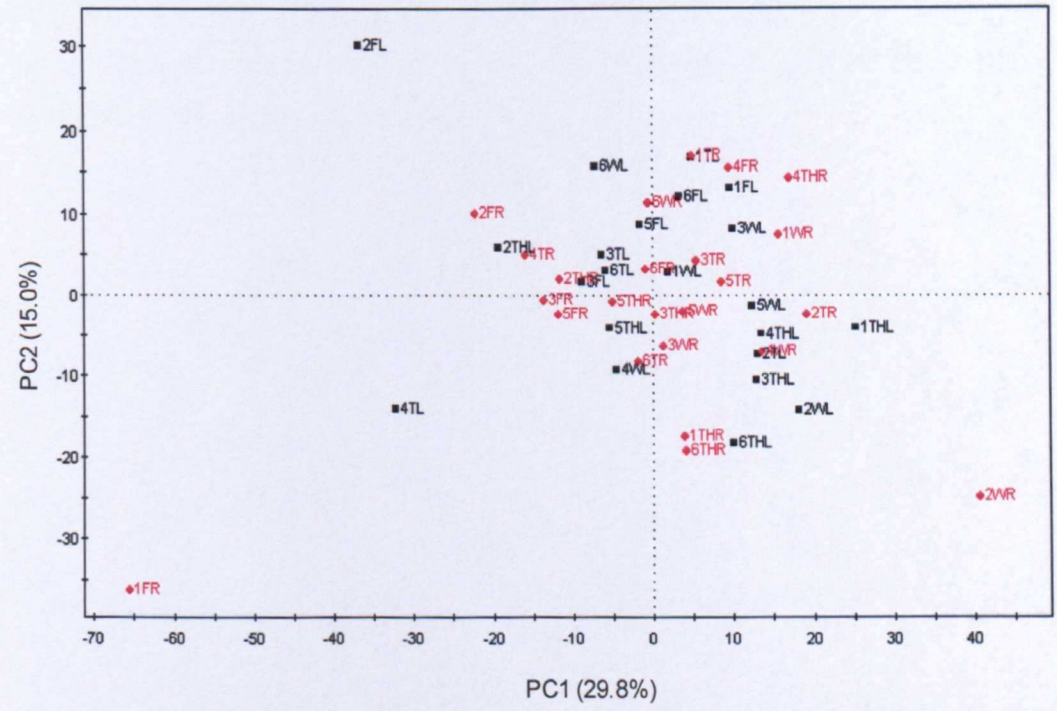



**Figure 5.14** PCA scores plot (UV scaled per person) of human apocrine sweat samples analysed by **A** NMR spectroscopy **B** UPLC-MS negative mode of six healthy male volunteers (left and right axillae measured separately) across four days. Coloured to highlight the difference between left (red) and right (black) axilla.

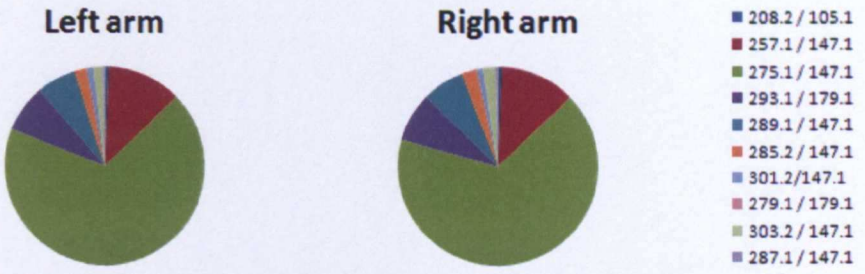



**Figure 5.15** Pie chart illustrating the relative difference between the left and right arm across all volunteers.

*Summary*

All the analytical methodologies were capable of detecting inter-individual differences in the axillary sweat metabolic profile. Furthermore, each technique (NMR, LC-MS, and LC-MS/MS) showed that there was minimal inter-day variation and that the composition of the apocrine sweat produced from left and right axilla was consistent with one another. Thus, all techniques used herein provided complementary information, as discrimination by $^{1}$H NMR and LC-MS was based on different sets of markers, especially in the case of the targeted approach were only ten metabolites were selected for analysis.

## Gln conjugates

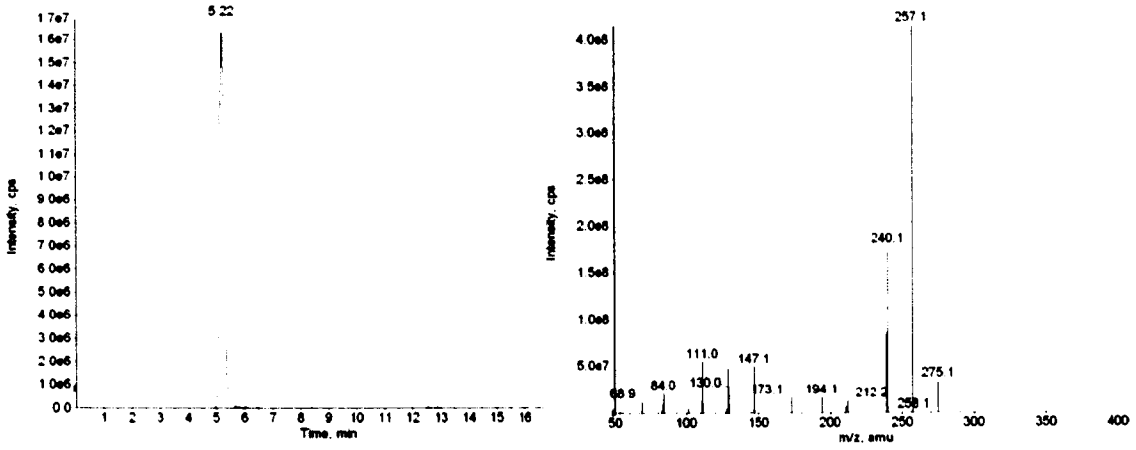



**Figure 5.16** Extracted ion chromatogram of *m/z* 275 and EPI spectrum of the peak at 5.2 min from an EPI triggered PI scan of *m/z* 147.

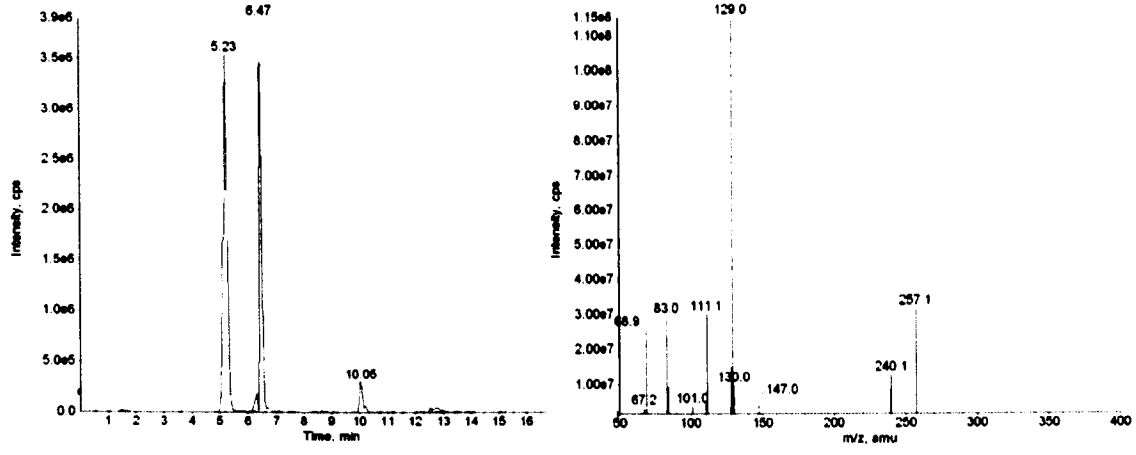



**Figure 5.17** Extracted ion chromatogram of *m/z* 257 and EPI spectrum of the peak at 6.4 min from an EPI triggered PI scan of *m/z* 147.

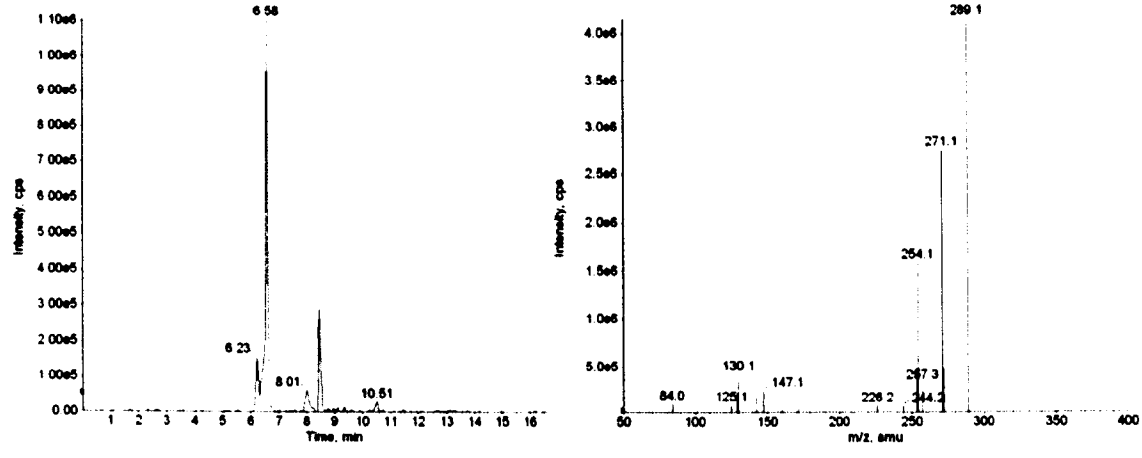



**Figure 5.18** Extracted ion chromatogram of *m/z* 289 and EPI spectrum of the peak at 6.5 min from an EPI triggered PI scan of *m/z* 147.

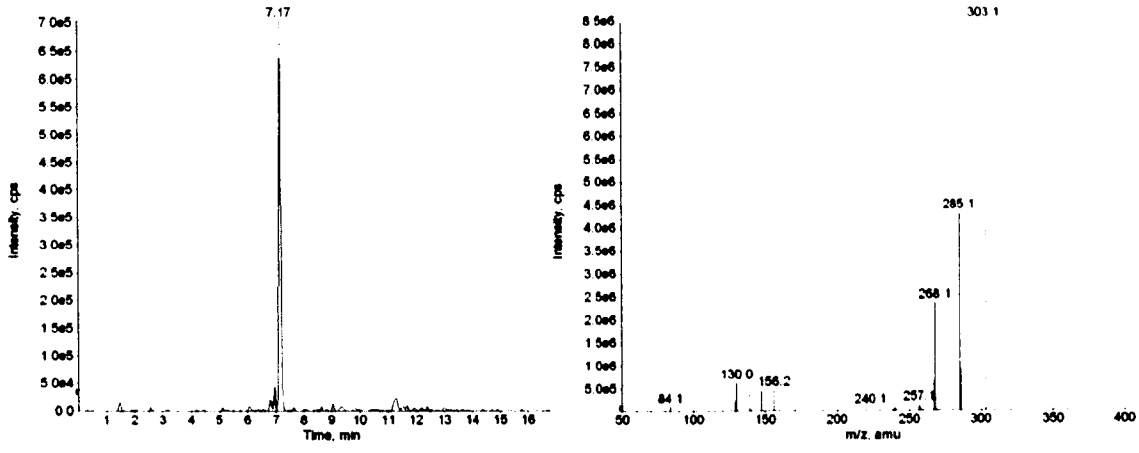



**Figure 5.19** Extracted ion chromatogram of *m/z* 303 and EPI spectrum of the peak at 7.1 min from an EPI triggered PI scan of *m/z* 147.

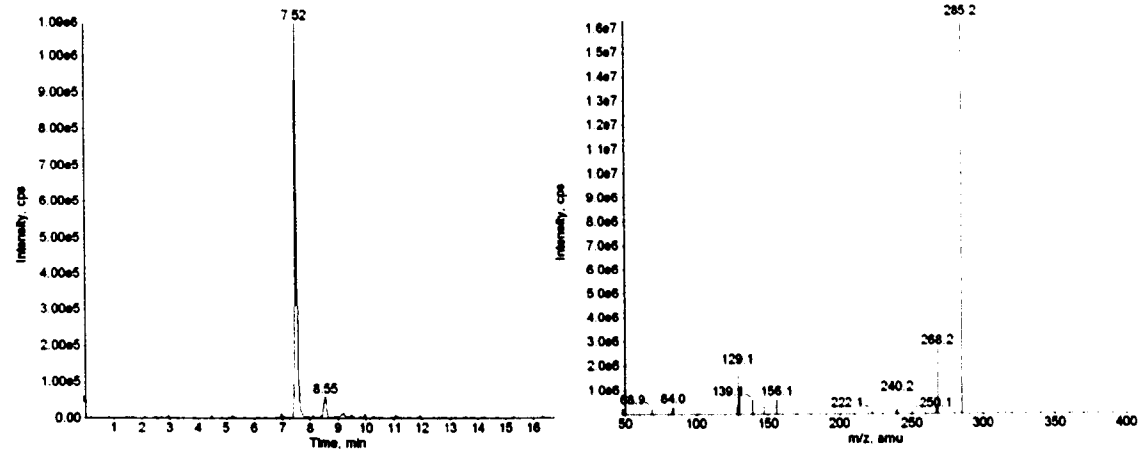



**Figure 5.20** Extracted ion chromatogram of *m/z* 285 and EPI spectrum of the peak at 7.5 min from an EPI triggered PI scan of *m/z* 147.

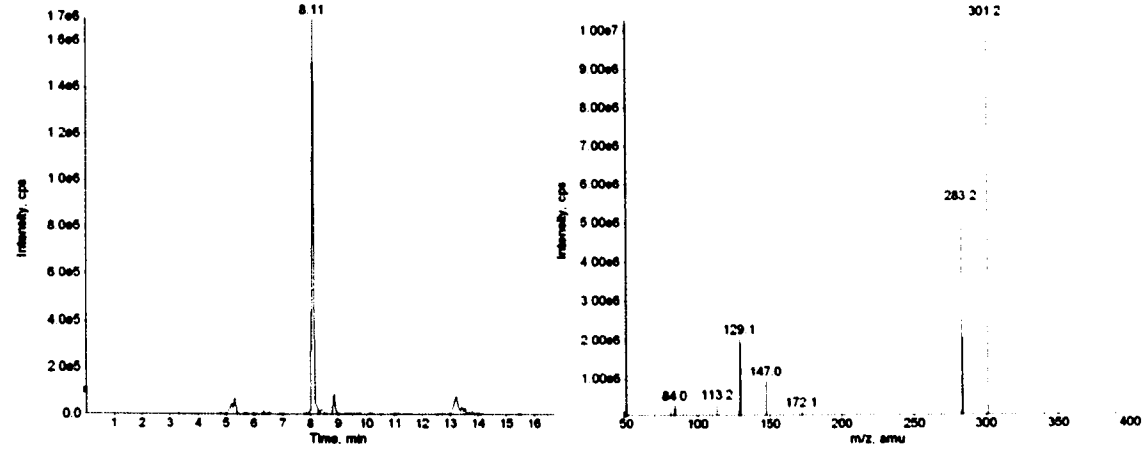



**Figure 5.21** Extracted ion chromatogram of *m/z* 301 and EPI spectrum of the peak at 8.1 min from an EPI triggered PI scan of *m/z* 147.

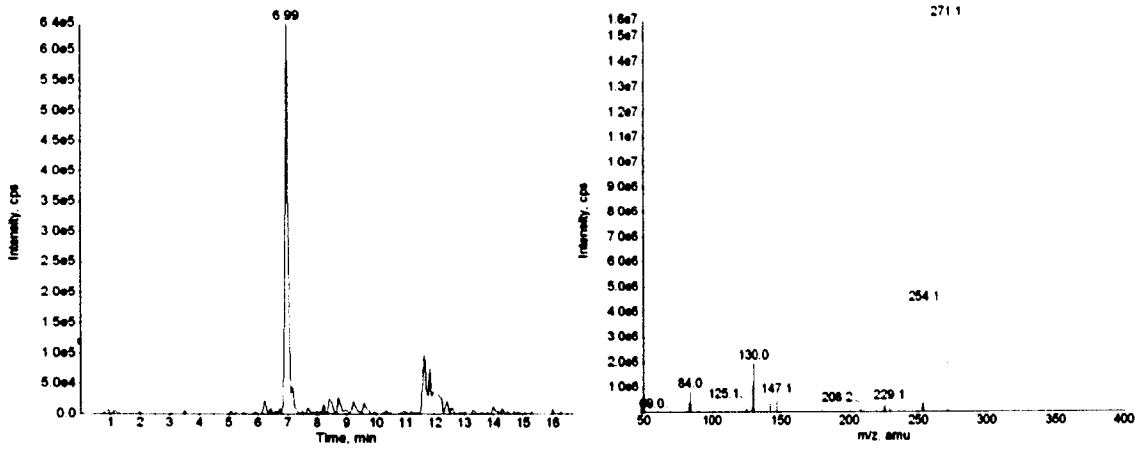



**Figure 5.22** Extracted ion chromatogram of *m/z* 271 and EPI spectrum of the peak at 6.9 min from an EPI triggered PI scan of *m/z* 147.

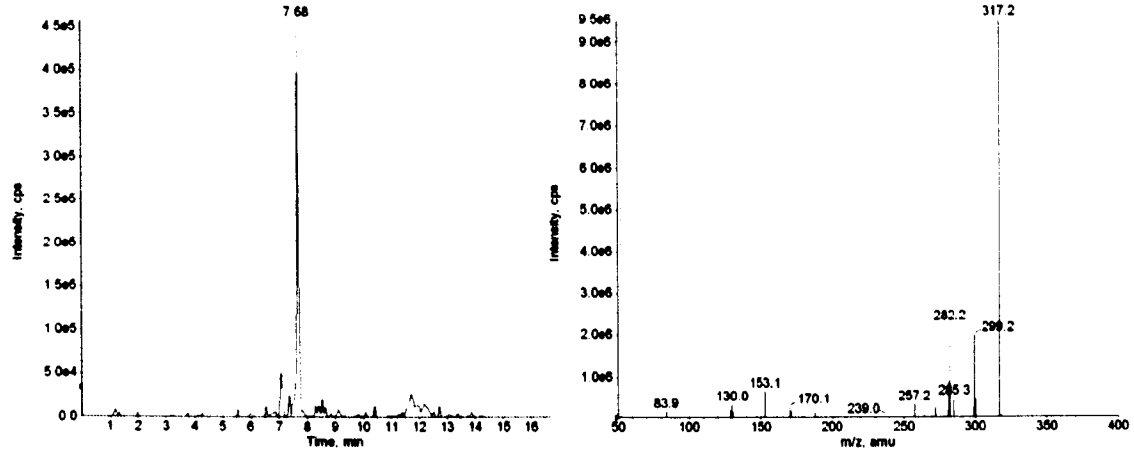



**Figure 5.23** Extracted ion chromatogram of *m/z* 317 and EPI spectrum of the peak at 7.6 min from an EPI triggered PI scan of *m/z* 147.

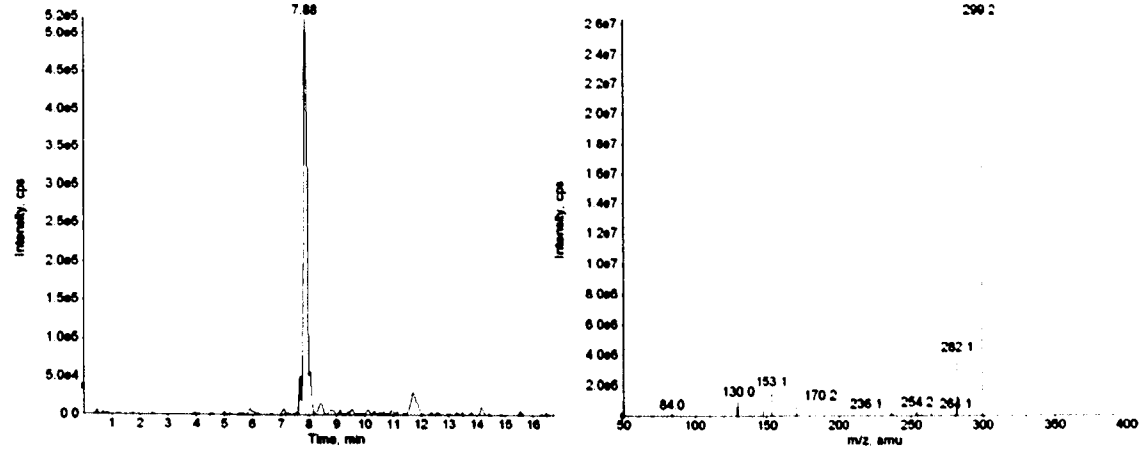



**Figure 5.24** Extracted ion chromatogram of *m/z* 299 and EPI spectrum of the peak at 7.9 min from an EPI triggered PI scan of *m/z* 147.

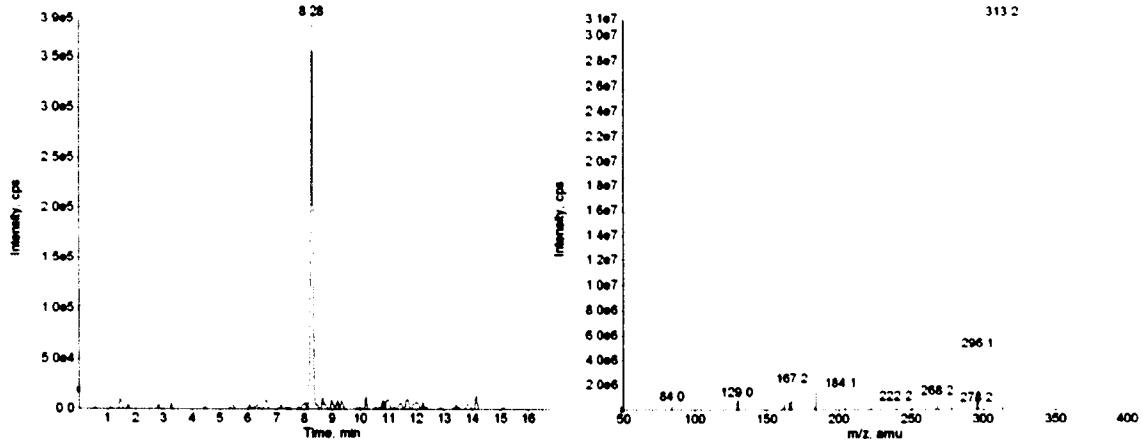



**Figure 5.25** Extracted ion chromatogram of *m/z* 313 and EPI spectrum of the peak at 8.2 min from an EPI triggered PI scan of *m/z* 147.

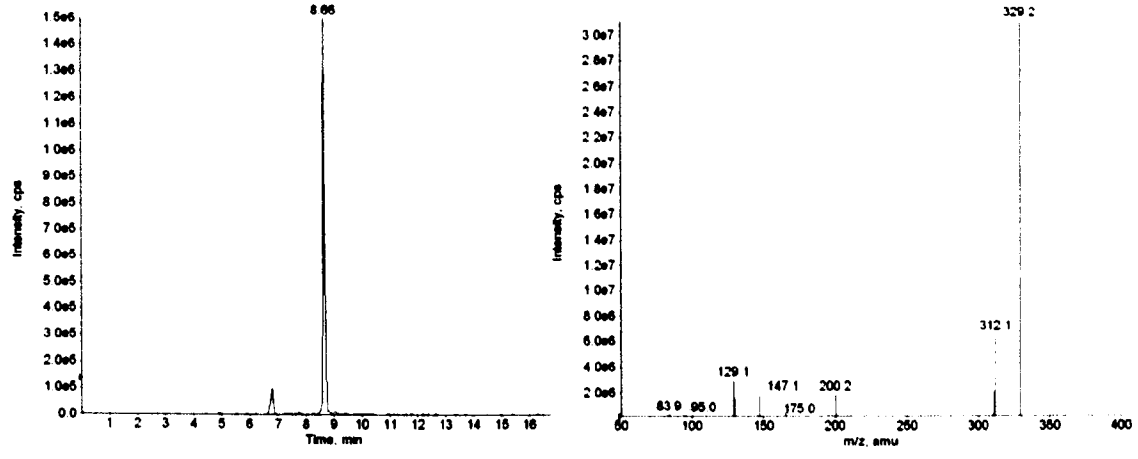



**Figure 5.26** Extracted ion chromatogram of *m/z* 329 and EPI spectrum of the peak at 8.7 min from an EPI triggered PI scan of *m/z* 147.

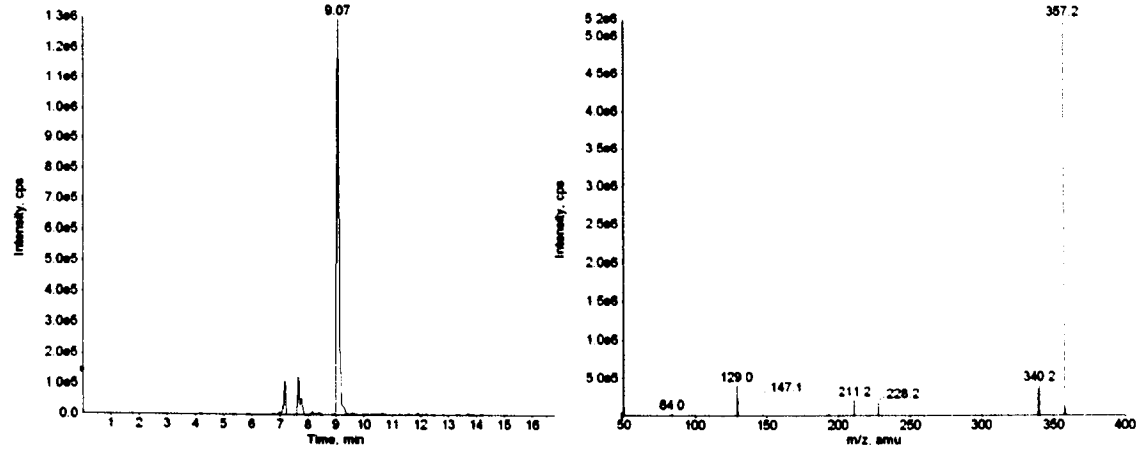



**Figure 5.27** Extracted ion chromatogram of *m/z* 357 and EPI spectrum of the peak at 9.0 min from an EPI triggered PI scan of *m/z* 147.

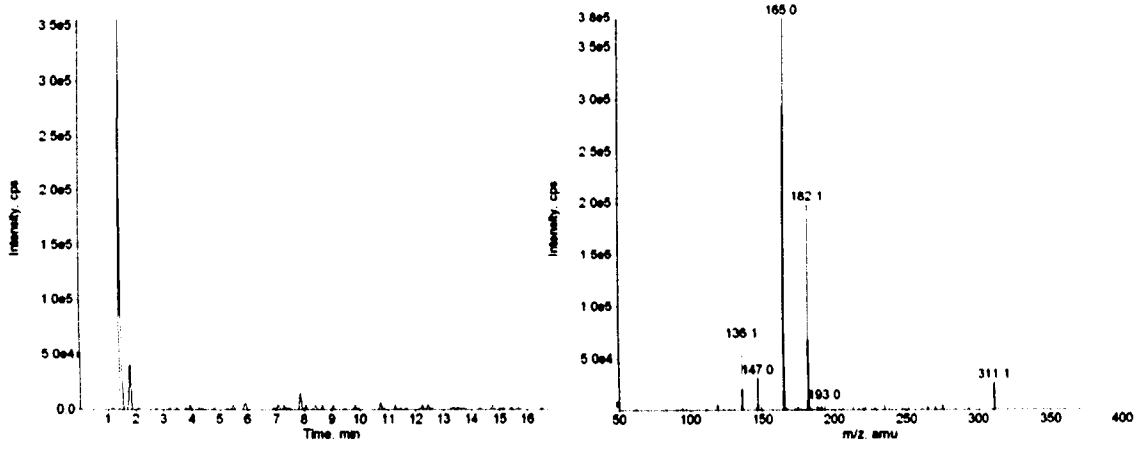



**Figure 5.28** Extracted ion chromatogram of *m/z* 311 and EPI spectrum of the peak at 1.4 min from an EPI triggered PI scan of *m/z* 147.

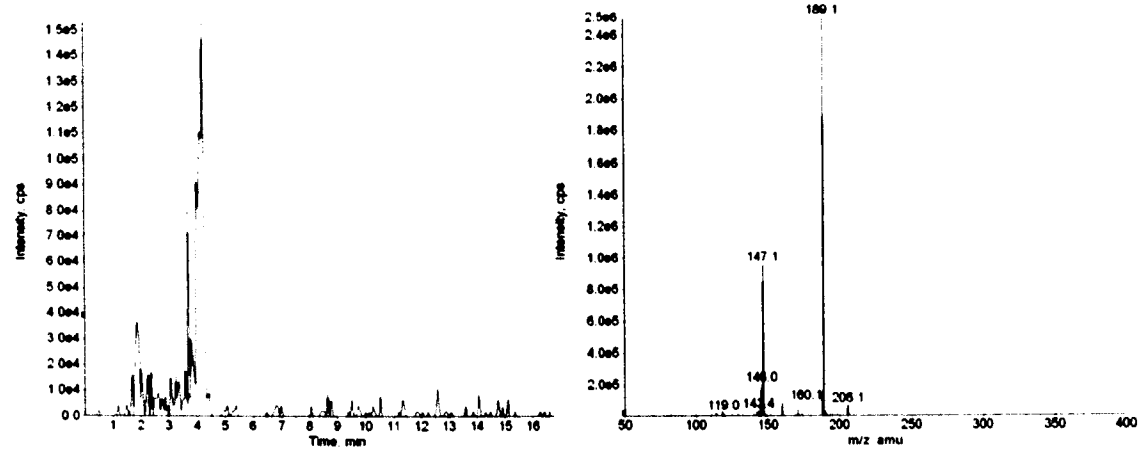



**Figure 5.29** Extracted ion chromatogram of *m/z* 206 and EPI spectrum of the peak at 4.1 min from an EPI triggered PI scan of *m/z* 147.

## Cys or Cys-Gly conjugates

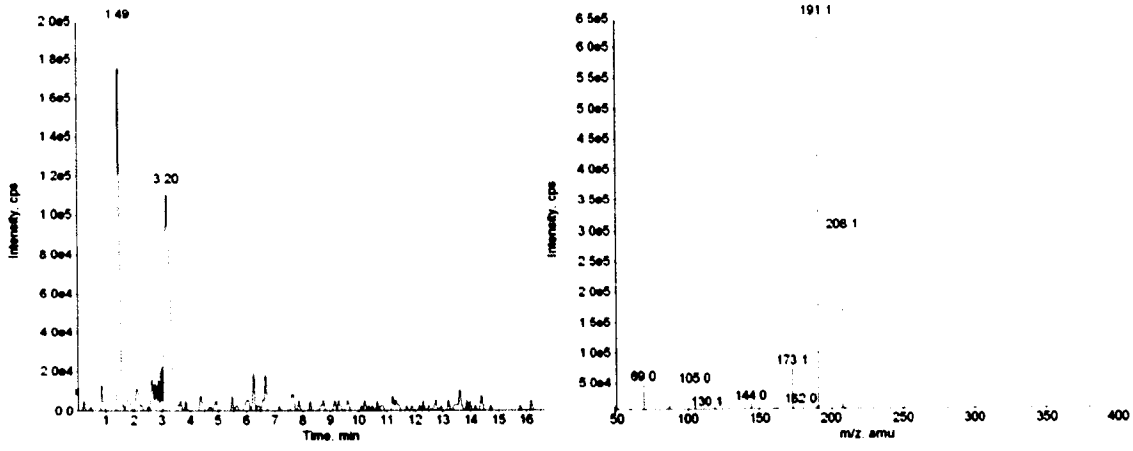



**Figure 5.30** Extracted ion chromatogram of *m/z* 208 and EPI spectrum of the peak at 3.2 min from an EPI triggered PI scan of *m/z* 105.

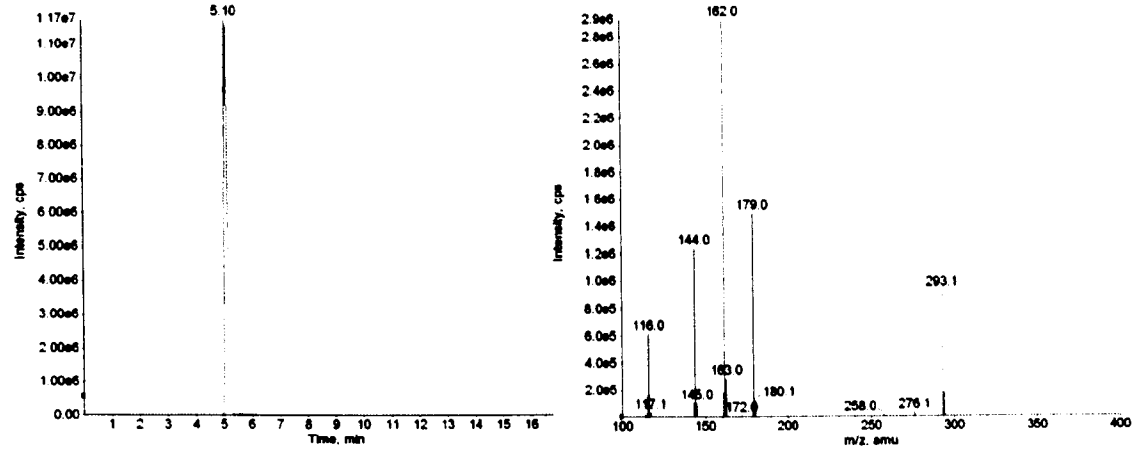



**Figure 5.31** Extracted ion chromatogram of *m/z* 293 and EPI spectrum of the peak at 5.1 min from an EPI triggered PI scan of *m/z* 179.

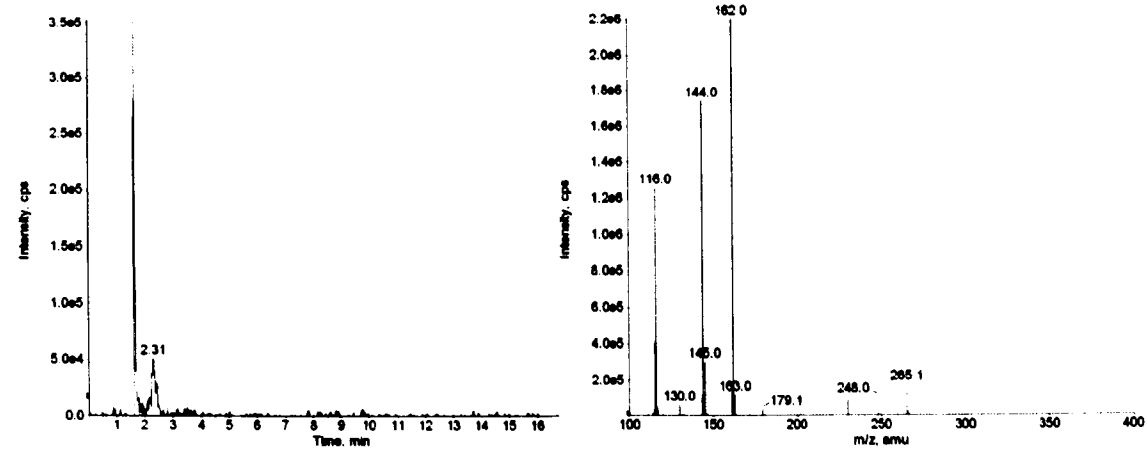



**Figure 5.32** Extracted ion chromatogram of *m/z* 265 and EPI spectrum of the peak at 1.6 min from an EPI triggered PI scan of *m/z* 179.

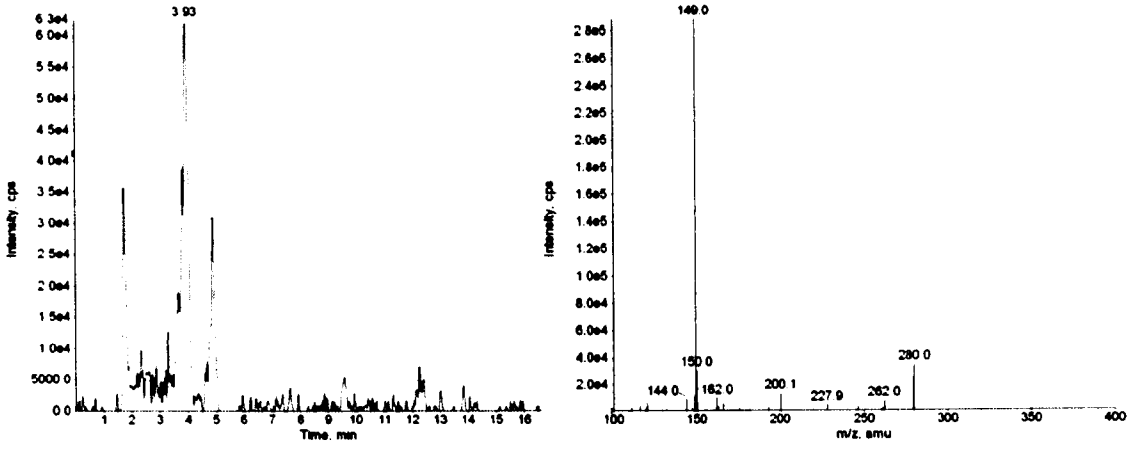



**Figure 5.33** Extracted ion chromatogram of *m/z* 279 and EPI spectrum of the peak at 3.6 min from an EPI triggered PI scan of *m/z* 179.

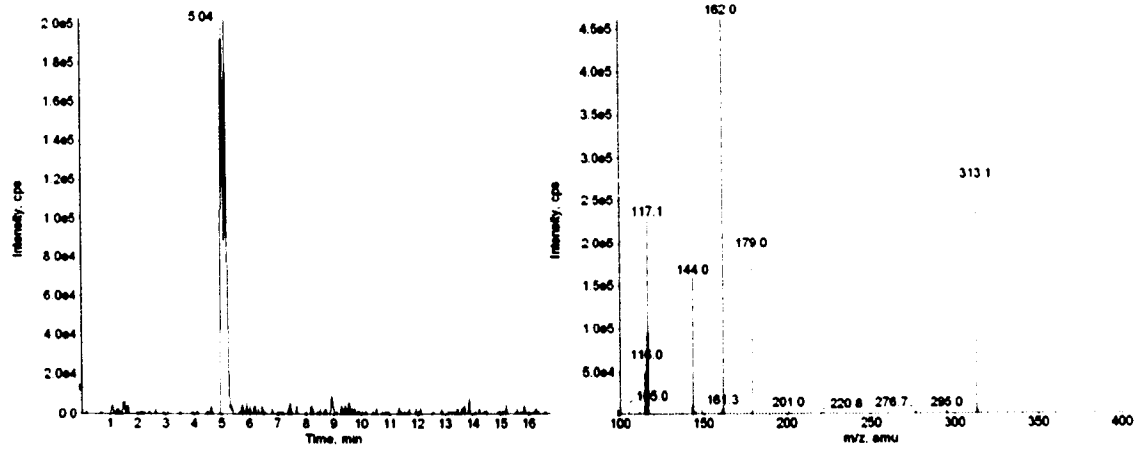



**Figure 5.34** Extracted ion chromatogram of *m/z* 313 and EPI spectrum of the peak at 5.1 min from an EPI triggered PI scan of *m/z* 179.

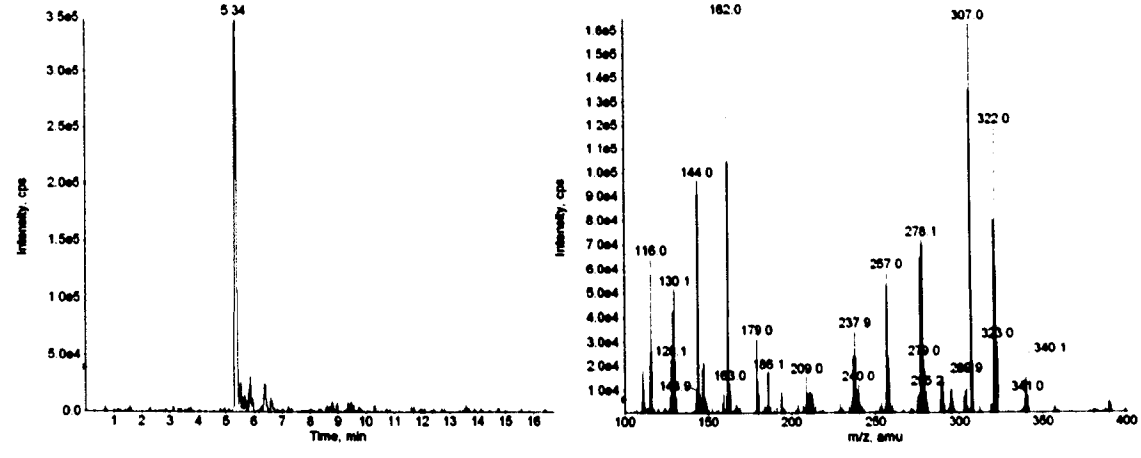



**Figure 5.35** Extracted ion chromatogram of *m/z* 307 and EPI spectrum of the peak at 5.3 min from an EPI triggered PI scan of *m/z* 179.

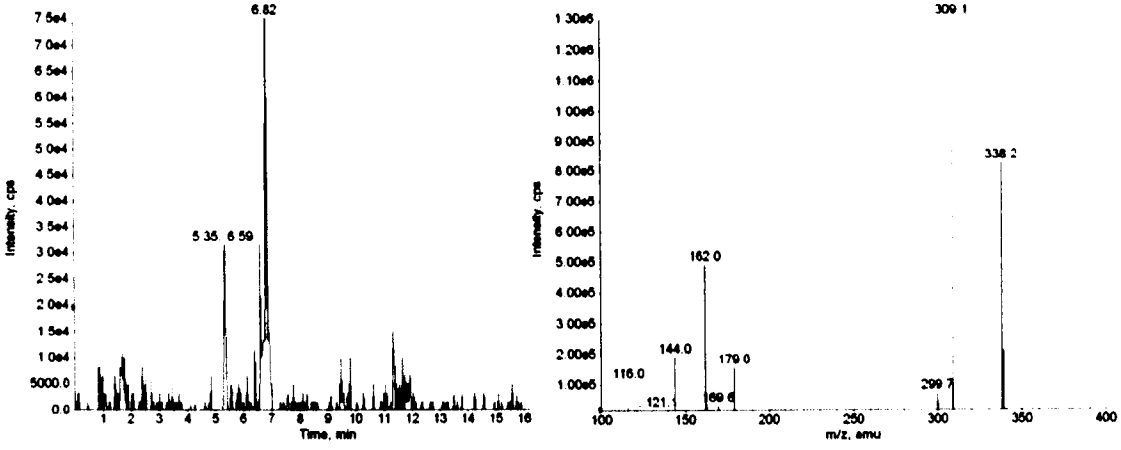



**Figure 5.36** Extracted ion chromatogram of *m/z* 309 and EPI spectrum of the peak at 6.8 min from an EPI triggered PI scan of *m/z* 179.

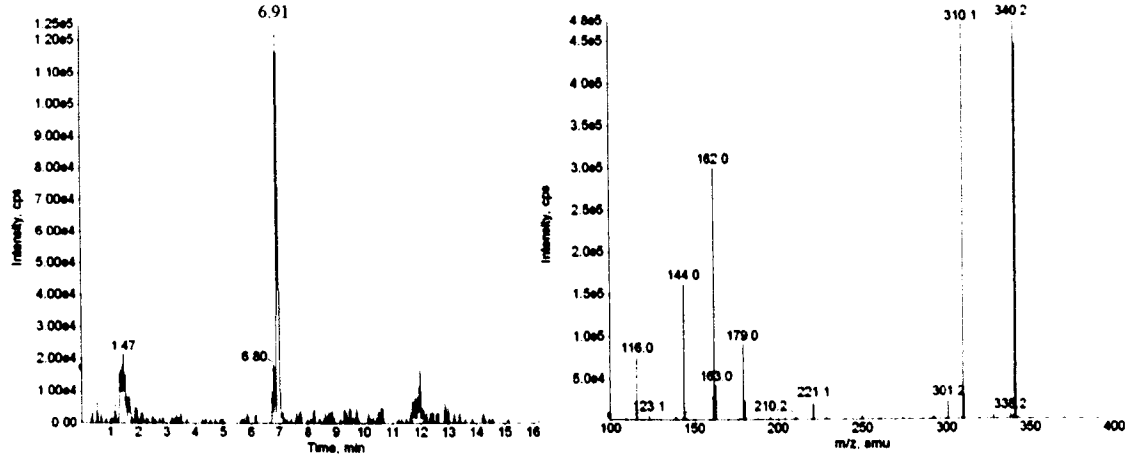



**Figure 5.37** Extracted ion chromatogram of *m/z* 310 and EPI spectrum of the peak at 6.9 min from an EPI triggered PI scan of *m/z* 179.

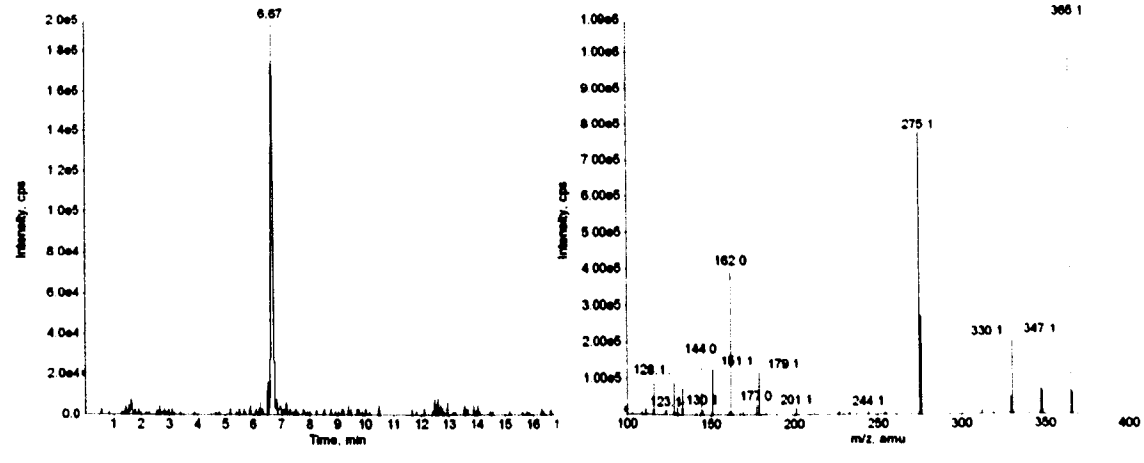



**Figure 5.38** Extracted ion chromatogram of *m/z* 365 and EPI spectrum of the peak at 6.6 min from an EPI triggered PI scan of *m/z* 179.

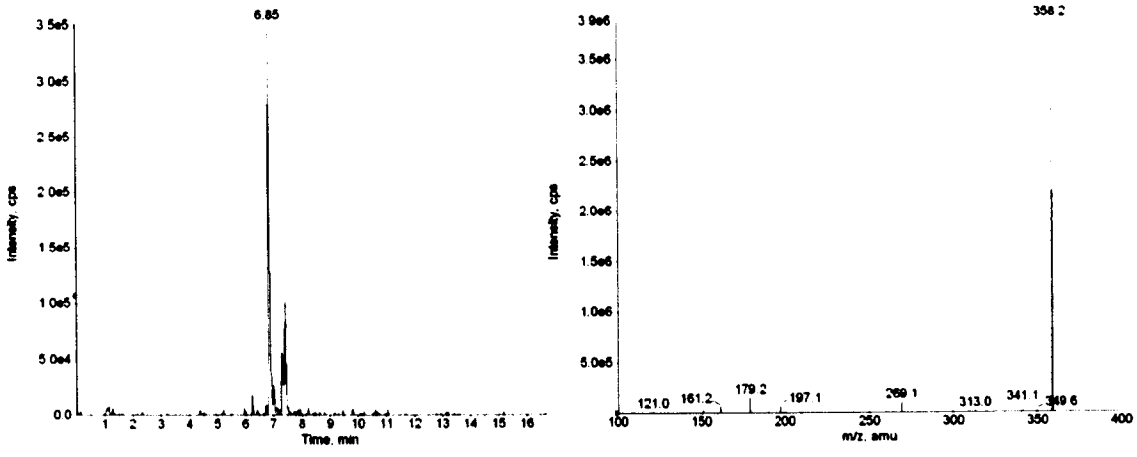



**Figure 5.39** Extracted ion chromatogram of *m/z* 358 and EPI spectrum of the peak at 6.8 min from an EPI triggered PI scan of *m/z* 179.

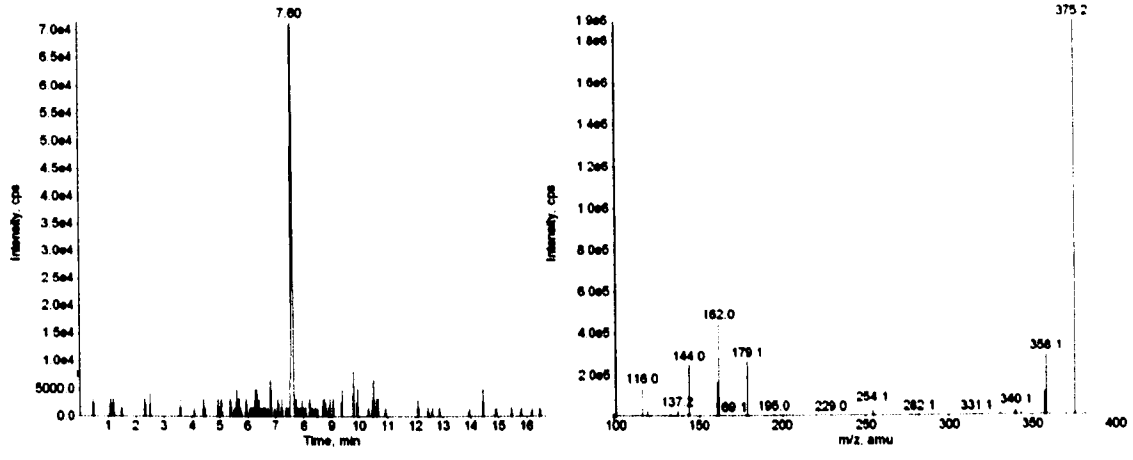



**Figure 5.40** Extracted ion chromatogram of *m/z* 375 and EPI spectrum of the peak at 7.6 min from an EPI triggered PI scan of *m/z* 179.

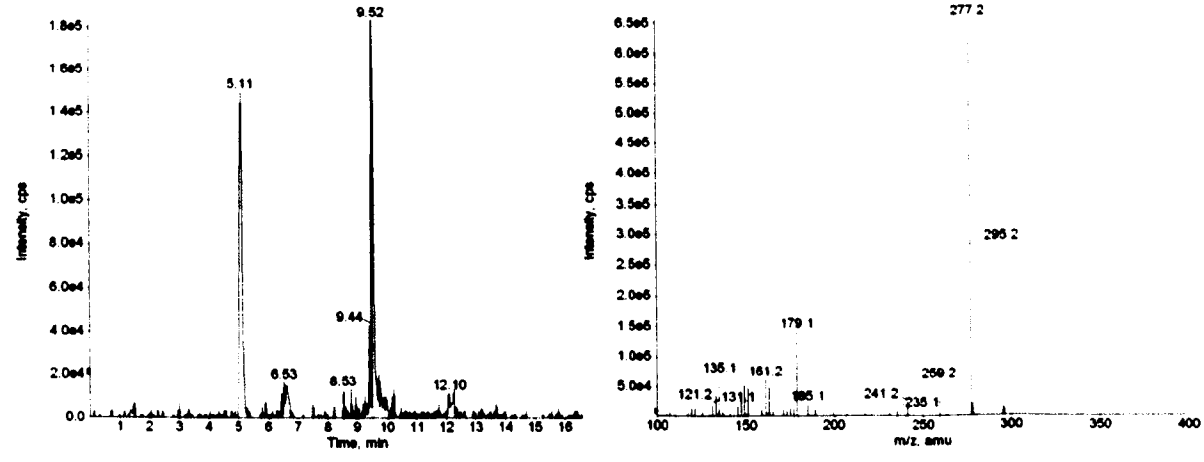



**Figure 5.41** Extracted ion chromatogram of *m/z* 295 and EPI spectrum of the peak at 9.5 min from an EPI triggered PI scan of *m/z* 179.

### 5.4.3 Overall Discussion and Conclusions

Previous analytical studies on human apocrine sweat have predominantly focused on individual 'odour profiles' which are detectable *via* GC-MS analysis often in combination with perceived odour intensity, rated by human volunteers. The work presented herein was more focused on the chemical precursors to 'body odour'.

To my knowledge this is the first NMR study on apocrine sweat samples, in which 25 metabolites could be tentatively identified. Furthermore, inter-person variation could be identified from both the NMR and LC-MS techniques, which is consistent with the reported literature in regards to the analysis of the volatile components (Bernier *et al.*, 1999; Curran *et al.*, 2005; Natsch *et al.*, 2006; Penn *et al.*, 2007; Sommerville *et al.*, 1994).

In total, 12 odour precursors have been identified across all LC-MS techniques with a further 20 being partially identified through the use of the targeted approach, due to the increase in sensitivity over both the Exactive and QTOF systems. Definitive identification was not always possible due to the lack of synthetic standards, however, strong evidence for identity has been provided *via* LC-MS/MS fragmentation as well as accurate mass identification.

Retention times of each of the amino acid odour precursors are expected to differ when comparing against different HPLC columns, due to the differences in the composition of the stationary phase, column length and particle size dimensions. However, in the case of RP columns, the order in which these odour precursors elute are expected to be consistent with one another. In regards to the HILIC column, the elution odour is considered to be opposite to that of RP systems.

The glutamine conjugates, 3M2H-Gln and HMHA-Gln as well as the 3M3SH-Cys-Gly conjugates, have been described as the key precursors to body odour (Emter and Natsch, 2008; Natsch *et al.*, 2006; Natsch *et al.*, 2003; Starkenmann *et al.*, 2005). All three conjugates were detectable across all samples, with HMHA being the most

dominant odour precursor detected. In previous reports, the 3M3SH-Cys-Gly precursor has been shown to vary between individuals, as reported here, however, in a recent paper by Trocas and co-workers; they reported a 2:1 ratio between males and females over a three year study (Troccaz *et al.*, 2009). Furthermore, they showed that the same microflora can generate distinctively different body odours with respect to the composition of apocrine sweat.

Moreover, these reported odour precursors (Natsch *et al.*, 2006; Natsch *et al.*, 2003) have been shown to occur in different ratios between different individuals, but whether these ratios are stable over time and whether they are genetically fixed has not been reported. From the work herein, it is shown that the sweat composition of each individual is consistent from one day to the next. However, it is not certain whether the sweat composition or in particular, the odour precursors, remain constant in an individual across the course of a day.

Both NMR and LC-MS showed that there were no side related differences, nor were there any differences between each 24 h time point. This is consistent with the work by Ferdenzi and co-workers (Ferdenzi *et al.*, 2009), where they concluded that that the left and right axillary can be considered as perceptually equivalent in attractiveness, intensity, or masculinity. However, if handedness is taken into account, there were reported differences between the left and right axillae in left-handed people only. It was reported that the odour, masculinity and intensity was more profound on the more active left side. This was in line with previous observations on asymmetry of axillary odour (Bird and Gower, 1982) and the rate of sweat production (Inaba and Inaba, 1992), with the view that side-related differences could be due to changes in the local environmental conditions caused by the increase in activity, favouring sweating rate or increased activity of microflora (Ferdenzi *et al.*, 2009). However, previous reports have shown the skin microflora to be quantitatively and qualitatively the same in the left and right axillae and not to be affected by handedness or sex (Leyden *et al.*, 1981). Thus, Ferdenzi's hypothesis *"that there exists an underlying activity-independent asymmetry that makes the left axilla smell stronger and that the superimposition of the activity-based asymmetry would balance both sides in right-handers but emphasize the asymmetry in left-handers"*, can neither be confirmed nor dismissed from the work presented here due to the limited sample

volume and lack of available biometric data. Nonetheless, the results presented herein are in agreement with the hypothesis that individual odour differences are more likely to be influenced both by genetics and microflora (Emter and Natsch, 2008; James *et al.*, 2004; Kuhn and Natsch, 2009; Penn and Potts, 1998b; Troccaz *et al.*, 2009). There is also evidence that MHC genes can influence microflora composition (Toivanen *et al.*, 2001).

Further examining odour production in relation to the genotype can offer many practical applications. Firstly, it would be advantageous for researchers if the left or right side could be used separately as identical stimuli (doubling the amount of samples) or as a control *vs* treated. Secondly, there is increasing interest in whether human odours could be used for forensic research (Roberts *et al.*, 2005), potentially non-invasive disease diagnostics (Mantini *et al.*, 2000; Penn and Potts, 1998b; Turner and Magan, 2004; Willis *et al.*, 2004) or reducing the risk of attracting mosquitoes and other disease vectors (Penn *et al.*, 2007). Finally, understanding of how chemonsensory individuality in humans is determined in part by MHC or other genes (Penn and Potts, 1998a; Penn, 2002) would provide rational for both a direct and indirect approach to mask or eliminate these odours.

The findings of this present study illustrate the complementary nature of these analytical techniques and demonstrate the potential value of employing both NMR and LC-MS, in both a global and targeted manner, to the analysis of apocrine sweat samples. Identification of components detected by $^{1}$H NMR spectroscopy was aided by the large body of information already available on metabolites identified in human biofluids. In comparison, the available data for metabolite identification by LC-MS is less extensive which reflects the major disadvantage of using LC-MS in metabolomics, as databases of compounds likely to be encountered in apocrine sweat have not yet been defined. However, through the use of high mass accuracy instruments, elemental compositions can be postulated. Nonetheless, further analysis would be required for structural elucidation of these metabolites *via* the application of LC-NMR-MS.

# Chapter 6

## 6 General Discussion

The main aim of this work was to develop a series of analytical methodologies (NMR, HILIC-MS, RP-UPLC-MS and RP-LC-MS/MS) that would provide further insight into the chemical composition of human apocrine secretions, with particular reference to odour precursors, as well as identifying intra- and inter-subject variation between six individuals. Although the methods were developed for the use of apocrine gland secretions, they have the potential to be adapted for the analysis of other biological fluids such as plasma and urine. Four analytical techniques were developed in tandem, in order to provide as much information as possible on the composition of human apocrine sweat. The most significant developments in each chapter are summarised in Table 6.1.

Analytical methodologies were initially developed with an artificial sweat matrix in order to test analytical procedures and were therefore not designed to replicate apocrine secretions *per se*, due to the current uncertainties about the exact composition. Firstly, NMR procedures were developed with hypoallergenic infant formula from Neocate (www.neocate.co.uk) as the exact composition has been fully characterised and therefore allowed accurate comparison of different NMR conditions. The data evaluated in chapter two showed that a 1 mm micro-volume probe coupled to a 400 MHz spectrometer was best suited to handling small samples volumes typically obtained from human apocrine sweat. The sensitivity enhancement was ~5.3 fold over data acquired using conventional 5 mm OD NMR tubes on a 400 MHz spectrometer, and provided similar sensitivity to that of a 500 MHz spectrometer coupled with a cryo-probe. Secondly, a HILIC-MS method was developed with blood plasma as the biological matrix in order to provide a novel global approach for the separation of the more polar compounds that would ultimately complement the NMR data. This was mostly focused on the separation of amino acids

due to the range of polarities they represent. Finally, two further RP-LC-MS/MS methods were developed with available standards, providing a more selective and sensitive approach for screening against odour precursors. Furthermore, there was the opportunity to analyse the apocrine sweat samples with an orbitrap MS using RP-UPLC. However, due to time constraints, the LC conditions used were developed by Dr. Catharine Ortori (University of Nottingham). The orbitrap is more advanced than the current QTOF instrument, thereby, providing higher mass resolution; which approaches that of FTICR (~100 000) in comparison to QTOF (~10 000); greater sensitivity and mass accuracy as well as having the ability of positive and negative switching during acquisition.

**Table 6.1** Summary of the main conclusions of the work carried out in each chapter

| Chapter | Development |
|---|---|
| Two | Artificial sweat matrix was used to develop NMR methodologies<br><br>1 mm micro-volume probe identified as the most suitable for the analysis of apocrine sweat |
| Three | Developed Global HILIC-MS approach in order to characterise apocrine gland secretions<br><br>Developed semi-targeted LC-MS/MS approach which is capable of screening for unknown odour precursors.<br><br>Developed targeted LC-MS/MS approach for the analysis of known reported odour precursors. |
| Four | Reviewed analytical methodologies with sweat produced *in vitro* using an ASG5 cell line<br><br>PCA and PC-DA was used to explore the metabolite differences between hormonal drug treatments<br><br>Both NMR and HILIC-MS data were able to differentiate between the different treatments, thus, showing complementary information on a different subset of metabolites<br><br>Putatively identified 275 metabolites ranging from amino acids, hydroxy acids, fatty acids, bile acids, nucleotides, lipids and vitamins |
| Five | PCA was used to explore the metabolite differences between volunteers<br><br>All methodologies showed complementary information on a different subset of metabolites<br><br>Left and right secretions were shown to be similar in composition<br><br>Identified 12 known odour precursors and possibly identified a further 20 odour precursors<br><br>Putatively identified 473 metabolites ranging from amino acids, hydroxy acids, fatty acids, bile acids, glucuronides, lipids and vitamins |

To the author's knowledge, this is the first time NMR profiling of human apocrine sweat has been achieved. However, previous studies on eccrine sweat have been reported (Harker *et al.*, 2006). Moreover, to the author's knowledge, this was the first metabolomics study that has used either HILIC or RP-UPLC methodologies for the global metabolite identification of human apocrine sweat. The majority of the literature is GC based (Hasegawa *et al.*, 2004; Natsch *et al.*, 2006; Natsch *et al.*, 2004; Penn *et al.*, 2007; Starkenmann *et al.*, 2005), with some work using targeted RP-LC-MS (Emter and Natsch, 2008; Natsch *et al.*, 2006; Troccaz *et al.*, 2009) for the identification of odour precursors. However, the main novelty of the MS/MS data presented herein, comes from the PI scanning methodology to selectively scan for unknown odour precursors. Nonetheless, one of the major limitations with the MS/MS methodology currently reported is the lack of synthetic standards or isotopically labelled standards to provide absolute identification. Isotopic labelled standards would have the same chemistry and retention time as their counterpart, but would be differentiated due to the difference in molecular weight, thus, providing different MRM transitions. However, under the circumstances, typically for the MS/MS methodologies, metabolite identification proposed was based on knowledge of fragmentation profiles of other standards. If time and funds were not placing such constraints, it may have been possible to organise synthesis of more of the amino acid conjugates (odour precursors). Presence of these compounds would allow the MS/MS methodology to be validated, allowing absolute quantification of these odour precursor metabolites.

Spectral variations obtained from global methodologies (NMR, HILIC-MS, and RP-UPLC-MS) were explored using both PCA and PC-DA, in which the variation was explained by the first three PCs. It was observed that inter-variation for each volunteer was greater than intra-variation. Moreover, samples collected from the left and right arm, as well as 24 h time points, were consistent with one another. This information was also echoed by RP-LC-MS/MS data, which only monitored the odour precursors. It is also noted that a 24 h time point is considered to be a long biological time period, thus, for future studies it would be interesting to identify whether these odour precursors are secreted at a consistent rate during a 24 h period. Nonetheless, this potentially has great benefits for future projects, as not only can one

double the sample volume obtained from a single volunteer, but there is also the potential to have a test and control side per individual. Identifying the metabolites contributing to inter-person variation could not be achieved, partly due to experimental design (small sample size) and the absence of available meta data for each volunteer, thereby, identifying a major limitation. Nonetheless, it was possible to provide further insight into the metabolite composition of human apocrine washes, contributing to the research area greatly, as well as highlighting the complementary nature of the developed methodologies.

The NMR data was able to identify 25 metabolites, with lactate being the most dominant, at ~13.2 mM, which is consistent with the literature (Meyer *et al.*, 2007; Sato *et al.*, 1989) and that reported in eccrine sweat (Harker *et al.*, 2006). Furthermore, comparison of the *in vitro* produced apocrine sweat from AGS cell lines (Burry *et al.*, 2008), was shown to be of similar composition to that of an *in vivo* apocrine sweat collected from human volunteers (see Table 4.1 and Table 5.3 on page 128 and 158 respectively). Thus, it can be concluded that the *in vitro* sweat would have provided a good sample type for method development and as a result would be recommended to use for any further method development. However, when analysing the *in vitro* samples by MS/MS, odour precursors were not shown to be present. Although, 3M2H-Gln has previously been identified through GC-MS with the AGS5 cells (Burry *et al.*, 2008), it is possible that the odour precursors were removed during the extraction procedure or that these compounds were below the limit of detection with the instrumentation used.

The biggest challenge in this project was post acquisition, as in metabolomics analysis there is the requirement of converting an unidentified feature into a known chemical entity i.e. a metabolite. Currently, both NMR and LC-MS have been developed to suit high-throughput analysis. However, these have not been developed to provide automatic identification of the many hundreds of features detected. Thus, identification is still a manual or semi-automated process, which is typically biased towards metabolites of interest rather than all metabolites. Currently, many lab groups quote the number of features, often thousands from LC-MS data, as the number of metabolites detected. This is expected to be many magnitudes lowers due to many metabolites producing more than one feature, for example, fragmentation products,

isotopes, multiply-charged species, or adducts (Brown *et al.*, 2009). Thus, it is not uncommon for one metabolite to produce 10 or more detectable features, adding to the complexity of the data (Dunn *et al.*, 2011b). To overcome these difficulties, a database of all reported sweat metabolites was sourced from available literature (see Appendix A), as well as using generic online databases, such as HMDB (see Table 5.2, page 155), to search for M+H as well as common adducts such as $Na^+$, $K^+$ and $NH_4^+$ to generate a putative identification. However, the majority of the reported literature is GC based, thus, leaving a large gap in identified metabolites *via* LC, as well as the available metabolite databases being far from a complete representation. To overcome these difficulties new MS software would need to be sourced in order to reduce the new number of detected features into a number of suitable *m/z* which could be used to search for hits or potential matches or to construct an accurate chemical formula based on the accurate mass. Nonetheless, 473 metabolites were putatively identified from the analysis of human apocrine sweat, with glucuronides, bile acids and sulphates being readily detected.

LC-MS/MS analyses readily identified 3M2H-Gln and HMHA-Gln, which are considered as the major odour precursor in apocrine sweat (Zeng *et al.*, 1991), as well as identifying 10 other known odour precursors, which are known to be present (Emter and Natsch, 2008; Natsch *et al.*, 2006; Natsch *et al.*, 2004; Starkenmann *et al.*, 2005; Starkenmann *et al.*, 2008). These odour precursors were also detected on both the HILIC and RP-LC-MS methodologies adding further evidence to their presence. Furthermore, the PI scanning approach was able to detect a further 20 metabolites, which have the potential to be unidentified odour precursors. In order to confirm their identities, other methodologies would need to be sourced, as the cost of sample collection, together with limited sample volumes, makes fraction collection followed by NMR analysis unfeasible in this instance. One approach would be to use an LTQ Orbitrap-Velos developed by Thermo Scientific, which would provide $MS^n$ accurate mass fragmentation and together with the knowledge that part of the metabolite is known (i.e. contains an amino acid residue), would allow the molecular formula and structure to be calculated. Once the structure is determined, a synthetic standard would need to be synthesised for absolute clarification.

# Chapter 7

## 7 References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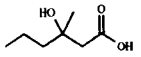
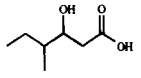
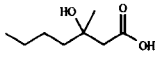
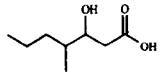
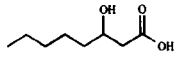
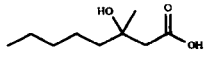
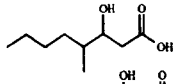
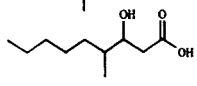
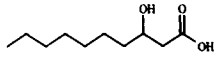
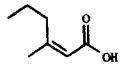
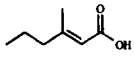
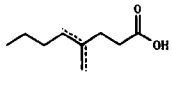
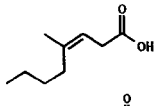
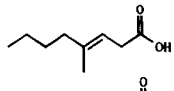
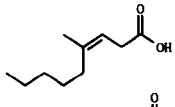
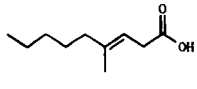
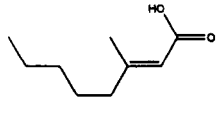
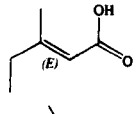
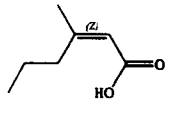
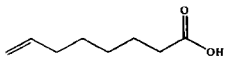
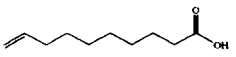
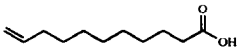
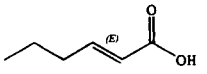
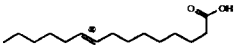
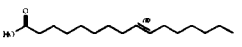
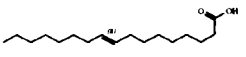
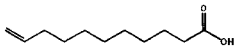
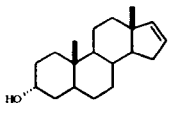
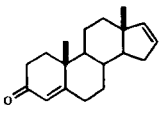
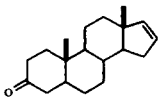
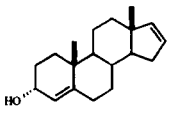
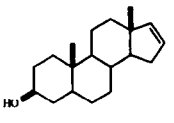
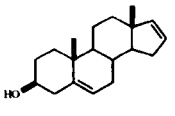
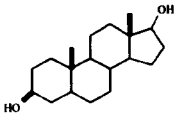
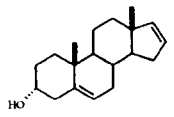
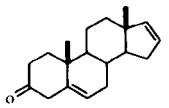
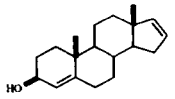
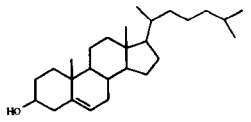
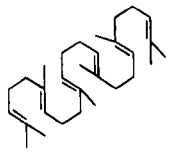
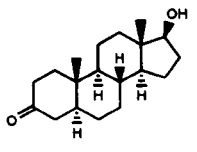
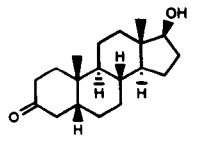
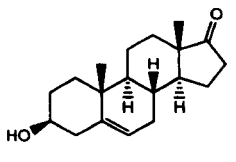
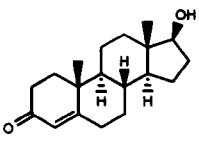
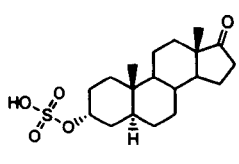
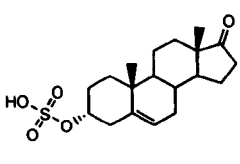
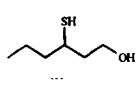
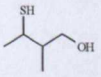
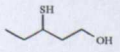
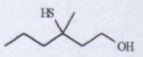
# Appendix A

A list of reported metabolites found in human apocrine sweat secretions. For further information see Appendix A on the attached CD

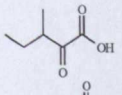
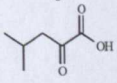
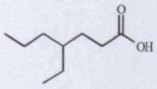
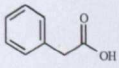
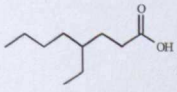
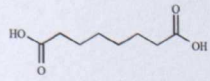
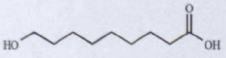
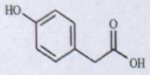
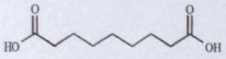
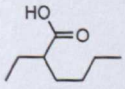
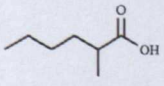
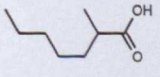
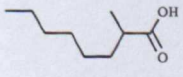
| Components | Structure | Molecule | Mass | M+H |
|---|---|---|---|---|
| **3-Hydroxy acids** | | | | |
| 3-Hydroxy-3-methylhexanoic acid |  | C7H14O3 | 146.0943 | 147.1021 |
| 3-Hydroxy-4-methylhexanoic acid |  | C7H14O3 | 146.0943 | 147.1021 |
| 3-Hydroxy-3-methylheptanoic acid |  | C8H16O3 | 160.1099 | 161.0628 |
| 3-Hydroxy-4-methylheptanoic acid |  | C8H16O3 | 160.1099 | 161.0628 |
| 3-Hydroxyoctanoic acid |  | C8H16O3 | 160.1099 | 161.0628 |
| 3-Hydroxy-3-methyloctanoic acid |  | C9H18O3 | 174.1256 | 175.1334 |
| 3-Hydroxy-4-methyloctanoic acid |  | C9H18O3 | 174.1256 | 175.1334 |
| 3-Hydroxy-4-methylnonanoic acid |  | C10H20O3 | 188.1412 | 189.1491 |
| 3-Hydroxydecanoic acid |  | C10H20O3 | 188.1412 | 189.1491 |
| **Unsaturated acids** | | | | |
| (Z)-3-Methylhex-2-enoic acid |  | C7H12O2 | 128.0837 | 129.0916 |
| (E)-3-Methylhex-2-enoic acid |  | C7H12O2 | 128.0837 | 129.0916 |
| 4-Methyloct-4-enoic acid |  | C9H16O2 | 156.1150 | 157.1229 |
| (Z)-4-Methyloct-3-enoic acid |  | C9H16O2 | 156.1150 | 157.1229 |
| (E)-4-Methyloct-3-enoic acid |  | C9H16O2 | 156.1150 | 157.1229 |
| (Z)-4-Methylnon-3-enoic acid |  | C10H18O2 | 170.1307 | 171.1385 |
| (E)-4-Methylnon-3-enoic acid |  | C10H18O2 | 170.1307 | 171.1385 |
| (E)-3-Methyl-2-octenoic acid |  | C9H16O2 | 156.1150 | 157.1229 |
| (E)-3-Methyl-2-pentenoic acid |  | C6H10O2 | 114.0681 | 115.0759 |
| (Z)-3-Methylhex-2-anoic acid |  | C7H12O2 | 128.0837 | 129.0916 |

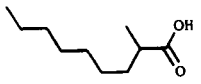
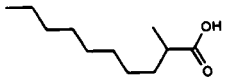
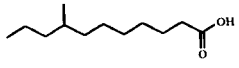
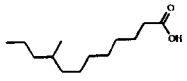
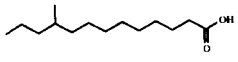
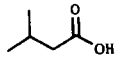
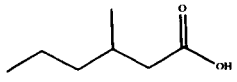
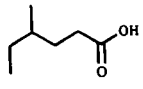
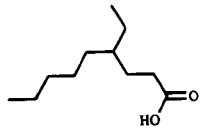
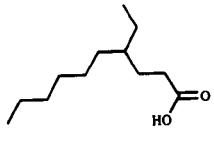
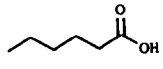
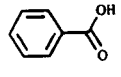
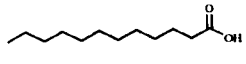
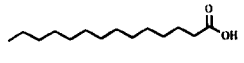
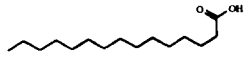
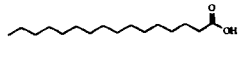
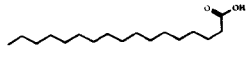
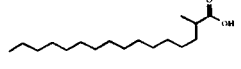
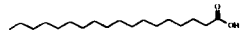
| | | | | |
|---|---|---|---|---|
| 7-Octenoic acid |  | C8H14O2 | 142.0994 | 143.1072 |
| 9-Decenoic acid |  | C10H18O2 | 170.1307 | 171.1385 |
| 10-Undecenoic acid |  | C11H20O2 | 184.1463 | 185.1542 |
| 2-Hexenoic acid |  | C6H10O2 | 114.0681 | 115.0759 |
| 9-Pentadecenoic acid |  | C15H28O2 | 240.2089 | 241.2168 |
| 9-Hexadecenoic acid |  | C16H30O2 | 254.2246 | 255.2324 |
| 9-Heptadecenoic acid |  | C17H32O2 | 268.2402 | 269.2481 |
| Oleic acid |  | C18H34O2 | 282.2559 | 283.2637 |
| Unsaturated C11 acid (10-undecenoic acid) |  | C11H20O2 | 184.1463 | 185.1542 |
| **Steroidal** | | | | |
| 5α-Androst-16-en-3α-ol |  | C19H30O | 274.2297 | 275.2375 |
| Androsta-4, 16-dien-3-one |  | C19H26O | 270.1984 | 271.2062 |
| 5α-Androst-16-en-3-one |  | C19H28O | 272.2140 | 273.2218 |
| Androsta-4, 16-dien-3α-ol |  | C19H28O | 272.2140 | 273.2218 |
| 5α-Androst-16-en-3β-ol |  | C19H30O | 274.2297 | 275.2375 |
| 5α-Androsta-5, 16-dien-3β-ol |  | C19H28O | 272.2140 | 273.2218 |
| Androst-5-ene-3β-17α/β-diols |  | C19H32O2 | 292.2402 | 293.2481 |

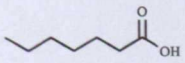
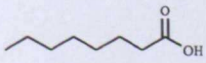
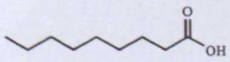
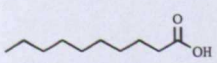
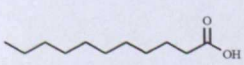
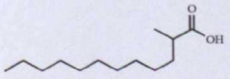
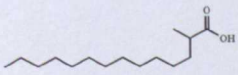
| | | | | |
|---|---|---|---|---|
| Androsta-5, 16-dien-3α-ol |  | C19H28O | 272.2140 | 273.2218 |
| Androsta-5, 16-dien-3-one |  | C19H26O | 270.1984 | 271.2062 |
| Androsta-4, 16-dien-3⊛-ol |  | C19H28O | 272.2140 | 273.2218 |
| Cholesterol |  | C27H46O | 386.3549 | 387.3627 |
| Squalene |  | C30H50 | 410.3913 | 411.3991 |
| 5α-Dihydrotestosterone |  | C19H30O2 | 290.2246 | 291.2324 |
| 5β-Dihydrotestosterone |  | C19H30O2 | 290.2246 | 291.2324 |
| Dehydrocpiandrosterone |  | C19H28O2 | 288.2089 | |
| Testosterone |  | C19H28O2 | 288.2089 | 289.2168 |
| 17-Oxo-5a-androstan-3α-yl sulfate |  | C19H30O5S | 370.1814 | 371.1892 |
| 17-Oxo-5-androsten-3α-yl sulfate |  | C19H28O5S | 368.1657 | 369.1736 |
| **Sulfanylalkanols** | | | | |
| 3-Sulfanylhexan-1-ol |  | C6H14OS | 134.0765 | 135.0844 |

| | | | | |
|---|---|---|---|---|
| 2-Methyl-3-sulfanylbutan-1-ol |  | C5H12OS | 120.0609 | 121.0687 |
| 3-Sulfanylpentan-1-ol |  | C5H12OS | 120.0609 | 121.0687 |
| 3-Methyl-3-sulfanylhexan-1-ol |  | C7H16OS | 148.0922 | 149.1000 |
| Dimethylsulfone |  | C2H6O2S | 94.0089 | 95.0167 |

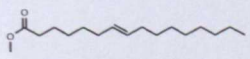
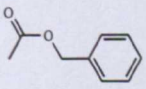
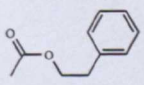
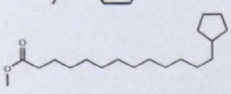
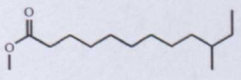
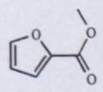
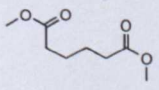
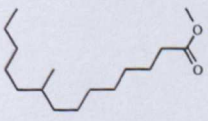
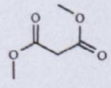
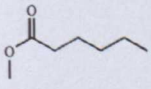
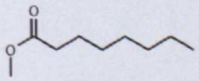
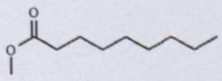
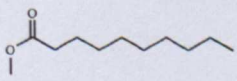
**Amino acids degradtion products and miscellaneous acids**

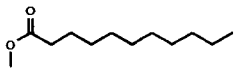
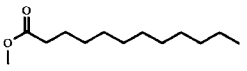
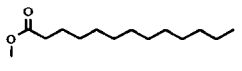
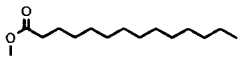
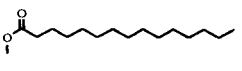
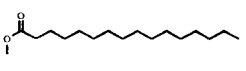
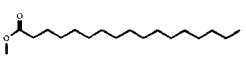
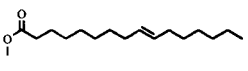
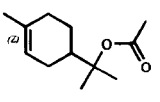
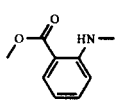
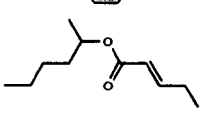
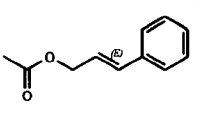
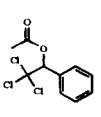
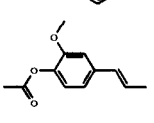
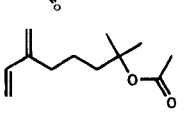
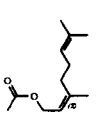
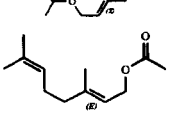
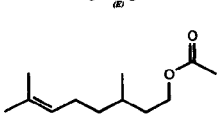
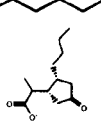
| | | | | |
|---|---|---|---|---|
| 2-Hydroxypropanoic acid |  | C3H6O3 | 90.0317 | 91.0395 |
| 3-Methyl-2-oxopentanoic acid |  | C6H10O3 | 130.0630 | 131.0708 |
| 4-Methyl-2-oxopentanoic acid |  | C6H10O3 | 130.0630 | 131.0708 |
| 4-Ethylheptanoic acid |  | C9H18O2 | 158.1307 | 159.1385 |
| Phenylacetic acid |  | C8H8O2 | 136.0524 | 137.0603 |
| 4-Ethyloctanoic acid ('goat acid') |  | C10H20O2 | 172.1463 | 173.1542 |
| 8-Hydroxyoctanoic acid |  | C8H16O3 | 160.1099 | 161.1178 |
| Octanedioic acid (suberic acid) |  | C8H14O4 | 174.0892 | 175.097 |
| 9-Hydroxynonanoic acid |  | C9H18O3 | 174.1256 | 175.1334 |
| (4-Hydroxyphenyl)acetic acid |  | C8H8O3 | 152.0473 | 153.0552 |
| Nonanedioic acid (azelaic acid) |  | C9H16O4 | 188.1049 | 189.1127 |
| 2-Ethylhexanoic acid |  | C8H16O2 | 144.115 | 145.1229 |
| 2-Piperidinone |  | C5H9NO | 99.0684 | 100.0762 |
| 2-Methylhexanoic acid |  | C7H14O2 | 130.0994 | 131.1072 |
| 2-Methylheptanoic acid |  | C8H16O2 | 144.115 | 145.1229 |
| 2-Methyloctanoic acid |  | C9H18O2 | 158.1307 | 159.1385 |

| | | | | |
|---|---|---|---|---|
| 2-Methylnonanoic acid |  | C10H20O2 | 172.1463 | 173.1542 |
| 2-Methyldecanoic acid |  | C11H22O2 | 186.162 | 187.1698 |
| 8-Methylundecanoic acid |  | C12H24O2 | 200.1776 | 201.1855 |
| 9-Methyldodecanoic acid |  | C13H26O2 | 214.1933 | 215.2011 |
| 10-Methyltridecanoic acid |  | C14H28O2 | 228.2089 | 229.2168 |
| Isovaleric acid |  | C5H10O2 | 102.0681 | 103.0759 |
| 3-Methylhexanoic acid |  | C7H14O2 | 130.0994 | 131.1072 |
| 4-Ethylpentanoic acid |  | C7H14O2 | 130.0994 | 131.1072 |
| 4-Ethylnonanoic acid |  | C11H22O2 | 186.162 | 187.1698 |
| 4-Ethyldecanoic acid |  | C12H24O2 | 200.1776 | 201.1855 |
| Propanoic acid |  | C3H6O2 | 74.0368 | 75.0446 |
| Hexanoic acid |  | C6H12O2 | 116.0837 | 117.0916 |
| Benzoic acid |  | C7H6O2 | 122.0368 | 123.0446 |
| Dodecanoic acid |  | C12H24O2 | 200.1776 | 201.1855 |
| Tetradecanoic acid |  | C14H28O2 | 228.2089 | 229.2168 |
| Pentadecanoic acid |  | C15H30O2 | 242.2246 | 243.2324 |
| Hexadecanoic acid |  | C16H32O2 | 256.2402 | 257.2481 |
| Heptadecanoic acid |  | C17H34O2 | 270.2559 | 271.2637 |
| 2-Methylhexadecanoic acid |  | C17H34O2 | 270.2559 | 271.2637 |
| Octadecanoic acid |  | C18H36O2 | 284.2715 | 285.2794 |

| | | | | |
|---|---|---|---|---|
| *n* -Heptanoic acid |  | C7H14O2 | 130.0994 | 131.1072 |
| *n* -Octanoic acid |  | C8H16O2 | 144.115 | 145.1229 |
| *n* -Nananoic acid |  | C9H18O2 | 158.1307 | 159.1385 |
| *n* -Decanoic acid |  | C10H20O2 | 172.1463 | 173.1542 |
| *n* -Undecanoic acid |  | C11H22O2 | 186.162 | 187.1698 |
| *n* - Methyldodecanoic acid |  | C13H26O2 | 214.1933 | 215.2011 |
| *n* -Methyltetradecanoic acid |  | C15H30O2 | 242.2246 | 243.2324 |

**Esters**

| | | | | |
|---|---|---|---|---|
| 7-Hexadecenoic acid methyl ester |  | C17H32O2 | 268.2402 | 269.2481 |
| Acetic acid phenylmethyl ester |  | C9H10O2 | 150.0681 | 151.0759 |
| 2-Phenylethyl acetate |  | C10H12O2 | 164.0837 | 165.0916 |
| Cyclopentanetridecanoic acid methyl ester |  | C19H36O2 | 296.2715 | 297.2794 |
| Dodecanoic acid, 10-methyl, methyl ester |  | C14H28O2 | 228.2089 | 229.2168 |
| Furancarboxylic acid methyl ester |  | C6H6O3 | 126.0317 | 127.0395 |
| Hexanedioic acid dimethyl ester |  | C8H14O4 | 174.0892 | 175.0970 |
| Methyl 9-methyltetradecanoate |  | C16H32O2 | 256.2402 | 257.2481 |
| Propanedioic acid dimethyl ester |  | C5H8O4 | 132.0423 | 133.0501 |
| Hexanoic acid methyl ester |  | C7H14O2 | 130.0994 | 131.1072 |
| Octanoic acid methyl ester |  | C9H18O2 | 158.1307 | 159.1385 |
| Nonanoic acid methyl ester |  | C10H20O2 | 172.1463 | 173.1542 |
| Decanoic acid methyl ester |  | C11H22O2 | 186.162 | 187.1698 |

| | | | | |
|---|---|---|---|---|
| Undecanoic acid methyl ester |  | C12H24O2 | 200.1776 | 201.1855 |
| Dodecanoic acid methyl ester |  | C13H26O2 | 214.1933 | 215.2011 |
| Tridecanoic acid methyl ester |  | C14H28O2 | 228.2089 | 229.2168 |
| Tetradecanoic acid methyl ester |  | C15H30O2 | 242.2246 | 243.2324 |
| Pentadecanoic acid methyl ester |  | C16H32O2 | 256.2402 | 257.2481 |
| Hexadecanoic acid methyl ester |  | C17H34O2 | 270.2559 | 271.2637 |
| Heptadecanoic acid methylester |  | C18H36O2 | 284.2715 | 285.2794 |
| 9-Hexadecenoic acid methyl ester |  | C17H32O2 | 268.2402 | 269.2481 |
| Terpinyl acetate |  | C12H20O2 | 196.1463 | 197.1542 |
| Methyl-*N*-methylanthranilate |  | C9H11NO2 | 165.079 | 166.0868 |
| 2-Hexyl 2-pentenoate |  | C11H20O2 | 184.1463 | 185.1542 |
| E-Cinnamyl acetate |  | C11H12O2 | 176.0837 | 177.0916 |
| α-Trichloromethyl-benzyl acetate |  | C10H9Cl3O2 | 265.9668 | 266.9746 |
| Isoeugenol acetate |  | C12H14O3 | 206.0943 | 207.1021 |
| Dihydromyrcenol acetate |  | C12H20O2 | 196.1463 | 197.1542 |
| Neryl acetate |  | C12H20O2 | 196.1463 | 197.1542 |
| Geranyl acetate |  | C12H20O2 | 196.1463 | 197.1542 |
| Citronellol acetate |  | C12H22O2 | 198.162 | 199.1698 |
| Methylcis-dihydrojasmonate |  | C12H19O3 | 211.1334 | 212.1412 |

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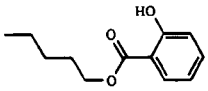
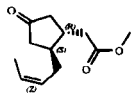
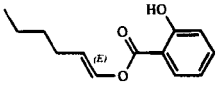
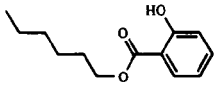
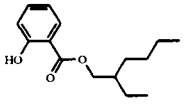
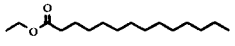
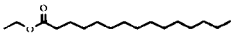
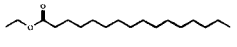
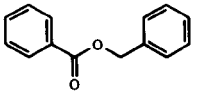
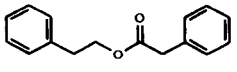
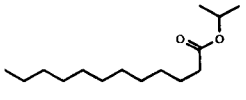
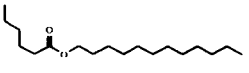
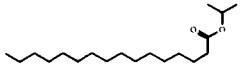
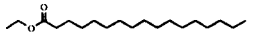
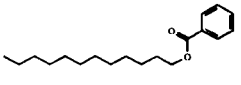
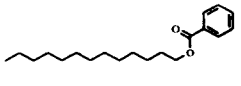
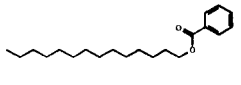
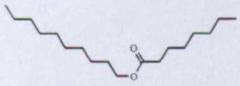
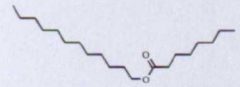
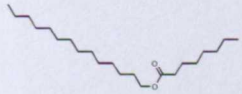
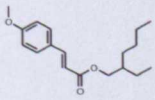
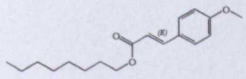
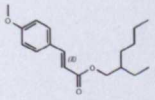
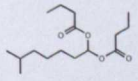
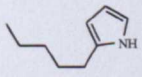
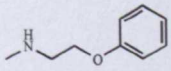
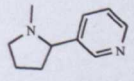
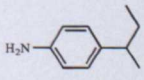
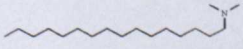
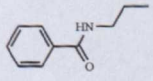
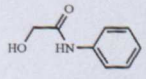
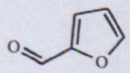
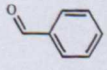
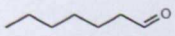
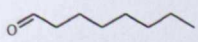
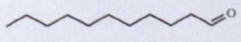
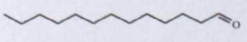
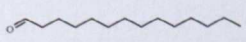
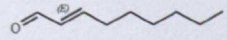
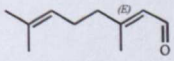
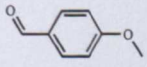
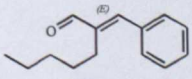
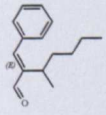
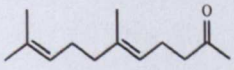
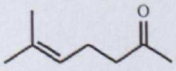
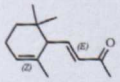
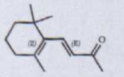
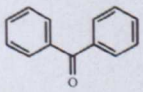
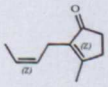
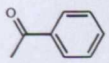
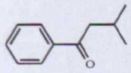
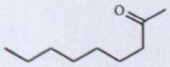
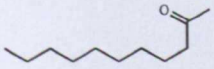
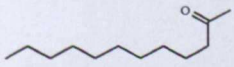
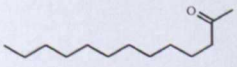
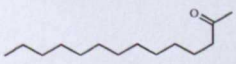
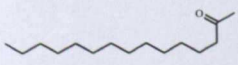
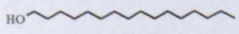
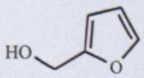
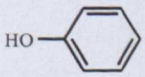
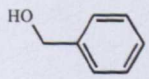
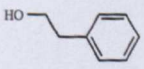
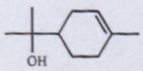
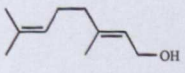
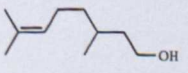
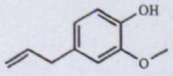
|---|---|---|---|---|
| Pentyl salicylate |  | C12H16O3 | 208.1099 | 209.1178 |
| Methyl trans-jasmonate |  | C12H18O3 | 210.1256 | 211.1334 |
| 1-Hexenyl salicylate |  | C13H16O3 | 220.1099 | 221.1178 |
| 1-Hexyl salicylate |  | C13H18O3 | 222.1256 | 223.1334 |
| 2-Ethylhexyl salicylate |  | C15H22O3 | 250.1569 | 251.1647 |
| Ethyl tetradecanoate |  | C16H32O2 | 256.2402 | 257.2481 |
| Ethyl pentadecanoate |  | C17H34O2 | 270.2559 | 271.2637 |
| Ethyl hexadecanoate |  | C18H36O2 | 284.2715 | 285.2794 |
| Benzyl benzoate |  | C14H12O2 | 212.0837 | 213.0916 |
| 2-phenylethyl phenylacetate |  | C16H16O2 | 240.1150 | 241.1229 |
| Isopropyl dodecanoate |  | C15H30O2 | 242.2246 | 243.2324 |
| Dodecyl hexanoate |  | C18H36O2 | 284.2715 | 285.2794 |
| Hexadecanoic acid isopropyl ester |  | C19H38O2 | 298.2872 | 299.2950 |
| Ethyl heptadecanoate |  | C19H38O2 | 298.2872 | 299.2950 |
| Dodecyl benzoate |  | C19H30O2 | 290.2246 | 291.2324 |
| Tridecyl benzoate |  | C20H32O2 | 304.2402 | 305.2481 |
| Tetradecyl benzoate |  | C21H34O2 | 318.2559 | 319.2637 |

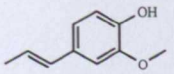
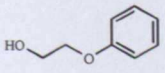
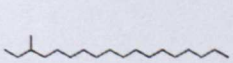
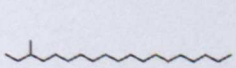
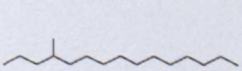
| | | | | |
|---|---|---|---|---|
| Decyl octanoate |  | C18H36O2 | 284.2715 | 285.2794 |
| Dodecyl octanoate |  | C20H40O2 | 312.3028 | 313.3107 |
| Tetradecyl octanoate |  | C22H44O2 | 340.3341 | 341.3420 |
| 2-Ethylhexyl 4-methoxycinnamate |  | C18H26O3 | 290.1882 | 291.1960 |
| 1-Octyl 4-methoxycinnamate |  | C18H26O3 | 290.1882 | 291.1960 |
| 2-Ethyl-hexyl 4-methoxycinnamate |  | C18H26O3 | 290.1882 | 291.1960 |
| Isooctanedioldibutyrate |  | C16H30O4 | 286.2144 | 287.2222 |
| **Amines** | | | | |
| 2-Pentylpyrrole |  | C9H15N | 137.1204 | 138.1283 |
| 2-Phenoxyethylmethylamine |  | C9H13NO | 151.0997 | 152.1075 |
| Nicotine |  | C10H14N2 | 162.1157 | 163.1235 |
| 4-Sec-butylaniline |  | C10H15N | 149.1204 | 150.1283 |
| N,N-Dimethyl-1-dodecylamine |  | C14H31N | 213.2457 | 214.2535 |
| N,N-Dimethyl-1-hexadecylamine |  | C18H39N | 269.3083 | 270.3161 |
| N,N-Dimethyl-1-octadecylamine |  | C20H43N | 297.3396 | 298.3474 |
| **Amides** | | | | |
| Methyl N,N-diethylthiocarbamate |  | C6H13NOS | 147.0718 | 148.0796 |
| n-Propylbenzamide |  | C10H13NO | 163.0997 | 164.1075 |
| Hydroxy acetanilide |  | C8H9NO2 | 151.0633 | 152.0712 |
| **Aldehydes** | | | | |
| 2-Furancarboxaldehyde |  | C5H4O2 | 96.0211 | 97.029 |
| Benzaldehyde |  | C7H6O | 106.0419 | 107.0497 |

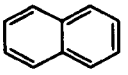
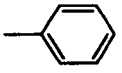
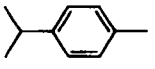
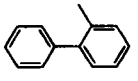
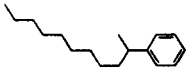
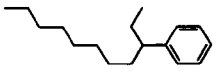
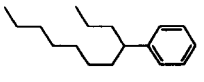
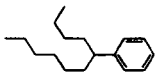
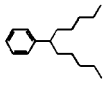
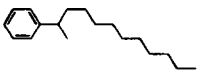
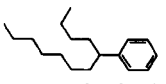
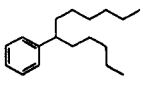
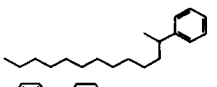
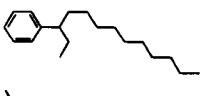
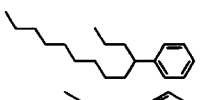
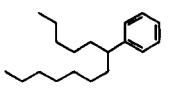
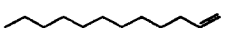
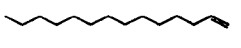
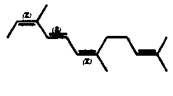
| | | | | |
|---|---|---|---|---|
| Hexanal |  | C6H12O | 100.0888 | 101.0966 |
| Heptanal |  | C7H14O | 114.1045 | 115.1123 |
| Octanal |  | C8H16O | 128.1201 | 129.1279 |
| Nonanal |  | C9H18O | 142.1358 | 143.1436 |
| Decanal |  | C10H20O | 156.1514 | 157.1592 |
| Undecanal |  | C11H22O | 170.1671 | 171.1749 |
| Dodecanal |  | C12H24O | 184.1827 | 185.1905 |
| Tridecanal |  | C13H26O | 198.1984 | 199.2062 |
| Tetradecanal |  | C14H28O | 212.214 | 213.2218 |
| Hexadecanal |  | C16H32O | 240.2453 | 241.2531 |
| (E)-2-Nonenal |  | C9H16O | 140.1201 | 141.1279 |
| Geranial |  | C10H16O | 152.1201 | 153.1279 |
| p-Anisaldehyde |  | C8H8O2 | 136.0524 | 137.0524 |
| Pentylcinnamaldehyde |  | C14H18O | 202.1358 | 203.1436 |
| E-2-Hexylcinnamaldehyde |  | C15H20O | 216.1514 | 217.1592 |

**Ketones**

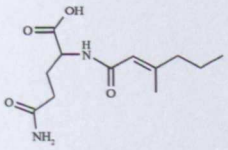
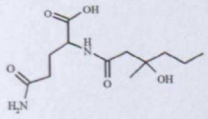
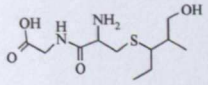
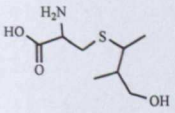
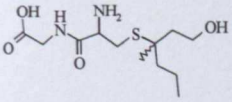
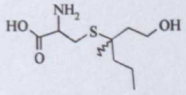
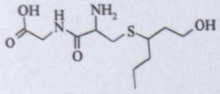
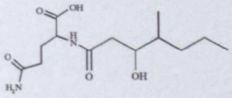
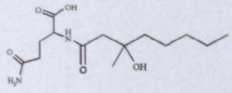
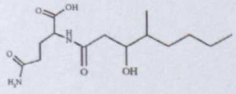
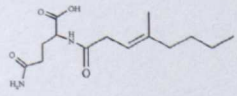
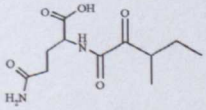
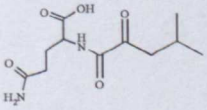
| | | | | |
|---|---|---|---|---|
| 6,10-Dimethyl-5,9-undecadien-2-one |  | C13H22O | 194.1671 | 195.1749 |
| 6-Methyl-5-hepten-2-one |  | C8H14O | 126.1045 | 127.1123 |
| α-Ionone |  | C13H20O | 192.1514 | 193.1592 |
| β-Ionone |  | C13H20O | 192.1514 | 193.1592 |
| Benzophenone |  | C13H10O | 182.0732 | 183.0810 |
| Jasmone |  | C11H16O | 164.1201 | 165.1279 |

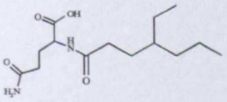
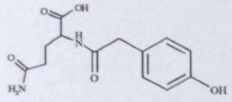
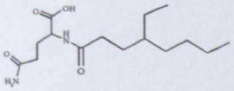
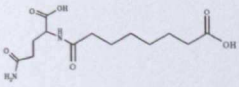
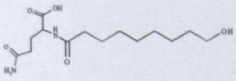
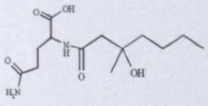
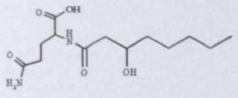
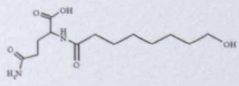
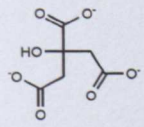
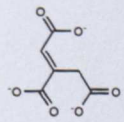
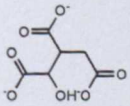
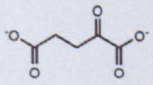
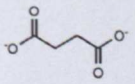
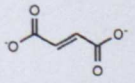
| | | | | |
|---|---|---|---|---|
| Acetophenone |  | C8H8O | 120.0575 | 121.0653 |
| Isopropylacetophenone |  | C11H14O | 162.1045 | 163.1045 |
| 2-Nonanone |  | C9H18O | 142.1358 | 143.1436 |
| 2-Undecanone |  | C11H22O | 170.1671 | 171.1749 |
| 2-Dodecanone |  | C12H24O | 184.1827 | 185.1905 |
| 2-Tridecanone |  | C13H26O | 198.1984 | 199.2062 |
| 2-Tetradecanone |  | C14H28O | 212.2140 | 213.2218 |
| 2-Pentadecanone |  | C15H30O | 226.2297 | 227.2375 |
| 2-Hexadecanone |  | C16H32O | 240.2453 | 241.2531 |
| **Alcohols** | | | | |
| Tridecan-1-ol |  | C13H28O | 200.2140 | 201.2218 |
| *n*-Tetradecanol |  | C14H30O | 214.2297 | 215.2375 |
| Pentadecanol |  | C15H32O | 228.2453 | 229.2531 |
| *n*-Hexadecanol |  | C16H34O | 242.2610 | 243.2688 |
| a Hexadecadienol |  | C16H30O | 238.2297 | 239.2375 |
| 2-Furanmethanol |  | C5H6O2 | 98.0368 | 99.0446 |
| Phenol |  | C6H6O | 94.0419 | 95.0497 |
| Benzyl Alcohol |  | C7H8O | 108.0575 | 109.0653 |
| 2-Phenylethanol |  | C8H10O | 122.0732 | 123.0810 |
| p-Menth-1-en-8-ol |  | C10H18O | 154.1358 | 155.1436 |
| Geraniol |  | C10H18O | 154.1358 | 155.1436 |
| Citronellol |  | C10H20O | 156.1514 | 157.1592 |
| Eugenol |  | C10H12O2 | 164.0837 | 165.0916 |

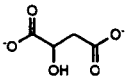
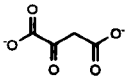
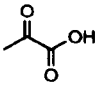
| | | | | |
|---|---|---|---|---|
| Isoeugenol |  | C10H12O2 | 164.0837 | 165.0916 |
| 2-Phenoxyethanol |  | C8H10O2 | 138.0681 | 139.0759 |
| **Aliphatic/Aromatic** | | | | |
| Nonane |  | C9H20 | 128.1565 | 129.1643 |
| Undecane |  | C11H24 | 156.1878 | 157.1956 |
| Dodecane |  | C12H26 | 170.2035 | 171.2113 |
| Tridecane |  | C13H28 | 184.2191 | 185.2269 |
| Tetradecane |  | C14H30 | 198.2348 | 199.2426 |
| Pentadecane |  | C15H32 | 212.2504 | 213.2582 |
| Hexadecane |  | C16H34 | 226.2661 | 227.2739 |
| Heptadecane |  | C17H36 | 240.2817 | 241.2895 |
| Octadecane |  | C18H38 | 254.2974 | 255.3052 |
| Nonadecane |  | C19H40 | 268.3130 | 269.3208 |
| Eicosane |  | C20H42 | 282.3287 | 283.3365 |
| Heneicosane |  | C21H44 | 296.3443 | 297.3521 |
| Docosane |  | C22H46 | 310.3600 | 311.3678 |
| Tricosane |  | C23H48 | 324.3756 | 325.3834 |
| Tetracosane |  | C24H50 | 338.3913 | 339.3991 |
| 3-Methyloctadecane |  | C19H40 | 268.3130 | 269.3208 |
| 3-Methylnonadecane |  | C20H42 | 282.3287 | 283.3365 |
| 4-Methylpentadecane |  | C16H34 | 226.2661 | 227.2739 |
| Nonane, 1-chloro- |  | C9H19Cl | 162.1175 | 163.1254 |
| Pyridine |  | C5H5N | 79.0422 | 80.0500 |

| | | | | |
|---|---|---|---|---|
| α-Pinene |  | C10H16 | 136.1252 | 137.1330 |
| Naphthalene |  | C10H8 | 128.0626 | 129.0704 |
| Cyclotetradecane |  | C14H28 | 196.2191 | 197.2269 |
| Toluene |  | C7H8 | 92.0626 | 93.0704 |
| p-Cymene |  | C10H14 | 134.1096 | 135.1174 |
| Methyl biphenyl |  | C13H12 | 168.0939 | 169.1017 |
| 2-Phenylundecane |  | C17H28 | 232.2191 | 233.2269 |
| 3-Phenylundecane |  | C17H28 | 232.2191 | 233.2269 |
| 4-Phenylundecane |  | C17H28 | 232.2191 | 233.2269 |
| 5-Phenylundecane |  | C17H28 | 232.2191 | 233.2269 |
| 6-Phenylundecane |  | C17H28 | 232.2191 | 233.2269 |
| 2-Phenyldodecane |  | C18H30 | 246.2348 | 247.2426 |
| 5-Phenyldodecane |  | C18H30 | 246.2348 | 247.2426 |
| 6-Phenyldodecane |  | C18H30 | 246.2348 | 247.2426 |
| 2-Phenyltridecane |  | C19H32 | 260.2504 | 261.2582 |
| 3-Phenyltridecane |  | C19H32 | 260.2504 | 261.2582 |
| 4-Phenyltridecane |  | C19H32 | 260.2504 | 261.2582 |
| 6-Phenyltridecane |  | C19H32 | 260.2504 | 261.2582 |
| 1-Dodecene |  | C12H24 | 168.1878 | 169.1956 |
| 1-Tetradecene |  | C14H28 | 196.2191 | 197.2269 |
| 1-Nonadecene |  | C19H38 | 266.2974 | 267.3052 |
| Farnesene |  | C15H24 | 204.1878 | 205.1956 |

**Precursors**

| | | | | |
|---|---|---|---|---|
| N-α-3-methylhex-2-enoyl-L-glutamine |  | C12H20N2O4 | 256.1423 | 257.1501 |
| N-α-3-hydroxy-3-methylhexanoyl- L-glutamine |  | C12H22N2O5 | 274.1529 | 275.1607 |
| S-[1-(2-hydroxy-1-methylethyl)-2-methylethyl]-L-cysteineylglycine |  | C11H22N2O4S | 278.1300 | 279.1379 |
| S-[1-(2-hydroxy-1-methylethyl)-1-ethyl]-L-cysteine |  | C8H17NO3S | 207.0929 | 208.1007 |
| S-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cysteinylglycine |  | C12H24N2O4S | 292.1457 | 293.1535 |
| S-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cystein |  | C10H21NO3S | 235.1242 | 236.1320 |
| S-[1-(2-hydroxyethyl)-butyl]-L-cysteinylglycine |  | C11H22N2O4S | 278.1300 | 279.1379 |
| N-α-3-hydroxy-4-methylhept anoyl- L-glutamine |  | C13H24N2O5 | 288.1685 | 289.1763 |
| N-α-3-hydroxy-3-methyloct anoyl- L-glutamine |  | C14H26N2O5 | 302.1842 | 303.1920 |
| N-α-3-hydroxy-4-methyloct anoyl- L-glutamine |  | C14H26N2O5 | 302.1842 | 303.1920 |
| N-α-4-methyl-3-oct-enoyl- L-glutamine |  | C14H24N2O4 | 284.1736 | 285.1814 |
| N-α-3-methyl-2-oxopent-anoyl- L-glutamine |  | C11H18N2O5 | 258.1216 | 259.1294 |
| N-α-4-methyl-2-oxopent-anoyl- L-glutamine |  | C11H18N2O5 | 258.1216 | 259.1294 |

| | | | | |
|---|---|---|---|---|
| N-α-4-ethyl-hept-anoyl- L-glutamine |  | $C_{14}H_{26}N_2O_4$ | 286.1893 | 287.1971 |
| N-α-4-hydroxyphenyl-acetyl- L-glutamine |  | $C_{13}H_{16}N_2O_5$ | 280.1059 | 281.1137 |
| N-α-4-ethyl-oct-anoyl- L-glutamine |  | $C_{15}H_{28}N_2O_4$ | 300.2049 | 301.2127 |
| N-α-7-carboxy-hept-anoyl- L-glutamine |  | $C_{13}H_{22}N_2O_6$ | 302.1478 | 303.1556 |
| N-α-9-hydroxy- non-anoyl- L-glutamine |  | $C_{14}H_{26}N_2O_5$ | 302.1842 | 303.1920 |
| **Hypothetical Precursors** | | | | |
| N-α-3-hydroxy- 3-methylhept-anoyl- L-glutamine |  | $C_{13}H_{24}N_2O_5$ | 288.1685 | 289.1763 |
| N-α-3-hydroxy-oct-anoyl- L-glutamine |  | $C_{13}H_{24}N_2O_5$ | 288.1685 | 289.1763 |
| N-α-8-hydroxy-oct-anoyl- L-glutamine |  | $C_{13}H_{24}N_2O_5$ | 288.1685 | 289.1763 |
| **TCA Cycle Intermediates** | | | | |
| Citrate |  | $C_6H_5O_7^{3-}$ | 189.0035 | 190.0114 |
| Aconitate |  | $C_6H_3O_6^{3-}$ | 170.9930 | 172.0008 |
| Isocitrate |  | $C_6H_5O_7^{3-}$ | 189.0035 | 190.0114 |
| α-Ketoglutarate |  | $C_5H_4O_5^{2-}$ | 144.0059 | 145.0137 |
| Succinate |  | $C_4H_4O_4^{2-}$ | 116.0110 | 117.0188 |
| Fumarate |  | $C_4H_4O_2^{2-}$ | 84.0211 | 85.0290 |

| | | | | |
|---|---|---|---|---|
| Malate |  | $C_4H_4O_5^{2-}$ | 132.0059 | 133.0137 |
| Oxaloacetate |  | $C_4H_2O_5^{2-}$ | 129.9902 | 130.9980 |
| Pyruvic acid |  | $C_3H_4O_3$ | 88.0160 | 89.0238 |

# Appendix B

A list of putitive identification of metabolites present in the *in vitro* ASG5 cell line sweat samples.

| m/z | rt (s) | ppm error | MF | Adduct | Metabolites | Class |
|---|---|---|---|---|---|---|
| **Carbohydrates** | | | | | | |
| 365.105 | 599.05 | 1.78 | C12H22O11 | Na | 1-alpha-D-Galactosyl-myo-inositol or related compounds | Carbohydrates |
| 689.2125 | 682.00 | 0.73 | C24H42O21 | Na | 1,1-kestotetraose or related compounds | Carbohydrates |
| 291.0692 | 444.26 | 2.71 | C9H16O9 | Na | 2(alpha-D-Mannosyl)-D-glycerate or related compounds | Carbohydrates |
| **Amines** | | | | | | |
| 232.1532 | 523.19 | 4.87 | C11H21NO4 | H | 3-(1-Aminoethyl)Nonanedioic Acid;Isobutyryl-L-carnitine;N(alpha)-t-Butoxycarbonyl-L-leucine;O-Butanoylcarnitine | Quaternary Amines |
| 282.2785 | 108.51 | 2.23 | C18H35NO | H | Oleamide | Fatty amides |
| 131.1175 | 482.98 | 2.97 | C6H14N2O | H | epsilon-aminocaproamide or related compounds | Polyamines |
| 137.0708 | 576.19 | 1.02 | C7H8N2O | H | N-Methylnicotinamide or related compounds | Cyclic Amines |
| **Amino Acids** | | | | | | |
| 291.1288 | 658.24 | 3.81 | C10H18N4O6 | H | Argininosuccinic acid;N(omega)-(L-Arginino)succinate | Amino Acids |
| 294.2411 | 113.38 | 2.58 | C16H33NO2 | Na | 2-amino-hexadecanoic acid or related compounds | Amino fatty acids |
| 126.0222 | 606.65 | 1.98 | C2H7NO3S | H | Taurine | Amino Acids |
| 90.0544 | 613.64 | 6.22 | C3H7NO2 | H | L-Alanine | Amino Acids |
| 134.0454 | 625.87 | 4.55 | C4H7NO4 | H | L-Aspartate | Amino Acids |
| 133.0608 | 639.57 | 0.23 | C4H8N2O3 | H | L-Asparagine | Amino Acids |
| 132.0764 | 613.03 | 2.73 | C4H9N3O2 | H | Creatine | Amino Acids |
| 120.0651 | 617.18 | 3.50 | C4H9NO3 | H | L-Threonine | Amino Acids |
| 147.0759 | 626.82 | 3.54 | C5H10N2O3 | H | L-Glutamine | Amino Acids |
| 150.0577 | 532.21 | 4.20 | C5H11NO2S | H | L-Methionine | Amino Acids |
| 116.0703 | 585.59 | 2.67 | C5H9NO2 | H | L-Proline | Amino Acids |
| 148.0602 | 621.57 | 1.62 | C5H9NO4 | H | L-Glutamate | Amino Acids |
| 130.0861 | 593.70 | 1.15 | C6H11NO2 | H | N4-Acetylaminobutanal or related compounds | Amino Acids |
| 132.1017 | 504.56 | 1.51 | C6H13NO2 | H | L-Isoleucine;L-Leucine | Amino Acids |
| 147.1125 | 597.33 | 2.04 | C6H14N2O2 | H | L-Leucyl-Hydroxylamine | Amino Acids |
| 175.1183 | 637.50 | 3.71 | C6H14N4O2 | H | L-Arginine | Amino Acids |
| 139.0495 | 556.97 | 5.11 | C6H6N2O2 | H | Urocanic acid or related compound | Amino Acids |
| 146.117 | 700.58 | 3.83 | C7H15NO2 | H | 2-amino-heptanoic acid or related compounds | Amino fatty acids |
| 138.0551 | 564.84 | 1.01 | C7H7NO2 | H | 2-nitrotoluene or related compounds | Amino Acids derivative |
| 152.0702 | 214.03 | 2.70 | C8H9NO2 | H | (E)-4-Hydroxyphenylacetaldehyde-oxime or related compound | Amino Acids |
| 182.0805 | 553.37 | 3.68 | C9H11NO3 | H | L-Tyrosine | Amino Acids |
| 220.117 | 523.81 | 0.59 | C9H17NO5 | H | Pantothenic acid | Amino Acids |
| **Bile Acids** | | | | | | |
| 433.248 | 512.78 | 0.30 | C24H38O3 | NaCl | (20S)-3beta-Hydroxychol-5-en-24-oic Acid or related compounds | Bile acids and derivatives |
| 391.284 | 103.24 | 0.69 | C24H38O4 | H | (22E)-3alpha,12alpha-Dihydroxy-5beta-chol-22-en-24-oic Acid or related compounds | Bile acids and derivatives |
| 516.3008 | 174.38 | 3.60 | C26H45NO7S | H | Taurallocholic acid or related compounds | Bile acids and derivatives |
| 549.341 | 118.52 | 2.24 | C32H41N3O4 | NH3 | 3-Amino-N-{4-[2-(2,6-Dimethyl-Phenoxy)-Acetylamino]-3-Hydroxy-1-Isobutyl-5-Phenyl-Pentyl}-Benzamide or related compounds | Bile acids and derivatives |
| **Glycerol** | | | | | | |
| 482.3598 | 466.40 | 1.43 | C24H52NO6P | H | 1-O-Hexadecyl-lyso-sn-glycero-3-phosphocholine | Glycerophosphocholines |
| 508.3765 | 464.47 | 0.71 | C26H54NO6P | H | PC(O-18:1(11Z)/0:0) or related compounds | Glycerophosphocholines |
| 575.3111 | 503.62 | 4.94 | C27H53O8P | K | PA(12:0/12:0) | Glycerophosphates |
| 606.3647 | 469.69 | 2.23 | C29H58NO6P | NaCl | PC(O-3:1(1E)/O-18:1(9Z))[S] or related compounds | Glycerophosphocholines |
| 665.4133 | 471.96 | 0.83 | C32H58NO10P | NH3 | 1-Palmitoyl-2-(5-keto-8-oxo-6-octenoyl)-sn-glycero-3- phosphatidylcholine | Glycerophosphates |
| 694.4177 | 481.95 | 1.12 | C33H66NO8P | NaCl | PC(10:0/15:0)[U] or related compounds | Glycerophosphocholines |
| 704.5168 | 130.30 | 4.61 | C35H74NO6P | HCOONa | PE-NMe2(O-14:0/O-14:0) | Glycerophosphocholines |
| 647.4851 | 112.18 | 0.93 | C36H66O5 | HCOONa | DG(15:0/18:2(9Z,12Z)/0:0) or related compounds | Glycerolipids |
| 676.4928 | 383.12 | 2.42 | C36H70NO8P | H | PC(10:0/18:1(9Z)) or related compounds | Glycerophosphocholines |
| 678.5077 | 394.42 | 1.31 | C36H72NO8P | H | PC(10:0/18:0) or related compounds | Glycerophosphocholines |
| 718.5324 | 129.42 | 4.59 | C36H76NO6P | HCOONa | PC(O-14:0/O-14:0) | Glycerophosphocholines |
| 610.5413 | 103.58 | 1.34 | C37H68O5 | NH3 | DG(14:0/20:2(11Z,14Z)/0:0) or related compounds | Glycerolipids |
| 754.5299 | 382.13 | 4.49 | C37H78NO6P | Na_HCOONa | PE(O-16:0/O-16:0) | Glycerophosphoethanolamines |
| 663.4711 | 113.23 | 2.19 | C38H68O5 | NaCl | DG(15:0/20:3(5Z,8Z,11Z)/0:0) or related compounds | Glycerolipids |
| 704.5228 | 380.02 | 3.90 | C38H74NO8P | H | PC(12:0/18:1(9Z)) or related compounds | Glycerophosphocholines |
| 706.5389 | 390.41 | 1.12 | C38H76NO8P | H | PC(11:0/19:0) or related compounds | Glycerophosphocholines |
| 706.5414 | 441.72 | 4.66 | C38H76NO8P | H | PC(10:0/20:0) or related compounds | Glycerophosphocholines |
| 692.5574 | 395.09 | 2.08 | C38H78NO7P | H | PC(O-14:0/16:0) | Glycerophosphocholines |
| 638.5733 | 104.49 | 2.38 | C39H72O5 | NH3 | DG(14:0/22:2(13Z,16Z)/0:0) or related compounds | Glycerolipids |
| 701.5597 | 448.85 | 0.74 | C39H74NO6P | NH3 | 1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine | Glycerophosphoethanolamines |
| 718.5387 | 219.08 | 4.18 | C39H76NO8P | H | 16:0-18:1-PE or related compounds | Glycerophosphocholines |
| 728.5542 | 420.27 | 3.06 | C39H80NO7P | Na | PE(O-18:0/16:0)[U] | Glycerophosphoethanolamines |
| 766.5288 | 190.92 | 1.33 | C39H82NO6P | KCl | PE-NMe2(O-16:0/O-16:0) | Glycerophosphoethanolamines |
| 782.5612 | 402.65 | 4.32 | C39H82NO6P | Na_HCOONa | PE-NMe2(O-16:0/O-16:0) | Glycerophosphoethanolamines |
| 709.4405 | 475.27 | 4.92 | C40H64O5 | HCOOK | DG(17:2(9Z,12Z)/20:5(5Z,8Z,11Z,14Z,17Z)/0:0)[iso2] | Glycerolipids |
| 730.5351 | 399.98 | 0.82 | C40H76NO8P | H | PC(14:0/18:2(11Z,14Z)) or related compounds | Glycerophosphocholines |
| 780.5472 | 342.59 | 4.83 | C40H84NO6P | KCl | PC(O-12:0/O-20:0)[U] or related compounds | Glycerophosphocholines |
| 742.5404 | 197.93 | 4.77 | C41H76NO8P | H | 18:0-18:3-PE or related compounds | Glycerophosphates |
| 770.5654 | 361.64 | 2.08 | C41H82NO8P | Na | PC(10:0/23:0) or related compounds | Glycerophosphocholines |
| 824.4862 | 252.37 | 2.89 | C42H76NO10P | K | 18:0-18:3-PS or related compounds | Glycerophosphoserines |
| 788.5418 | 324.67 | 0.79 | C42H78NO10P | H | 18:0-18:2-PS or related compounds | Glycerophosphoserines |
| 826.4996 | 497.21 | 0.16 | C42H78NO10P | K | PS(18:0/18:2(9Z,12Z)) or related compounds | Glycerophosphoserines |
| 770.5506 | 366.21 | 1.43 | C42H79NO8 | Na_Na | Galactosylceramide (d18:1/18:1(9Z));Glucosylceramide (d18:1/9Z-18:1) | Glycolipids |
| 784.5799 | 406.43 | 3.49 | C42H84NO8P | Na | PC(10:0/24:0) or related compounds | Glycerophosphocholines |
| 776.5909 | 398.33 | 0.41 | C42H86NO6P | Na_Na | PC(O-16:/O-18:1(9Z))[U] or related compounds | Glycerophosphocholines |
| 772.5976 | 385.56 | 0.58 | C42H88NO6P | K | PC(O-16:0/O-18:0) | Glycerophosphocholines |
| 753.4658 | 480.16 | 2.44 | C43H66O5 | Na_HCOONa | DG(18:3(6Z,9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0) or related compounds | Glycerolipids |
| 775.4798 | 481.19 | 2.17 | C43H73O6P | NaCl | DG(17:0/22:4(7Z,10Z,13Z,16Z)/0:0)[iso2] or related compounds | Glycolipids |
| 763.5828 | 355.45 | 2.66 | C43H82O5 | HCOOK | DG(16:1(9Z)/24:0/0:0) or related compounds | Glycerolipids |
| 763.5885 | 364.87 | 4.81 | C43H82O5 | HCOOK | DG(16:0/24:1(15Z)/0:0) or related compounds | Glycerolipids |
| 824.5581 | 385.15 | 3.52 | C44H84NO8P | K | PC(14:0/22:2(13Z,16Z)) or related compounds | Glycerophosphocholines |
| 774.5427 | 179.11 | 2.48 | C45H76NO7P | H | PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/dm18:1(11Z)) or related compounds | Glycerophosphoethanolamines |
| 763.6 | 360.23 | 2.96 | C45H84O5 | NaCl | DG(18:1(11Z)/24:1(15Z)/0:0) or related compounds | Glycerolipids |
| 806.5843 | 136.27 | 2.36 | C45H86NO6P | K | 1-(10-methylhexadecanyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphocholine | Glycerophosphocholines |
| 822.5794 | 143.75 | 2.53 | C45H86NO7P | K | PE(22:1(13Z)/dm18:1(11Z)) or related compounds | Glycerophosphocholines |
| 808.5851 | 336.63 | 3.04 | C46H82NO8P | H | PC(16:0/22:5(4E,7E,10E,13E,16E))[U] or related compounds | Glycerophosphocholines |
| 812.6202 | 340.75 | 2.45 | C46H86NO8P | H | PC(16:0/22:3(13Z,16Z,19Z))[U] or related compounds | Glycerophosphocholines |
| 763.6155 | 364.36 | 4.13 | C46H86O5 | Na_Na | DG(21:0/22:2(13Z,16Z)/0:0)[iso2] | Glycerolipids |
| 730.5395 | 364.07 | 1.90 | C47H68O5 | NH3 | DG(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)* | Glycerophosphocholines |
| 834.6022 | 329.38 | 2.31 | C48H84NO8P | H | PC(18:0/22:6(4E,7E,10E,13E,16E,19E))[U] or related compounds | Glycerophosphocholines |
| 894.6423 | 146.39 | 0.86 | C48H91NO8 | HCOOK | Galactosylceramide (d18:1/24:1(15Z)) or related compounds | Glycolipids |
| 792.7049 | 124.71 | 3.32 | C49H90O6 | NH3 | TG(16:1(9Z)/14:0/16:1(9Z))[iso3] | Glycerolipids |
| 915.6705 | 102.32 | 4.93 | C53H92O6 | Na_HCOONa | TG(16:1(9Z)/16:1(9Z)/18:3(9Z,12Z,15Z))[iso3] or related compounds | Triacylglycerols |
| 929.6858 | 102.78 | 4.47 | C56H92O6 | HCOONa | TG(16:1(9Z)/17:2(9Z,12Z)/20:5(5Z,8Z,11Z,14Z,17Z))[iso6] or related compounds | Triacylglycerols |
| **Fatty Acids** | | | | | | |
| 184.1325 | 193.79 | 3.80 | C10H14O2 | NH3 | 1-(3,4-dimethylphenyl)ethane-1,2-diol or related compounds | Fatty Acids and Conjugates |
| 202.1799 | 492.63 | 1.24 | C11H20O2 | NH3 | 11-Undecanolactone or related compounds | Fatty Acids and Conjugates |

| | | | | | | |
|---|---|---|---|---|---|---|
| 208.1328 | 436.45 | 1.92 | C12H17NO2 | H | 2-sec-Butylphenyl N-methylcarbamate;3-(Dimethylamino)propyl benzoate;"3,4-Methylenedioxy-N-ethylamphetamine | Fatty Acids and Conjugates |
| 229.1535 | 680.98 | 1.61 | C12H24O | Na_Na | 10-dodecen-1-ol or related compounds | Fatty alcohols |
| 241.1542 | 555.30 | 1.95 | C13H24O | Na_Na | 2-tridecenal | Fatty aldehydes |
| 239.1049 | 691.73 | 2.09 | C14H16O2 | Na | 10E,12E-tetradecadiene-4,6-diynoic acid | Fatty Acids and Conjugates |
| 235.206 | 113.43 | 1.57 | C16H26O | H | 4,6,11-hexadecatrienal | Fatty aldehydes |
| 268.2271 | 110.85 | 0.00 | C16H26O2 | NH3 | 4,7,10-hexadecatrienoic acid or related compounds | Fatty Acids and Conjugates |
| 254.2471 | 312.60 | 2.87 | C16H28O | NH3 | 10,12-hexadecadienal or related compounds | Fatty aldehydes |
| 256.263 | 109.00 | 1.87 | C16H30O | NH3 | 6E,11Z-hexadecadien-1-ol or related compounds | Fatty alcohols |
| 261.2201 | 115.22 | 4.71 | C16H30O | Na | 10-propyl-5,9-tridecadien-1-ol or related compounds | Fatty alcohols |
| 258.2781 | 254.89 | 3.99 | C16H32O | NH3 | 1,2-Epoxyhexadecane;11Z-hexadecen-1-ol or related compounds | Fatty alcohols |
| 274.2733 | 263.19 | 2.73 | C16H32O2 | NH3 | 13-methyl-pentadecanoic acid or related compounds | Fatty Acids and Conjugates |
| 288.2889 | 239.09 | 2.77 | C17H34O2 | NH3 | (+)-14-methyl palmitic acid;10-methyl-hexadecanoic acid or related compounds | Fatty Acids and Conjugates |
| 275.1998 | 112.20 | 2.73 | C18H26O2 | H | 13E-octadecene-9,11-diynoic acid or related compounds | Fatty Acids and Conjugates |
| 298.2727 | 115.34 | 4.53 | C18H32O2 | NH3 | 10E,12E-octadecadienoic acid or related compounds | Fatty Acids |
| 284.2942 | 273.47 | 2.04 | C18H34O | NH3 | 11-octadecenal or related compounds | Fatty alcohols |
| 300.2906 | 297.59 | 3.00 | C18H34O2 | NH3 | (11E)-Octadecenoic acid or related compounds | Fatty Acids and Conjugates |
| 304.262 | 109.01 | 3.06 | C18H35NO | Na | Oleamide | Fatty amides |
| 302.3048 | 255.27 | 1.82 | C18H36O2 | NH3 | (+)-Isostearic acid;10-methyl-heptadecanoic acid or related compounds | Fatty Acids and Conjugates |
| 302.3048 | 314.89 | 1.82 | C18H36O2 | NH3 | 15-methyl-heptadecanoic acid or related compounds | Fatty Acids and Conjugates |
| 316.3203 | 229.21 | 2.18 | C19H38O2 | NH3 | (+)-16-methyl stearic acid;11-methyl-octadecanoic acid or related compounds | Fatty Acids and Conjugates |
| 330.3358 | 248.00 | 2.54 | C20H40O2 | NH3 | 17-methyl-nonadecanoic acid or related compounds | Fatty Acids and Conjugates |
| 340.32 | 241.18 | 2.91 | C21H38O2 | NH3 | 12Z,15Z-heneicosadienoic acid or related compounds | Fatty Acids and Conjugates |
| 342.3362 | 239.30 | 1.29 | C21H40O2 | NH3 | 12Z-heneicosenoic acid or related compounds | Fatty Acids and Conjugates |
| 358.3678 | 241.70 | 0.39 | C22H44O2 | NH3 | 19-methyl-heneicosanoic acid or related compounds | Fatty Acids and Conjugates |
| 480.3083 | 429.95 | 0.31 | C25H43NO2 | Na_HCOONa | (-)N-(1R-methyl-2-hydroxy-ethyl) alpha,alpha-dimethylarachidonoyl amine or related compounds | Fatty amides |
| 677.5591 | 446.20 | 0.12 | C38H76O9 | H | 1-(O-alpha-D-glucopyranosyl)-(1,3R,29S,31R)-dotriacontanetetraol | Fatty acyl glycosides |
| 704.5297 | 441.47 | 2.19 | C40H73N3O4 | Na_Na | N-ornithinyl-35-aminobacteriohopane-32,33,34-triol | Hopanoids |
| 782.4773 | 493.77 | 4.95 | C41H73NO8 | KCl | bacteriohopane-,32,33,34-triol-35-cyclitol;bacteriohopanetetrol cyclitol | Hopanoids |
| 87.0443 | 657.38 | 2.76 | C4H6O2 | H | 1,4-Lactone or related compounds | Fatty Acids |
| 89.0595 | 161.09 | 2.36 | C4H8O2 | H | 2-Methylpropanoate or related compounds | Fatty Acids |
| 160.1325 | 718.21 | 4.37 | C8H14O2 | NH3 | 2-ethyl-3E-hexenoic acid or related compounds | Fatty Acids and Conjugates |
| 156.1376 | 111.09 | 4.42 | C9H14O | NH3 | (3Z,6Z)-nonadienal or related compounds | Fatty aldehydes |
| 155.106 | 119.97 | 4.19 | C9H14O2 | H | 2,6-nonadienoic acid or related compounds | Fatty Acids |
| 172.1325 | 120.17 | 4.07 | C9H14O2 | NH3 | 2,6-nonadienoic acid or related compounds | Fatty Acids and Conjugates |
| 146.0594 | 496.09 | 4.38 | C9H7NO | H | 1(2H)-Isoquinolinone or related compounds | Fatty aldehydes |
| **Hydroxy acids** | | | | | | |
| 250.0928 | 166.87 | 0.80 | C10H19NO2S2 | H | S-Acetyldihydrolipoamide | Lipoamides and Derivatives |
| 223.1071 | 510.95 | 2.78 | C11H11NO3 | NH3 | 5-Methoxyindolacetate;Indolelactate | Indoles and Indole Derivatives |
| 239.1047 | 605.62 | 1.25 | C11H20O3 | K | 2-hydroxy-10-undecenoic acid or related compounds | Hydroxy fatty acids |
| 226.1438 | 435.97 | 0.13 | C12H16O3 | NH3 | 12-oxo-5E,8E,10Z-dodecatrienoic acid or related compounds | Oxo fatty acids |
| 304.2852 | 148.51 | 1.94 | C17H34O3 | NH3 | 17-hydroxy-heptadecanoic acid or related compounds | Hydroxy fatty acids |
| 304.2858 | 187.96 | 3.91 | C17H34O3 | NH3 | 2-hydroxy-heptadecanoic acid or related compounds | Hydroxy fatty acids |
| 310.2375 | 115.40 | 0.52 | C18H28O3 | NH3 | (-)-8-hydroxy-11E,17-octadecadien-9-ynoic acid or related compounds | Hydroxy fatty acids |
| 332.3145 | 181.15 | 4.24 | C19H38O3 | NH3 | 19-hydroxy-nonadecanoic acid; 2-hydroxy-nonadecanoic acid | Hydroxy fatty acids |
| 387.2633 | 112.88 | 0.85 | C20H40O3 | NaCl | 20-hydroxy-eicosanoic acid;2-hydroxy-eicosanoic acid;3-hydroxy-eicosanoic acid;L-2-hydroxyphytanate | Hydroxy fatty acids |
| 137.045 | 294.53 | 4.01 | C4H8O5 | H | D-threonic acid | Hydroxy fatty acids |
| 133.0855 | 160.75 | 3.16 | C6H12O3 | H | (R)-3-Hydroxyhexanoic acid or related compounds | Hydroxy Acids |
| 219.0051 | 638.54 | 1.46 | C9H8O4 | K | 2,4-Dihydroxy-Trans Cinnamic Acid or related compounds | Hydroxy Acids |
| **Isoprenoids** | | | | | | |
| 368.4239 | 220.63 | 3.15 | C25H50 | NH3 | 4-pentacosene;6-pentacosene;9-pentacosene;C25 Monocyclic highly branched isoprenoid;C25:1 Highly branched isoprenoid | Isoprenoids |
| 447.3383 | 114.34 | 4.25 | C30H48 | K | 4,4'-Diapophytoene;"4,4'-Diapophytoene/ Dehydrosqualene" | Isoprenoids |
| 489.3617 | 113.79 | 1.37 | C30H54 | KCl | Tetrahydrosqualene | Isoprenoids |
| 489.35 | 114.72 | 1.74 | C35H46 | Na | 4'-Apo-3,4-didehydrolycopene/ (4-Apo-3',4'-didehydrolycopene) | Isoprenoids |
| **Phospholipids** | | | | | | |
| 468.3079 | 465.94 | 1.17 | C22H46NO7P | H | LysoPC(14:0);PC(0:0/14:0) or related compounds | Phospholipids |
| 494.3238 | 464.08 | 0.61 | C24H48NO7P | H | 1-16:1-lysoPC or related compounds | Phospholipids |
| 480.3443 | 456.83 | 1.12 | C24H50NO6P | H | LysoPC(dm16:0);PC(O-16:1(11Z)/0:0) or related compounds | Phospholipids |
| 496.3401 | 463.31 | 0.71 | C24H50NO7P | H | LysoPC(16:0) or related compounds | Phospholipids |
| 520.3404 | 461.86 | 1.25 | C26H50NO7P | H | LysoPC(18:2(9Z,12Z)) or related compounds | Phospholipids |
| 544.3384 | 461.39 | 1.95 | C28H50NO7P | H | LysoPC(20:4(5Z,8Z,11Z,14Z)) or related compounds | Phospholipids |
| 692.4062 | 212.33 | 4.87 | C33H64NO8P | NaCl | PE(14:0/14:1(9Z)) or related compounds | Phospholipids |
| 688.4941 | 208.14 | 4.27 | C37H70NO8P | H | PE(14:0/18:2(9Z,12Z)) or related compounds | Phospholipids |
| 674.5119 | 197.75 | 0.00 | C37H72NO7P | H | PE(14:0/dm18:1(11Z)) or related compounds | Phospholipids |
| 690.5069 | 210.44 | 3.62 | C37H72NO8P | H | PE(14:0/18:1(11Z)) or related compounds | Phospholipids |
| 675.5451 | 450.32 | 2.32 | C37H75N2O6P | H | SM(d18:1/14:0) | Phospholipids |
| 736.4311 | 223.45 | 0.39 | C38H68NO8P | K | PE(15:0/18:4(6Z,9Z,12Z,15Z)) or related compounds | Phospholipids |
| 738.4443 | 488.35 | 3.71 | C38H70NO8P | K | PE(15:0/18:3(6Z,9Z,12Z)) or related compounds | Phospholipids |
| 702.5098 | 372.47 | 4.26 | C38H72NO8P | H | PC(14:1(9Z)/16:1(9Z)) or related compounds | Phospholipids |
| 700.5287 | 193.51 | 1.66 | C39H74NO7P | H | PE(16:1(9Z)/dm18:1(11Z)) or related compounds | Phospholipids |
| 716.5237 | 204.02 | 1.73 | C39H74NO8P | H | 16:0-18:2-PE or related compounds | Phospholipids |
| 702.5441 | 198.47 | 1.30 | C39H76NO7P | H | PE(18:1(11Z)/dm16:0) or related compounds | Phospholipids |
| 702.5408 | 421.81 | 3.40 | C39H76NO7P | H | PE(16:0/dm18:1(9Z)) or related compounds | Phospholipids |
| 702.5442 | 507.59 | 1.44 | C39H76NO7P | H | 1-Hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3- phosphonoethanolamine or related compounds | Phospholipids |
| 728.5561 | 520.51 | 0.45 | C39H80NO7P | Na | PE(O-18:0/16:0)[U] | Phospholipids |
| 728.526 | 348.01 | 4.86 | C40H74NO8P | H | PC(14:0/18:3(6Z,9Z,12Z)) or related compounds | Phospholipids |
| 716.5567 | 438.82 | 2.99 | C40H78NO7P | H | PC(14:0/dm18:1(9Z)) or related compounds | Phospholipids |
| 732.554 | 371.08 | 3.62 | C40H78NO8P | H | PC(16:0/16:1(9Z)) or related compounds | Phospholipids |
| 732.5537 | 408.42 | 3.21 | C40H78NO8P | H | PC(14:0/18:1(9Z)) or related compounds | Phospholipids |
| 732.5549 | 443.06 | 4.85 | C40H78NO8P | H | PC(14:0/18:1(9E))[U] or related compounds | Phospholipids |
| 732.5547 | 503.26 | 4.57 | C40H78NO8P | H | PC(14:0/18:1(11Z)) or related compounds | Phospholipids |
| 718.5741 | 378.11 | 0.54 | C40H80NO7P | H | PC(14:0/dm18:0) or related compounds | Phospholipids |
| 722.5098 | 186.64 | 3.78 | C41H72NO7P | H | PE(18:4(6Z,9Z,12Z,15Z)/dm18:1(11Z)) or related compounds | Phospholipids |
| 782.4689 | 493.25 | 0.51 | C41H72NO8P | Na_Na | PE(14:0/22:5(4Z,7Z,10Z,13Z,16Z)) or related compounds | Phospholipids |
| 724.5276 | 184.40 | 3.41 | C41H74NO7P | H | PE(18:3(6Z,9Z,12Z)/dm18:1(11Z)) or relea tec ompounds | Phospholipids |
| 726.5429 | 189.44 | 2.92 | C41H76NO7P | H | PE(18:2(9Z,12Z)/dm18:1(11Z)) or related compounds | Phospholipids |
| 728.5606 | 191.66 | 2.42 | C41H78NO7P | H | PE(18:1(11Z)/dm18:1(11Z)); PE(18:1(11Z)/dm18:1(9Z)) or related compounds | Phospholipids |
| 744.5539 | 166.55 | 0.19 | C41H78NO8P | H | PE(14:0/22:2(13Z,16Z)) or related compounds | Phospholipids |
| 744.5557 | 202.25 | 2.61 | C41H78NO8P | H | 18:0-18:2-PE or related compounds | Phospholipids |
| 744.5551 | 395.76 | 1.80 | C41H78NO8P | H | PC(15:0/18:2(9Z,12Z)) or related compounds | Phospholipids |
| 756.5543 | 353.49 | 3.90 | C42H78NO8P | H | PC(14:0/20:3(5Z,8Z,11Z)) or related compound | Phospholipids |
| 756.5505 | 438.70 | 1.12 | C40H80NO8P | Na | PC(10:0/22:0) or related compounds | Phospholipids |
| 775.5495 | 109.36 | 1.50 | C42H79O10P | H | PG(18:0/18:2(9Z,12Z)) or related compounds | Phospholipids |
| 742.5756 | 342.73 | 4.74 | C42H80NO7P | H | PC(16:1(9Z)/dm18:1(11Z)) or related compounds | Phospholipids |
| 758.5702 | 358.48 | 1.04 | C42H80NO8P | H | PC(14:0/20:2(11Z,14Z)) or related compounds | Phospholipids |
| 744.5895 | 352.48 | 0.86 | C42H82NO7P | H | PC(16:0/dm18:1(11Z)) or related compounds | Phospholipids |
| 760.585 | 365.23 | 3.10 | C42H82NO8P | H | PC(14:1(9Z)/20:0) or related compounds | Phospholipids |
| 760.583 | 428.20 | 0.47 | C42H82NO8P | H | PC(14:0/20:1(11Z)) or related compounds | Phospholipids |
| 746.6046 | 373.88 | 1.59 | C42H84NO7P | H | PC(16:0/dm18:0) or related compounds | Phospholipids |
| 780.4597 | 237.21 | 2.69 | C43H68NO8P | Na | PE(18:4(6Z,9Z,12Z,15Z)/20:5(5Z,8Z,11Z,14Z,17Z)) or related compounds | Phospholipids |
| 750.5424 | 184.27 | 2.16 | C43H76NO7P | H | PE(20:4(5Z,8Z,11Z,14Z)/dm18:1(11Z)) or related compounds | Phospholipids |
| 768.5906 | 335.00 | 3.73 | C44H82NO7P | H | PC(18:2(9Z,12Z)/dm18:1(11Z)) or related compounds | Phospholipids |
| 784.5877 | 347.40 | 3.38 | C44H82NO8P | H | PC(14:1(9Z)/22:2(13Z,16Z)) or related compounds | Phospholipids |
| 770.6083 | 344.05 | 3.26 | C44H84NO7P | H | PC(18:1(11Z)/dm18:1(11Z)) or related compounds | Phospholipids |

| | | | | | | |
|---|---|---|---|---|---|---|
| 824.5595 | 342.49 | 3.54 | C44H84NO8P | K | PC(14:0/22:2(13Z,16Z)) or related compounds | Phospholipids |
| 786.5984 | 381.69 | 2.92 | C44H84NO8P | H | PC(14:1(9Z)/22:1(13Z)) or related compounds | Phospholipids |
| 786.6028 | 427.54 | 2.67 | C44H84NO8P | H | PC(16:0/20:2(11E,14E))[U] or related compounds | Phospholipids |
| 786.5982 | 451.86 | 3.18 | C44H84NO8P | H | PC(18:1(10Z)/18:1(10Z)) or related compounds | Phospholipids |
| 786.6026 | 505.34 | 2.42 | C44H84NO8P | H | PC(18:0/18:2(2E,4E)) or related compounds | Phospholipids |
| 786.6042 | 594.01 | 4.45 | C44H84NO8P | H | PC(18:0/18:2(6Z,9Z)) or related compounds | Phospholipids |
| 786.6028 | 675.87 | 2.67 | C44H84NO8P | H | PC(18:0/18:2(10Z,12Z)) or related compounds | Phospholipids |
| 772.618 | 360.33 | 4.45 | C44H86NO7P | H | PC(18:0/dm18:1(11Z)) or related compounds | Phospholipids |
| 776.5602 | 182.06 | 4.66 | C45H78NO7P | H | PE(22:5(4Z,7Z,10Z,13Z,16Z)/dm18:1(11Z)) or related compounds | Phospholipids |
| 810.6021 | 333.87 | 4.70 | C46H84NO8P | H | 20:2-18:2-PC or related compounds | Phospholipids |
| 836.596 | 141.97 | 3.62 | C46H88NO7P | K | PC(20:1(11Z)/dm18:1(11Z)) or related compounds | Phospholipids |
| 814.634 | 426.34 | 2.46 | C46H88NO8P | H | PC(14:1(9Z)/24:1(15Z)) or related compounds | Phospholipids |
| 850.6111 | 140.03 | 2.92 | C47H90NO7P | K | PE(24:1(15Z)/dm18:1(11Z)) or related compounds | Phospholipids |
| 880.6235 | 146.68 | 2.25 | C48H92NO8P | K | PC(16:1(9Z)/24:1(15Z)) or related compounds | Phospholipids |
| 924.7379 | 103.23 | 1.34 | C52H104NO8P | Na | PC(18:0/26:0) or related compounds | Phospholipids |
| 188.175 | 580.12 | 3.88 | C9H21N3O | H | N1-Acetylspermidine or related compounds | Polyamines |
| 188.175 | 623.03 | 3.93 | C9H21N3O | H | N8-Acetylspermidine or related compounds | Polyamines |
| **Polypeptides** | | | | | | |
| 273.0825 | 294.95 | 1.25 | C10H16N2O4 | Na_Na | (S)-ATPA;Prolylhydroxyproline | Polypeptides |
| 308.0904 | 615.60 | 2.21 | C10H17N3O6S | H | Glutathione;Reduced glutathione | Polypeptides |
| 229.1539 | 541.82 | 3.36 | C11H20N2O3 | H | L-isoleucyl-L-proline;L-leucyl-L-proline | Polypeptides |
| 427.0954 | 664.31 | 4.40 | C13H22N4O8S2 | H | Cysteineglutathione disulfide | Polypeptides |
| 613.1595 | 649.09 | 0.16 | C20H32N6O12S2 | H | Glutathione disulfide;Oxidized glutathione | Polypeptides |
| 681.1999 | 294.13 | 3.21 | C22H42N4O8S2 | NaCl_HCOONa | D-Pantethine | Polypeptides |
| 179.0482 | 614.98 | 1.62 | C5H10N2O3S | H | Cys-Gly;Cysteine-S-Acetamide | Polypeptides |
| 188.1751 | 477.34 | 3.40 | C9H21N3O | H | N1-Acetylspermidine;N8-Acetylspermidine | Polyamines |
| **Nucleotides and Derivatives** | | | | | | |
| 275.0738 | 250.68 | 4.62 | C10H12N4O4 | Na | Deoxyinosine;Nebularine;Purine Riboside | Nucleoside Analogues |
| 306.0804 | 507.19 | 1.57 | C10H13N5O5 | Na | 8-Hydroxy-2'-Deoxyguanosine;8-Hydroxy-deoxyguanosine;Crotonoside;Guanosine; | Nucleoside Analogues |
| 338.0497 | 548.33 | 0.12 | C10H13N5O6 | K | 8-Hydroxyguanosine | Nucleoside Analogues |
| 364.0643 | 642.29 | 2.69 | C10H14N5O8P | H | 8-Oxo-2'-Deoxy-Guanosine-5'-Monophosphate or related compounds | Nucleotides |
| 298.0957 | 156.40 | 3.82 | C11H15N5O3S | H | 5'-Methylthioadenosine or related compounds | Nucleoside Analogues |
| 565.0832 | 696.81 | 0.30 | C16H26N2O16P2 | H | dTDP-D-galactose or related compounds | Nucleotides |
| 608.0896 | 610.25 | 1.23 | C17H27N3O17P2 | H | UDP-GlcNAc or related compounds | Nucleotides |
| 664.1178 | 617.75 | 0.72 | C21H27N7O14P2 | H | Nicotinamide adenine dinucleotide or related compounds | Nucleotides |
| 113.0341 | 185.11 | 4.07 | C4H4N2O2 | H | Uracil | Pyrimidines and Derivatives |
| 129.0765 | 524.80 | 4.57 | C4H5N3O | NH3 | Cytosine | Pyrimidines and Derivatives |
| 152.0561 | 508.74 | 4.93 | C4H6O5 | NH3 | 3-Dehydro-L-threonate;Malate | Purines and Purine Derivatives |
| 152.056 | 642.92 | 4.27 | C4H6O5 | NH3 | 3-Dehydro-L-threonate;Malate | Purines and Purine Derivatives |
| 137.0454 | 446.55 | 2.85 | C5H4N4O | H | Hypoxanthine | Purines and Purine Derivatives |
| 153.0412 | 314.70 | 3.27 | C5H4N4O2 | H | 6,8-Dihydroxypurine;Alloxanthine;Oxypurinol | Purines and Purine Derivatives |
| 136.0617 | 615.68 | 0.51 | C5H5N5 | H | Adenine | Purines and Purine Derivatives |
| 136.0615 | 252.64 | 1.98 | C5H5N5 | H | Adenine | Purines and Purine Derivatives |
| 136.0612 | 229.73 | 4.19 | C5H5N5 | H | Adenine | Purines and Purine Derivatives |
| 136.0621 | 157.31 | 2.43 | C5H5N5 | H | Adenine | Purines and Purine Derivatives |
| 152.0561 | 461.72 | 4.93 | C5H5N5O | H | 2-Hydroxyadenine;8-Hydroxyadenine;Guanine | Purines and Purine Derivatives |
| 129.0657 | 629.63 | 1.24 | C5H8N2O2 | H | 5,6-Dihydrothymine or related compounds | Pyrimidines and Derivatives |
| 405.0095 | 616.31 | 4.07 | C9H14N2O12P2 | H | UDP | Nucleotides |
| **Quaternary Amines** | | | | | | |
| 244.1538 | 126.45 | 2.17 | C12H21NO4 | H | Tiglylcarnitine | Quaternary Amines |
| 246.1694 | 499.59 | 2.36 | C12H23NO4 | H | 2-Methylbutyroylcarnitine;Isovalerylcarnitine | Quaternary Amines |
| 330.2638 | 119.83 | 3.03 | C18H35NO4 | H | 4,8 dimethylnonanoyl carnitine | Quaternary Amines |
| 398.3262 | 440.68 | 2.59 | C23H43NO4 | H | trans-Hexadec-2-enoyl carnitine | Quaternary Amines |
| 400.3418 | 440.34 | 0.80 | C23H45NO4 | H | L-Palmitoylcarnitine | Quaternary Amines |
| 426.357 | 436.45 | 1.81 | C25H47NO4 | H | Elaidic carnitine;octadecenoyl carnitine;Vaccenyl carnitine;; | Quaternary Amines |
| 162.1119 | 653.66 | 3.52 | C7H15NO3 | H | L-Carnitine | Quaternary Amines |
| 204.1222 | 590.80 | 4.07 | C9H17NO4 | H | L-Acetylcarnitine | Quaternary Amines |
| **Sphingoid bases** | | | | | | |
| 252.2303 | 113.30 | 2.10 | C14H31NO | Na | Lauryl Dimethylamine-N-Oxide;Xestoaminol C;; | Sphingoid bases |
| 310.1746 | 712.63 | 4.93 | C15H23N3O4 | H | Polyproline | Sphingoid bases |
| 442.1864 | 609.22 | 4.95 | C17H38NO5P | KCl | C17 Sphinganine-1-phosphate | Sphingoid bases |
| 318.2399 | 110.04 | 1.38 | C18H33NO2 | Na | (4E,8E,10E-d18:3)sphingosine;; | Sphingoid bases |
| 320.2555 | 111.94 | 1.53 | C18H35NO2 | Na | (4E,8E,d18:2) sphingosine or related compounds | Sphingoid bases |
| 648.38 | 200.39 | 3.87 | C32H63NO5S | KCl | N-(tetradecanoyl)-deoxysphing-4-enine-1-sulfonate | Sphingolipids |
| 703.575 | 449.57 | 0.26 | C39H79N2O6P | H | SM(d18:1/16:0) | Sphingolipids |
| 785.6536 | 446.72 | 0.67 | C45H89N2O6P | H | SM(d18:1/22:1(13Z)) | Sphingolipids |
| **Vitamines** | | | | | | |
| 417.2769 | 274.31 | 0.86 | C27H38O2 | Na | 25-hydroxy-16,17,23,23,24,24-hexadehydrovitamin D3 / 25-hydroxy-16,17,23,23,24,24-hexadehydrocholecalciferol or related compounds | Vitamin D2 and derivatives |
| 447.3106 | 115.06 | 1.45 | C27H42O5 | H | (23R)-1alpha,23,25-trihydroxy-24-oxovitamin D3 / (23R)-1alpha,23,25-trihydroxy-24-oxocholecalciferol or related compounds | Vitamin D2 and derivatives |
| 433.3304 | 103.54 | 3.67 | C27H44O4 | H | (20S)-1alpha,20,25-trihydroxyvitamin D3 / (20S)-1alpha,20,25-trihydroxycholecalciferol or related compounds | Vitamin D3 and derivatives |
| 447.3466 | 103.15 | 0.60 | C28H46O4 | H | (20S)-1alpha,20,25-trihydroxy-24a-homovitamin D3 / (20S)-1alpha,20,25-trihydroxy-24a-homocholecalciferol or related compounds | Vitamin D2 and derivatives |
| 503.3072 | 191.93 | 3.93 | C29H48O2 | KCl | (22alpha)-hydroxy-isofucosterol or related compounds | Vitamin D2 and derivatives |
| 461.3626 | 103.90 | 0.17 | C29H48O4 | H | (20S)-14alpha,20,25-trihydroxy-26,27-dimethylvitamin D3 / (20S)-1alpha,20,25-trihydroxy-26,27-dimethylcholecalciferol or related compounds | Vitamin D2 and derivatives |
| 601.3268 | 506.60 | 4.31 | C36H50O | K | 11-(4-acetoxymethylphenyl)-1alpha,25-dihydroxy-9,11-didehydrovitamin D3 / 11-(4-acetoxymethylphenyl)-1alpha,25-dihydroxy-9,11-didehydrocholecalciferol or related compounds | Vitamin D2 and derivatives |
| 725.4475 | 131.38 | 0.65 | C39H64O12 | H | 1-Hydroxyvitamin D3 cellobioside;; | Vitamin D3 and derivatives |
| **Miscellaneous** | | | | | | |
| 224.125 | 193.91 | 3.17 | C10H19NO3 | Na | Capryloylglycine | Acyl Glycines |
| 188.0704 | 496.37 | 1.12 | C11H9NO2 | H | Indoleacrylic acid | Indoles and Indole Derivatives |
| 275.1412 | 408.38 | 1.53 | C15H24O2 | K | Capsidiol;dihydroartemisinate;Farnesoic acid;Helminthosporol;Hernandulcin;Latia luciferin;Lubimin;Sirenin;; | Alcohols and Polyol |
| 289.1792 | 103.80 | 2.14 | C18H24O3 | H | 16,17-Epiestriol or related compounds | Steroids and Steroid Derivatives |
| 300.3246 | 260.32 | 4.93 | C19H38O | NH3 | 2-nonadecanone;Pristanal | Aldehydes |
| 357.2622 | 113.06 | 3.75 | C20H36O5 | H | 13,14-dihydro PGE1 or related compounds | Prostanoids |
| 478.3214 | 198.74 | 4.41 | C21H43N5O7 | H | Gentamicin | Drug |
| 142.0261 | 697.79 | 1.90 | C2H8NO4P | H | 1-Hydroxy-2-aminoethylphosphonate;Ethanolamine phosphate;; | Acyl Phosphates |
| 604.354 | 189.84 | 2.25 | C30H52O7P2 | NH3 | all-trans-Hexaprenyl diphosphate | Alkanes and Alkenes |
| 790.5238 | 186.10 | 2.76 | C46H73N3O4 | NaCl | N-tryptophanyl-35-aminobacteriohopane-32,33,34-triol | Hopanoids |
| 104.1064 | 573.42 | 5.67 | C5H13NO | H | Choline | Aldehydes |
| 123.0549 | 152.27 | 3.17 | C6H6N2O | H | Nicotinamide | Cyclic Amines |
| 107.0492 | 483.94 | 0.56 | C7H6O | H | Benzaldehyde | Aldehydes |
| 187.1076 | 646.46 | 0.64 | C8H11NO3 | NH3 | 5-Hydroxydopamine or related compounds | Catecholamines and Derivatives |
| 204.0861 | 610.01 | 2.55 | C8H13NO5 | H | N2-acetyl-alpha-aminoadipate or related compounds | Pterins |
| 147.0436 | 552.77 | 2.72 | C9H6O2 | H | Phenylpropiolic acid | Alkyl Chlorides |

# Appendix C

A list of putitive identification of metabolites in human apocrine sweat. Bold text represents matches found in the HMDB urine database.

| m/z | rt (s) | ppm error | MF | Adduct | Metabolites | Class | Ionmode |
|---|---|---|---|---|---|---|---|
| **Amino Acids** | | | | | | | |
| **249.0615** | **84.30** | **1.73** | **C11H12N2O2** | **Na_Na** | **L-Tryptophan** | **Amino Acids** | **pos** |
| **369.1512** | **469.14** | **0.84** | **C12H24N2O8** | **HCOOH** | **Galactosylhydroxylysine** | **Sugar Amino Acids** | **neg** |
| **369.1531** | **504.24** | **4.47** | **C12H24N2O9** | **HCOOH** | **Galactosylhydroxylysine** | **Sugar Amino Acids** | **neg** |
| **543.1576** | **444.30** | **0.31** | **C18H34N2O13** | **NaCl** | **Glucosylgalactosyl hydroxylysine** | **Sugar Amino Acids** | **neg** |
| **199.9543** | **804.54** | **1.00** | **C2H7NO3S** | **KCl** | **Taurine** | **Amino Acids** | **pos** |
| **172.0220** | **67.20** | **4.48** | **C3H7NO3** | **HCOONa** | **L-Serine** | **Amino Acids** | **neg** |
| 183.9914 | 80.16 | 3.86 | C3H7NO6S | H | L-Serine O-sulfate | Amino Acids | neg |
| **164.0296** | **68.46** | **1.22** | **C4H9NO3** | **Na_Na (HCOONa)** | **L-Allothreonine;L-Homoserine;L-Threonine** | **Amino Acids** | **pos/neg** |
| 252.0148 | 66.48 | 0.16 | C4H9NO5S; | HCOONa | L-Methionine sulfone | Amino Acids | pos |
| **191.0406** | **68.46** | **1.41** | **C5H10N2O3** | **Na_Na** | **L-Glutamine** | **Amino Acids** | **pos** |
| **169.0583** | **385.80** | **0.35** | **C5H10N2O3** | **Na** | **3-Ureidoisobutyrate** | **Amino Acids** | **pos** |
| **238.0142** | **63.84** | **4.58** | **C5H11NO2S** | **Na_HCOONa** | **L-Methionine** | **Amino Acids** | **neg** |
| **177.0612** | **64.56** | **1.13** | **C5H12N2O2** | **Na_Na (HCOONa)** | **L-Ornithine** | **Amino Acids** | **pos/neg** |
| **191.0768** | **64.56** | **0.84** | **C6H14N2O2** | **Na_Na** | **L-Lysine** | **Amino Acids** | **pos** |
| **185.9932** | **76.80** | **3.71** | **C5H7NO3** | **NaCl** | **Pyroglutamic acid** | **Amino Acids** | **neg** |
| **231.9888** | **66.48** | **1.64** | **C5H9NO2** | **NaClx2** | **L-Proline** | **Amino Acids** | **pos** |
| **220.0669** | **66.54** | **0.05** | **C6H13N3O3** | **Na_Na** | **L-Citrulline** | **Amino Acids** | **pos** |
| **200.0408** | **64.56** | **0.95** | **C6H9N3O2** | **Na_Na (HCOONa)** | **L-Histidine** | **Amino Acids** | **pos/neg** |
| 240.0486 | 81.12 | 2.92 | C7H11NO4 | HCOONa | (2s,5s)-5-Carboxymethylproline or related compounds | Amino Acids | neg |
| **144.1019** | **430.92** | **0.21** | **C7H13NO2** | **H** | **Proline Betaine** | **Amino Acids** | **pos** |
| 252.9794 | 81.12 | 2.10 | C8H8N2O3 | KCl | Nicotinurate | Cyclic Amino Acids | neg |
| **226.0454** | **74.28** | **1.86** | **C9H11NO3** | **Na_Na** | **L-Tyrosine** | **Amino Acids** | **pos** |
| **307.0345** | **81.00** | **2.41** | **C9H16N2O5S** | **Na_Na** | **N-Acetylcystathionine** | **Amino Acids** | **neg** |
| **Di/Polypeptides** | | | | | | | |
| 293.1468 | 404.58 | 4.09 | C10H17N3O6 | NH3 | Norophthalmic acid | Polypeptides | pos |
| **319.1248** | **87.96** | **2.38** | **C11H20N2O3** | **Na_HCOONa (HCOOH)** | **L-isoleucyl-L-proline;L-leucyl-L-proline** | **Polypeptides** | **pos/neg** |
| **319.1240** | **146.64** | **0.03** | **C11H20N2O3** | **Na_HCOONa (HCOOH)** | **L-leucyl-L-proline** | **Polypeptides** | **pos/neg** |
| **273.1454** | **169.86** | **0.73** | **C11H20N2O3** | **HCOOH** | **L-isoleucyl-L-proline;L-leucyl-L-proline** | **Polypeptides** | **neg** |
| **273.1452** | **200.76** | **1.32** | **C11H20N2O3** | **HCOOH** | **L-leucyl-L-proline** | **Polypeptides** | **neg** |
| **273.1453** | **232.32** | **0.99** | **C11H20N2O3** | **HCOOH** | **L-leucyl-L-proline** | **Polypeptides** | **neg** |
| 277.1224 | 682.08 | 1.30 | C11H22N2O4S | H | Pantetheine | Polypeptides | neg |
| **363.1782** | **414.48** | **1.82** | **C16H22N6O4** | **H** | **Thyrotropin releasing hormone** | **Polypeptides** | **pos** |
| 533.2586 | 383.58 | 4.01 | C22H40N8O5 | K | Postin | Polypeptides | neg |
| 581.2786 | 418.26 | 3.72 | C22H40N8O5 | HCOOK | Postin | Polypeptides | pos |
| **177.0331** | **463.98** | **4.69** | **C5H10N2O3S** | **H** | **Cys-Gly;Cysteine-S-Acetamide** | **Polypeptides** | **neg** |
| **187.0716** | **424.98** | **4.17** | **C7H12N2O4** | **H** | **L-glycyl-L-hydroxyproline;N-Acetylglutamine** | **Polypeptides** | **neg** |
| 233.0509 | 191.58 | 3.86 | C7H12N2O4 | Na_Na | L-glycyl-L-hydroxyproline;N-Acetylglutamine | Polypeptides | pos |
| **Polyamines** | | | | | | | |
| **239.1647** | **428.52** | **1.38** | **C10H26N4** | **K** | **Spermine** | **Polyamines** | **neg** |
| **301.1766** | **388.14** | **3.32** | **C12H28N4O** | **NaCl** | **N1-Acetylspermine** | **Polyamines** | **neg** |
| 331.2094 | 422.16 | 4.29 | C14H30N4O2 | Na_Na | N1,N12-Diacetylspermine | Polyamines | pos |
| **Quaternary Amines** | | | | | | | |
| 338.2300 | 465.90 | 0.47 | C17H33NO4 | Na | Decanoylcarnitine;O-Decanoyl-L-carnitine | Quaternary Amines | pos |
| 330.2639 | 444.30 | 3.39 | C18H35NO4 | H | 4,8 dimethylnonanoyl carnitine | Quaternary Amines | pos |
| 344.2793 | 457.86 | 0.58 | C19H37NO4 | H | Dodecanoylcarnitine | Quaternary Amines | pos |
| 372.3106 | 478.02 | 0.64 | C21H41NO4 | H | Tetradecanoylcarnitine | Monoacylglycerophosphates | pos |
| 398.3263 | 485.88 | 0.45 | C23H43NO4 | H | trans-Hexadec-2-enoyl carnitine | Quaternary Amines | pos |
| 430.3164 | 425.28 | 0.33 | C23H43NO6 | H | Hexadecanedioic acid mono-L-carnitine ester | Quaternary Amines | pos |
| **400.3421** | **501.54** | **0.05** | **C23H45NO4** | **H** | **L-Palmitoylcarnitine** | **Quaternary Amines** | **pos** |
| 414.3581 | 508.68 | 0.77 | C24H47NO4 | H | Heptadecanoyl carnitine | Quaternary Amines | pos |
| 424.3422 | 491.70 | 0.16 | C25H45NO4 | H | Linoelaidyl carnitine;Linoleyl carnitine | Quaternary Amines | pos |
| 448.3396 | 508.68 | 0.25 | C25H47NO4 | Na | Elaidic carnitine or related compounds | Quaternary Amines | pos |
| 428.3739 | 518.04 | 1.05 | C25H49NO4 | H | Stearoylcarnitine | Quaternary Amines | pos |
| 584.4885 | 696.00 | 1.71 | C33H65NO4 | HCOOH | Hexacosanoyl carnitine | Quaternary Amines | neg |
| 356.0420 | 66.54 | 0.42 | C9H15N3O2S | HCOONa | Ergothioneine | Quaternary Amines | pos |
| **Carbohydrates** | | | | | | | |
| **365.1060** | **70.44** | **4.41** | **C12H22O11** | **Na** | **Alpha-Lactose;D-Maltose;Melibiose;Sucrose** | **Carbohydrates** | **pos** |
| 382.1343 | 458.82 | 2.25 | C14H23NO11 | H | N-Acetyl-9-O-lactoylneuraminic acid | Carbohydrates | pos |
| 485.1127 | 587.46 | 3.59 | C15H26N2O12 | NaCl | Chitobiose | Carbohydrates | pos |
| **201.0374** | **83.46** | **3.38** | **C6H12O6** | **Na** | **D-Fructose;D-Galactose;D-Glucose;D-Mannose;Myoinositol** | **Carbohydrates** | **neg** |
| 253.0140 | 169.44 | 4.58 | C7H14O5S | Na_Na | 4-Methylthio-Alpha-D-Mannose;O1-Methyl-4-Deoxy-4-Thio-Alpha-D-Glucose;O1-Methyl-4-Deoxy-4-Thio-Beta-D-Glucose | Amino ketones | neg |
| **Fatty Acids and Conjugates** | | | | | | | |
| 173.1535 | 604.14 | 0.58 | C10H20O2 | H | 2-methyl nonaoic acid or related compounds | Fatty Acids and Conjugates | pos |
| 173.1536 | 648.06 | 0.23 | C10H20O2 | H | 3-methyl-nonanoic acid or related compounds | Fatty Acids and Conjugates | pos |
| 173.1536 | 703.80 | 0.23 | C10H20O2 | H | 7-methyl-nonanoic acid or related compounds | Fatty Acids and Conjugates | pos |
| 173.1536 | 739.08 | 0.29 | C10H20O2 | H | 2-methyl nonaoic acid or related compounds | Fatty Acids and Conjugates | pos |
| 185.1535 | 440.52 | 0.43 | C11H20O2 | H | 11-Undecanolactone;2-hendecenoic acid or related compounds | Fatty Acids and Conjugates | pos |
| 213.1848 | 461.64 | 0.42 | C13H24O2 | H | 12-tridecenoic acid;2-methyl-2-dodecenoic acid or related compounds | Fatty Acids and Conjugates | pos |
| **295.1885** | **601.20** | **1.05** | **C14H28O2** | **HCOONa** | **Myristic acid** | **Fatty Acids;** | **neg** |
| 311.1974 | 459.72 | 0.64 | C16H25NO4 | NH3 | 10E-heptadecenoic acid;10-methyl-9-hexadecenoic acid or related compounds | Fatty Acids and Conjugates | neg |
| 251.2005 | 446.22 | 0.20 | C16H26O2 | H | 4,7,10-hexadecatrienoic acid or related compounds | Fatty Acids and Conjugates | pos |
| **255.2318** | **476.16** | **0.04** | **C16H30O2** | **H** | **Palmitoleic acid** | **Fatty Acids and Conjugates** | **pos** |
| **253.2169** | **615.90** | **1.66** | **C16H30O2** | **H** | **10-hexadecenoic acid or related compounds** | **Fatty Acids and Conjugates** | **neg** |
| **297.1817** | **447.96** | **1.01** | **C16H30O2** | **Na_Na** | **Palmitoleic acid** | **Fatty Acids and Conjugates** | **neg** |
| **274.2739** | **448.20** | **0.55** | **C16H32O2** | **NH3 (Na_Na)** | **Palmitic acid** | **Fatty Acids and Conjugates** | **pos/neg** |
| **255.2325** | **656.88** | **1.92** | **C16H32O2** | **H** | **13-methyl-pentadecanoic acid or related compounds** | **Fatty Acids and Conjugates** | **neg** |
| 267.2326 | 641.82 | 1.27 | C17H32O2 | H | 10E-heptadecenoic acid or related compounds | Fatty Acids and Conjugates | neg |
| 311.1427 | 562.86 | 2.67 | C18H26O2 | K | 13E-octadecene-9,11-diynoic acid or related compounds | Fatty Acids and Conjugates | neg |
| **279.2318** | **438.66** | **0.25** | **C18H30O2** | **H** | **Alpha-Linolenic acid** | **Fatty Acids** | **pos** |
| 311.2215 | 466.44 | 0.58 | C18H30O4 | H | (9Z,11E,14Z)-(13S)-hydroperoxyoctadeca-(9,11,14)-trienoate or related compounds | Fatty Acids and Conjugates | pos |
| 341.2444 | 488.82 | 0.64 | C19H38O2 | Na_Na | (+)-16-methyl stearic acid or related compounds | Fatty Acids | neg |
| **347.2200** | **633.30** | **1.04** | **C18H32O2** | **HCOONa** | **10E,12E-octadecadienoic acid or related compounds** | **Fatty Acids and Conjugates** | **neg** |
| **325.2115** | **634.44** | **2.03** | **C18H32O2** | **Na_Na** | **Linoleic acid** | **Fatty Acids and Conjugates** | **pos** |
| **295.2276** | **443.04** | **0.95** | **C18H32O3** | **H** | **13S-hydroxyoctadecadienoic acid** | **Fatty Acids;** | **neg** |
| 313.2371 | 474.30 | 0.67 | C18H32O4 | H | (9Z,11E)-(13S)-13-Hydroperoxyoctadeca-9,11-dienoic acid or related compounds | Fatty Acids | pos |
| **283.2631** | **499.50** | **0.32** | **C18H34O2** | **H** | **Oleic acid** | **Fatty Acids** | **pos** |
| **281.2483** | **509.04** | **1.07** | **C18H34O2** | **H** | **(11E)-Octadecenoic acid or related compounds** | **Fatty Acid** | **neg** |
| **283.2632** | **663.66** | **0.32** | **C18H34O2** | **H** | **Oleic acid** | **Fatty Acids** | **pos** |
| **327.2273** | **667.62** | **1.74** | **C18H34O2** | **Na_Na (H)** | **(11E)-Octadecenoic acid;(6Z)-Octadecenoic acid or related compounds** | **Fatty Acids** | **pos/neg** |
| **327.2286** | **479.10** | **1.59** | **C18H36O2** | **Na_Na** | **Stearic acid** | **Fatty Acids** | **neg** |
| 399.1968 | 477.06 | 2.08 | C18H36O2S | HCOOK | 2-mercapto-octadecanoic acid | Fatty Acids and Conjugates | neg |
| 325.2373 | 481.86 | 0.03 | C19H32O4 | H | methyl 15-hydroperoxy-9Z,12Z,16E-octadecatrienoate or related compounds | Fatty Acids and Conjugates | pos |
| 374.2539 | 400.68 | 0.53 | C19H32O6 | NH3 | methyl 10,12-dihydroperoxy-8E,13E,15Z-octadecatrienoate or related compounds | Fatty Acids and Conjugates | pos |
| 327.2529 | 485.88 | 0.15 | C19H34O4 | H | 11-methoxy-12,13-epoxy-9-octadecenoic acid or related compounds | Fatty Acids and Conjugates | pos |
| 369.1955 | 443.70 | 1.65 | C19H36O2 | KCl | 10E-nonadecenoic acid or related compounds | Fatty Acids and Conjugates | neg |
| 339.2286 | 488.82 | 0.91 | C19H36O2 | Na_Na | 10E-nonadecenoic acid;10-methylene-octadecanoic acid or related compounds | Fatty Acids and Conjugates | neg |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 297.2790 | 513.18 | 0.64 | C19H36O2 | H | 10E-nonadecenoic acid;10-methylene-octadecanoic acid;10Z-nonadecenoic acid or related compounds | Fatty Acids and Conjugates | pos |
| 371.2116 | 465.72 | 2.51 | C19H38O2 | KCl | (+)-16-methyl stearic acid;"(2R,6S,10S)-pristanate";11-methyl-octadecanoic acid or related compounds | Fatty Acids and Conjugates | neg |
| 316.3212 | 478.02 | 0.76 | C19H38O2 | NH3 | (+)-16-methyl stearic acid;"(2R,6S,10S)-pristanate";11-methyl-octadecanoic acid or related compounds | Fatty Acids and Conjugates | pos |
| **305.2473** | **448.14** | **0.62** | **C20H32O2** | **H** | **Arachidonic acid** | **Fatty Acids** | **pos** |
| 307.2640 | 686.28 | 0.94 | C20H36O2 | H | 11,14-eicosadienoic acid or related compounds | Fatty Acids and Conjugates | neg |
| 311.2943 | 528.90 | 0.42 | C20H38O2 | H | 13-eicosenoic acid or related compounds | Fatty Acids and Conjugates | pos |
| 357.2644 | 484.86 | 0.56 | C20H38O5 | H | 11,13-dimethoxy-12-hydroxy-9-octadecenoic acid;"13,14-Dihydro PGF-1a"; or related compounds | Fatty Acids and Conjugates | neg |
| 369.2069 | 488.34 | 0.57 | C22H28O2 | HCOOH | 4,7,10,13-Docosatetraynoic acid or related compounds | Fatty Acids and Conjugates | neg |
| **329.2473** | **455.88** | **0.58** | **C22H32O2** | **H** | **Docosahexaenoic acid** | **Fatty Acids and Conjugates** | **pos/neg** |
| 381.2754 | 531.84 | 0.87 | C22H42O2 | Na_Na | (13Z)-Docosenoic acid or related compounds | Fatty Acids and Conjugates | neg |
| 383.2911 | 533.82 | 1.12 | C22H44O2 | Na_Na | 19-methyl-heneicosanoic acid;3-methyl-heneicosanoic acid;Docosanoic acid;Isobehenic acid | Fatty Acids and Conjugates | neg |
| 465.3456 | 801.42 | 1.01 | C23H49N2O5P | H | Sphingosyl-phosphocholine;;"2,4,6-trimethyl-2,15-tetracosadienoic acid";; | Fatty Acids and Conjugates | pos |
| 425.2574 | 592.38 | 4.63 | C23H44O2 | KCl | 14E-tricosenoic acid or related compounds | Fatty Acids and Conjugates | neg |
| 409.3039 | 460.80 | 3.44 | C24H44O2 | Na_Na | 5,9-tetracosadienoic acid | Fatty Acids and Conjugates | pos |
| **187.0717** | **75.90** | **1.87** | **C8H16O2** | **Na_Na** | **Caprylic acid** | **Fatty Acids and Conjugates** | **neg** |
| **187.0717** | **169.44** | **4.01** | **C8H16O2** | **Na_Na** | **Caprylic acid** | **Fatty Acids and Conjugates** | **neg** |
| **187.0710** | **203.52** | **1.98** | **C8H16O2** | **Na_Na** | **Caprylic acid** | **Fatty Acids and Conjugates** | **neg** |
| **187.0709** | **234.24** | **2.46** | **C8H16O2** | **Na_Na** | **Caprylic acid** | **Fatty Acids and Conjugates** | **neg** |
| **187.0717** | **388.14** | **4.01** | **C8H16O2** | **Na_Na** | **Caprylic acid** | **Fatty Acids and Conjugates** | **neg** |
| **271.0246** | **169.86** | **4.02** | **C9H16O2** | **NaClx2** | **2-amyl 3-butenoic acid;2E-nonenoic acid or related compounds** | **Fatty Acids and Conjugates** | **neg** |
| **271.0245** | **200.76** | **4.46** | **C9H16O2** | **NaClx2** | **2-amyl 3-butenoic acid;2E-nonenoic acid or related compounds** | **Fatty Acids and Conjugates** | **neg** |
| **271.0247** | **374.22** | **3.91** | **C9H16O2** | **NaClx2** | **2-amyl 3-butenoic acid;2E-nonenoic acid or related compounds** | **Fatty Acids and Conjugates** | **neg** |
| **Fatty aldehydes** | | | | | | | |
| 167.1430 | 440.52 | 0.30 | C11H18O | H | 2,5-undecadienal | Fatty aldehydes | pos |
| 209.1898 | 466.44 | 0.81 | C14H24O | H | 5,8-tetradecadienal | Fatty aldehydes | pos |
| 240.2321 | 462.54 | 0.37 | C15H26O | NH3 | "(1E,4S,5E,7R)-Germacra-1(10) or related compounds | Fatty aldehydes | pos |
| 270.2794 | 458.88 | 0.96 | C17H32O | NH3 | 14-Methyl-8E-hexadecenal or related compounds | Fatty aldehydes | pos |
| 309.2431 | 561.06 | 1.20 | C18H32O | HCOOH | 9,12-octadecadienal | Fatty aldehydes | neg |
| 367.2159 | 576.72 | 4.49 | C20H38O | KCl | 11Z-Eicosenal | Fatty aldehydes | neg |
| 169.0609 | 169.44 | 0.71 | C8H14O | Na_Na | 2-octenal or related compounds | Fatty aldehydes | neg |
| **Fatty alcohols** | | | | | | | |
| 205.1587 | 430.92 | 0.24 | C14H20O | H | 13-tetradecen-2,4-diyn-1-ol | Fatty alcohols | pos |
| 253.2139 | 562.02 | 0.43 | C14H30O2 | Na | 2,2,9,9-tetramethyl-undecan-1,10-diol | Fatty alcohols | pos |
| 297.2398 | 599.22 | 0.74 | C15H32O | HCOONa | 3S,7S-dimethyl-2S-tridecanol | Fatty alcohols | pos |
| 258.2794 | 454.92 | 1.12 | C16H32O | NH3 | 1,2-Epoxyhexadecane;11Z-hexadecen-1-ol or related compounds | Fatty alcohols | pos |
| 311.2554 | 626.88 | 0.90 | C16H34O | HCOONa | 14-methyl-1-pentadecanol;1-Hexadecanol;"3S,7S-dimethyl-2S-tetradecanol" | Fatty alcohols | pos |
| 343.1316 | 555.12 | 2.94 | C17H22O2 | HCOOK | "1,9Z,16-heptadecatrien-4,6-diyn-3,8-diol";"5,8,11-heptadecatriynoic acid";Cicutoxin;; | Fatty alcohols | pos |
| 325.2711 | 587.46 | 0.65 | C17H36O | HCOONa | 14-methyl-1-hexadecanol | Fatty alcohols | pos |
| 313.2494 | 480.78 | 1.63 | C18H38O | Na_Na | 3S,7S-dimethyl-2-hexadecanol;Octadecanol | Fatty alcohols | neg |
| 371.2705 | 465.72 | 1.83 | C20H42O2 | NaCl | 1,2-eicosanediol; 13-methyl-1,2-nonadecanediol | Fatty alcohols | neg |
| 371.2895 | 465.72 | 3.21 | C21H44O2 | Na_Na | 1,2-heneicosanediol;"13-methyl-1,2-eicosanediol";"15-methyl-1,2-eicosanediol";; | Fatty alcohols | pos/neg |
| 143.0452 | 463.98 | 1.47 | C6H12O | Na_Na | 2E-hexenol;2-Oxohexane; or Unsaturated alcohol | Fatty alcohols | neg |
| 145.0609 | 424.98 | 1.24 | C6H14O | Na_Na | 3-Methylpentan-1-ol;Hexan-1-ol | Fatty alcohols | neg |
| **Hydroxy-oxo fatty acids** | | | | | | | |
| 263.1618 | 430.92 | 0.04 | C14H24O3 | Na | (1R,2R)-3-oxo-2-pentyl-cyclopentanebutanoic acid or related compounds | Hydroxy fatty acids | pos |
| 285.1434 | 501.54 | 1.05 | C14H24O3 | Na_Na | 6(R)-hydroxy-tetradeca-2E,8Z-dienoate or related compounds | Hydroxy fatty acids | pos |
| 287.1611 | 374.22 | 2.23 | C14H28O3 | Na_Na | 10-hydroxy-tetradecanoic acid or related compounds | Hydroxy fatty acids | neg |
| 301.1410 | 558.18 | 0.53 | C16H22O4 | Na | 2-Ethylhexyl phthalate;3'-carboxy-alpha-chromanol;4-prenylphlorisovalerophenone;Alpha-CEHC;Dibutyl phthalate;Diisobutyl phthalate | Hydroxy fatty acids | pos |
| 265.1806 | 442.20 | 1.28 | C16H26O3 | H | (2E,6E)-(10R,11S)-10,11-Epoxy-3,7,11-trimethyltrideca-2,6-dienoic acid or related compounds | Hydroxy fatty acids | neg |
| 265.1804 | 549.78 | 1.96 | C16H26O3 | H | (9R,13R)-1a,1b-dinor-10,11-dihydro-12-oxo-15-phytoenoic acid or related compounds | Hydroxy fatty acids | neg |
| 337.1985 | 444.30 | 0.68 | C16H28O3 | HCOONa | (1R,2R)-3-oxo-2-pentyl-cyclopentanehexanoic acid";"(1S,2S)-3-oxo-2-pentyl-cyclopentanehexanoic acid | Hydroxy fatty acids | pos |
| 291.1929 | 446.22 | 0.41 | C16H28O3 | Na | (1S,2S)-3-oxo-2-pentyl-cyclopentanehexanoic acid or related compounds | Hydroxy fatty acids | pos |
| 313.1767 | 404.40 | 0.57 | C16H30O3 | Na_Na | (+)-12-hydroxy-9Z-hexadecenoic acid;10-keto palmitic acid or related compounds | Hydroxy fatty acids | neg |
| 269.2119 | 510.30 | 1.19 | C16H30O3 | H | 10-keto palmitic acid or related compounds | Hydroxy fatty acids | neg |
| 269.2119 | 570.84 | 1.08 | C16H30O3 | H | 10-oxo-14-methyl-pentadecanoic acid or related compounds | Hydroxy fatty acids | neg |
| 315.1924 | 424.98 | 0.57 | C16H32O3 | Na_Na | 11-hydroxy palmitic acid or related compounds | Hydroxy fatty acids | neg |
| 271.2277 | 598.26 | 0.70 | C16H32O3 | H | 14-hydroxy palmitic acid or related compounds | Hydroxy fatty acids | neg |
| 355.2097 | 567.90 | 1.32 | C16H32O4 | HCOONa | 10,16-Dihydroxyhexadecanoic acid or related compounds | Hydroxy fatty acids | neg |
| 309.2043 | 627.54 | 1.39 | C16H32O4 | Na | 3,12-dihydroxy palmitic acid or related compounds | Hydroxy fatty acids | neg |
| 367.2106 | 467.94 | 1.20 | C17H32O4 | HCOONa | 2-methyl-hexadecanedioic acid or related compounds | Hydroxy fatty acids | neg |
| 369.2253 | 443.70 | 1.44 | C17H34O4 | HCOONa | MG(0:0/14:0/0:0);MG(14:0/0:0/0:0) | Hydroxy fatty acids | neg |
| 369.2254 | 487.26 | 1.19 | C17H34O4 | HCOONa | MG(0:0/14:0/0:0);MG(14:0/0:0/0:0) | Hydroxy fatty acids | neg |
| 347.1828 | 430.92 | 3.31 | C18H28O5 | Na | 12-oxo-14,18-dihydroxy-9Z,13E,15Z-octadecatrienoic acid | Oxo fatty acids | pos |
| 293.2121 | 435.36 | 0.34 | C18H30O3 | H | (9R,13R)-10,11-dihydro-12-oxo-15-phytoenoic acid or related compounds | Hydroxy fatty acids | neg |
| **319.2245** | **548.52** | **1.19** | **C18H32O3** | **Na (H)** | **(1R,2R)-3-oxo-2-pentyl-cyclopentaneoctanoic acid or related compounds** | **Hydroxy fatty acids** | **pos/neg** |
| 341.2080 | 450.36 | 1.85 | C18H34O3 | Na_Na | (6R,7S)-6,7-Epoxyoctadecanoic acid;(9Z)-(12S)-Hydroxyoctadecenoic acid;10-hydroxy-8-octadecenoic acid or related compounds | Hydroxy fatty acids | neg |
| 297.2431 | 572.82 | 1.45 | C18H34O3 | H | 10R-hydroxy-8E-octadecenoic acid or related compounds | Hydroxy fatty acids | neg |
| 299.2588 | 565.02 | 1.30 | C18H36O3 | H | (R)-2-Hydroxystearate or related compounds | Hydroxy fatty acids | neg |
| 315.2535 | 518.10 | 1.78 | C18H36O4 | H | (9R,10R)-Dihydroxyoctadecanoic acid or related compounds | Hydroxy fatty acids | neg |
| 385.2562 | 536.70 | 0.39 | C18H36O4 | HCOONa | (9S,10S)-9,10-Dihydroxyoctadecanoate or related compounds | Hydroxy fatty acids | pos |
| 383.2413 | 588.42 | 0.50 | C18H36O4 | HCOONa | 10,11-dihydroxy stearic acid or related compounds | Hydroxy fatty acids | neg |
| 337.2357 | 679.98 | 3.83 | C18H36O4 | Na | 11,12-dihydroxy stearic acid or related compounds | Hydroxy fatty acids | neg |
| **399.2373** | **571.86** | **2.35** | **C18H36O5** | **HCOONa** | **18-hydroxy-9S,10R-dihydroxy-stearic acid; 9,10,18-trihydroxystearate or related compounds** | **Hydroxy fatty acids** | **neg** |
| 371.2407 | 495.60 | 0.78 | C18H36O6 | Na | Sativic acid | Hydroxy fatty acids | pos |
| 371.2320 | 465.72 | 3.85 | C19H38O3 | NaCl | 19-hydroxy-nonadecanoic acid;2-hydroxy-nonadecanoic acid | Hydroxy fatty acids | neg |
| **369.2751** | **518.10** | **0.16** | **C21H42O2** | **Na_Na** | **(+)-18-methyl-eicosanoic acid;19-methyl-eicosanoic acid;"2,6-dimethyl-nonadecanoic acid";Heneicosanoic acid;Homophytanic acid** | **Methyl branched fatty acids** | **neg** |
| 372.3476 | 505.38 | 1.13 | C22H42O3 | NH3 | 10-oxo-docosanoic acid or related compounds | Oxo fatty acids | pos |
| 400.3789 | 536.70 | 0.92 | C24H46O3 | NH3 | 24-hydroxy-10Z-tetracosenoic acid;2-hydroxy-15Z-tetracosenoic acid;3-oxo-tetracosanoic acid;Hydroxynervonic acid | Hydroxy fatty acids | pos |
| **133.0865** | **577.68** | **4.51** | **C6H12O3** | **H** | **5-Hydroxyhexanoic acid;Hydroxyisocaproic acid** | **Hydroxy Acids** | **pos** |
| **Eicosanoids** | | | | | | | |
| 303.2317 | 448.14 | 0.43 | C20H30O2 | H | (+)-Pimaric acid or related compounds | Eicosanoids | pos |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **319.2276** | **452.82** | **4.39** | **C20H32O3** | **H** | **(15S)-15-Hydroxy-5,8,11-cis-13-trans-eicosatetraenoate or related compounds** | **Eicosanoids** | **neg** |
| 321.2432 | 455.22 | 0.84 | C20H34O3 | H | 12-HETrE,12R-HETrE; 15-oxo-11Z,13E-eicosadienoic acid or related compounds | Eicosanoids | neg |
| 356.2793 | 459.72 | 0.73 | C20H34O4 | NH3 | 10,11-dihydro-12-epi-leukotriene B4;"11,12-DHET";11-deoxy-PGE1;11-deoxy-PGF2a;11-deoxy-PGF2beta;12-keto-tetrahydro-Leukotriene B4;"14,15-DHET";"15-hydroperoxyeicosa-8Z,11Z,13E-trienoate";"5,6-DHET";"5,6-dihydroxy-8,11,14-eicosatrienoic acid";"6,7-dihydro-12-epi-leukotriene B4";"8,9-DHET";"8,9-dihydroxy-5,11,14-eicosatrienoic acid";Aphidicolin;S 1033;Zoapatanol;; | Eicosanoids | pos |
| 339.2530 | 489.78 | 0.12 | C20H34O4 | H | 10,11-dihydro-12-epi-leukotriene B4;"11,12-DHET";11-deoxy-PGE1;11-deoxy-PGF2a;11-deoxy-PGF2beta;12-keto-tetrahydro-Leukotriene B4;"14,15-DHET";"15-hydroperoxyeicosa-8Z,11Z,13E-trienoate";"5,6-DHET";"5,6-dihydroxy-8,11,14-eicosatrienoic acid";"6,7-dihydro-12-epi-leukotriene B4";"8,9-DHET";"8,9-dihydroxy-5,11,14-eicosatrienoic acid";Aphidicolin;S 1033;Zoapatanol | Eicosanoids | pos |
| 583.1303 | 463.56 | 0.65 | C20H36O12 | NaCl*2 | 3'-O-N-Octanoyl-a-D-Glucopyranosyl-B-D-Fructofuranoside,"N-Octanoyl-B-D-Fructofuranosyl-a-D-Glucopyranoside,Sucrose Monocaproylate" | Eicosanoids | neg |
| 369.2634 | 443.70 | 3.25 | C20H36O3 | HCOOH | 11(R)-HEDE or related compounds | Eicosanoids | neg |
| 369.2634 | 490.74 | 3.41 | C20H36O3 | HCOOH | 11S-HEDE or related compounds | Eicosanoids | neg |
| **Fatty amides** | | | | | | | |
| 256.2632 | 448.14 | 0.94 | C16H33NO | H | Palmitic amide | Fatty amides | pos |
| 294.2405 | 552.24 | 0.37 | C16H33NO2 | Na | N-(Tetradecanoyl)-ethanolamine/2-amino-hexadecanoic acid | Fatty amides/Amino fatty acid | pos |
| 282.2790 | 474.30 | 0.60 | C18H35NO | H | Elaidoylamide;Oleamide | Fatty amides | pos |
| 348.2874 | 610.02 | 0.37 | C20H39NO2 | Na | N-Oleoylethanolamine | Fatty amides | pos |
| 374.3031 | 597.24 | 0.45 | C22H41NO2 | Na | Anandamide (20:2, n-6) | Fatty amides | pos |
| 382.3068 | 494.88 | 0.16 | C22H45NO | Na_Na | Docosanamide | Fatty amides | neg |
| 430.2857 | 511.26 | 2.00 | C22H45NO2 | KCl | Eicosanoyl-EA | Fatty amides | pos |
| 504.2398 | 429.72 | 0.10 | C25H43NO2 | NaClx2 | "(-)N-(1R-methyl-2-hydroxy-ethyl) alpha,alpha-dimethylarachidonoyl amine" or related compounds | Fatty amides | neg |
| 512.3043 | 483.78 | 4.57 | C25H51NO2 | NaClx2 | Tricosanoyl-EA | Fatty amides | neg |
| 510.2523 | 469.80 | 5.00 | C29H43NO2 | KCl | (+/-)N-(1-methyl-2-hydroxy-2-phenyl-ethyl) arachidonyl amine | Fatty amides | neg |
| **Fatty acyl glycosides** | | | | | | | |
| 545.3663 | 542.58 | 0.64 | C26H52O7 | HCOONa | 1-O-alpha-D-glucopyranosyl-1,2-eicosandiol | Fatty acyl glycosides | pos |
| 671.3632 | 571.62 | 1.16 | C32H58O13 | Na | 25-O-(2"-beta-D-glucopyranosyl-beta-D-glucopyranosyl)-25-hydroxy-11E-eicosenoic acid | Fatty acyl glycosides | neg |
| 691.3669 | 599.22 | 2.94 | C32H62O8 | NaClx2 | 1-(O-alpha-D-glucopyranosyl)-3-keto-(1,25R)-hexacosanediol | Fatty acyl glycosides | pos |
| 655.4203 | 600.24 | 1.57 | C34H66O9 | K | 1-(O-alpha-D-glucopyranosyl)-25-keto-(1,3R,27R)-octacosanetriol or related compounds | Fatty acyl glycosides | neg |
| **Carboxylic acids** | | | | | | | |
| 307.1886 | 592.38 | 1.50 | C16H30O4 | Na | 10-hydroxy-16-oxo-hexadecanoic acid;"2,3-Dihydroxycyclopentaneundecanoic acid";9-hydroxy-16-oxo-hexadecanoic acid;Hexadecanedioic acid | Dicarboxylic acids | neg |
| 299.2215 | 464.46 | 0.70 | C17H30O4 | H | 8E-Heptadecenedioic acid | Dicarboxylic acids | pos |
| 369.3013 | 443.70 | 0.87 | C22H42O4 | H | Di(2-ethylhexyl) adipate;Docosanedioic acid | Dicarboxylic acids | neg |
| 393.2978 | 757.68 | 0.79 | C22H42O4 | Na | Di(2-ethylhexyl) adipate;Docosanedioic acid | Dicarboxylic acids | pos |
| 402.3577 | 499.50 | 0.10 | C23H44O4 | NH3 | Tricosanedioic acid | Dicarboxylic acids | pos |
| 416.3737 | 513.18 | 0.65 | C24H46O4 | NH3 | Tetracosanedioic acid | Dicarboxylic acids | pos |
| 444.4049 | 554.22 | 0.36 | C26H50O4 | NH3 | Hexacosanedioic acid | Dicarboxylic acids | pos |
| 543.2937 | 615.90 | 4.58 | C26H50O4 | NaClx2 | Hexacosanedioic acid | Dicarboxylic acids | pos |
| 449.3599 | 788.94 | 0.56 | C26H50O4 | Na | Hexacosanedioic acid | Dicarboxylic acids | pos |
| **193.0351** | **64.56** | **0.05** | **C6H8O7** | **H** | **Citric acid;Isocitric acid** | **Tricarboxylic Acids** | **pos** |
| **Steroids and Derivatives** | | | | | | | |
| **339.1573** | **571.44** | **3.45** | **C18H22O2** | **HCOONa** | **Estrone** | **Steroids and Steroid Derivatives** | **pos** |
| **355.1528** | **562.02** | **1.63** | **C18H22O3** | **HCOONa (HCOOK)** | **2-Hydroxyestrone** | **Steroids and Steroid Derivatives** | **pos/neg** |
| **357.1473** | **566.94** | **2.86** | **C18H24O2** | **HCOOK** | **17a-Estradiol; Estradiol** | **Steroids and Steroid Derivatives** | **pos** |
| **277.2162** | **434.82** | **0.14** | **C18H28O2** | **H** | **19-Norandrosterone; 19-Noretiocholanolone** | **Steroids and Steroid Derivatives** | **pos** |
| **371.1626** | **573.84** | **4.58** | **C19H26O2** | **HCOOK** | **Androstenedione** | **Steroids and Steroid Derivatives** | **pos** |
| 273.2213 | 441.54 | 0.11 | C19H28O | H | 3-Oxo-delta1-steroid; 5a-Androst-3-en-17-one | Steroids and Steroid Derivatives | pos |
| **373.1986** | **442.38** | **0.13** | **C19H28O3** | **HCOONa** | **11-Ketoetiocholanolone;16a-Hydroxydehydroisoandrosterone;;** | **Steroids and Steroid Derivatives** | **pos** |
| **361.2349** | **485.88** | **0.17** | **C19H32O2** | **HCOONa** | **Androstanediol** | **Steroids and Steroid Derivatives** | **pos** |
| **311.2007** | **566.04** | **4.37** | **C21H28O2** | **H** | **16-Dehydroprogesterone or related compounds** | **Steroids and Steroid Derivatives** | **neg** |
| 383.1636 | 429.00 | 4.31 | C21H28O4 | K | 11-Dehydrocorticosterone or related compounds | Steroids and Steroid Derivatives | pos |
| **383.1837** | **581.58** | **1.77** | **C21H28O5** | **Na** | **Aldosterone;Cortisone** | **Steroids and Steroid Derivatives** | **pos** |
| **385.1787** | **583.80** | **3.06** | **C21H30O4** | **K** | **Cortexolone** | **Steroids and Steroid Derivatives** | **pos** |
| **401.2297** | **455.88** | **0.77** | **C21H32O3** | **HCOONa** | **16-a-Hydroxypregnenolone;21-Hydroxypregnenolone;;** | **Steroids and Steroid Derivatives** | **pos** |
| 417.2248 | 438.66 | 0.34 | C21H32O4 | HCOONa | 11b,21-Dihydroxy-5b-pregnane-3,20-dione or related compounds | Steroids and Steroid Derivatives | pos |
| **403.2455** | **463.56** | **0.67** | **C21H34O3** | **HCOONa** | **Tetrahydrodeoxycorticosterone** | **Steroids and Steroid Derivatives** | **pos** |
| **419.2403** | **444.30** | **0.17** | **C21H34O4** | **HCOONa** | **5a-Tetrahydrocorticosterone;"5beta-Pregnane-3alpha,17alpha,20alpha-triol-11-one";9-deoxy-9-methylene-PGE2;Tetrahydrocorticosterone** | **Steroids and Steroid Derivatives** | **pos** |
| **384.2743** | **430.92** | **0.39** | **C21H34O5** | **NH3** | **5a-Tetrahydrocortisol;Cortolone;Tetrahydrocortisol** | **Steroids and Steroid Derivatives** | **pos** |
| **367.2484** | **467.94** | **4.93** | **C21H36O5** | **H** | **Beta-Cortol;Cortol** | **Steroids and Steroid Derivatives** | **neg** |
| 377.1968 | 567.96 | 2.44 | C21H28O6 | H (HCOOH) | 18-Oxocortisol | Steroids and Steroid Derivatives | pos/neg |
| 504.2750 | 476.10 | 0.67 | C26H45NO | NaClx2 | 25-Azacholesterol | Steroids and Steroid Derivatives | pos |
| 455.3346 | 659.76 | 0.75 | C26H46O6 | H | 27-norcholestanehexol | Steroids and Steroid Derivatives | pos |
| 511.2906 | 447.96 | 1.27 | C27H44O9 | H | (24R)-11alpha,20,24-trihydroxyecdysone;"(25R)-11alpha,20,26-trihydroxyecdysone","(25S)-11alpha,20,26-trihydroxyecdysone";; | Cholesterol and derivatives | neg |
| 417.3455 | 513.18 | 3.07 | C27H48 | Na_Na | 5beta-cholestane | Cholesterol and derivatives | pos |
| 454.3897 | 544.56 | 1.28 | C27H48O4 | NH3 | 3alpha,7alpha,12alpha,26-Tetrahydroxy-5beta-cholestane or related compounds | Steroids and Steroid Derivatives | pos |
| 497.2937 | 527.88 | 4.28 | C30H48O | KCl | 4alpha,14alpha-dimethyl-24-methylene-cholest-7,9(11)-dien-3beta-ol | Ergosterols and C24-methyl derivatives | neg |
| 466.3895 | 548.52 | 0.99 | C28H48O4 | NH3 | Teasterone;Typhasterol | Ergosterols and C24-methyl derivatives | pos |
| **459.3559** | **526.92** | **4.03** | **C29H50O** | **Na_Na** | **Beta-Sitosterol** | **Steroids and Steroid Derivatives** | **pos** |
| 541.3406 | 619.20 | 4.53 | C29H52O | NaCl_HCOONa | 23R,24R-dimethylcholestan-3b-ol or related compounds | Ergosterols and C24-methyl derivatives | neg |
| 553.2842 | 549.78 | 4.18 | C33H38N4O4 | H | Integerressine | Steroids and Steroid Derivatives | neg |
| 693.5314 | 712.62 | 4.79 | C44H74O2 | NaCl | episteryl palmitoleate,fecosteryl palmitoleate;; | Ergosterols and C24-methyl derivatives | pos |
| 733.5092 | 504.24 | 2.13 | C46H76O2 | KCl | ergosteryl oleate | Ergosterols and C24-methyl derivatives | neg |
| **Bile acids** | | | | | | | |
| 473.2511 | 518.04 | 3.63 | C22H33NO9 | NH3 | (23R)-23-Hydroxy-3,7-dioxo-5beta-cholan-24-oic Acid or related compounds | Bile acids and derivatives | pos |
| 414.3210 | 464.46 | 0.84 | C23H40O5 | NH3 | 24-Nor-5beta-cholane-3alpha,7alpha,12alpha,22,23-pentol;PGF2alpha isopropyl ester | Bile acids and derivatives | pos |
| 367.2293 | 467.94 | 3.89 | C24H32O3 | H | 3-Oxocholа-1,4,6-trien-24-oic Acid | Bile acids and derivatives | neg |
| 383.2236 | 588.42 | 0.42 | C24H32O4 | H | (22E)-12alpha-Hydroxy-3-oxocholа-1,4,22-trien-24-oic Acid or related compounds | Bile Acids | neg |
| 399.2181 | 571.86 | 0.80 | C24H32O5 | H | 3,7,12-Trioxo-5beta-chol-1-en-24-oic Acid or related compounds | Bile Acids | neg |
| 355.2630 | 474.30 | 0.42 | C24H34O2 | H | 5beta-Chola-3,8(14),11-trien-24-oic Acid | Bile acids and derivatives | pos |
| 369.2441 | 443.70 | 1.65 | C24H34O3 | H | (20S,22E)-3beta-Hydroxychola-5,16,22-trien-24-oic Acid or related compounds | Bile Acids | neg |
| 373.2735 | 474.30 | 0.56 | C24H36O3 | H | (20S)-3beta-Hydroxychola-5,16-dien-24-oic Acid or related compounds | Bile acids and derivatives | pos |
| 449.2541 | 520.02 | 0.82 | C24H36O5 | HCOOH | (23R)-23-Hydroxy-3,7-dioxo-5beta-cholan-24-oic Acid or related compounds | Bile Acids | neg |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 413.2662 | 752.82 | 0.31 | C24H38O4 | Na | (22E)-3alpha,12alpha-Dihydroxy-5beta-chol-22-en-24-oic Acid or related compounds | Bile acids and derivatives | pos |
| 445.2558 | 453.96 | 2.31 | C24H38O6 | Na | (3r,5r)-7-((1r,2r,6s,8r,8as)-2,6-Dimethyl-8-{[(2r)-2-Methylbutanoyl]Oxy}-1,2,6,7,8,8a-Hexahydronaphthalen-1-Yl)-3,5-Dihydroxyheptanoic Acid or related compounds | Bile acids and derivatives | pos |
| **391.2847** | **512.22** | **1.69** | **C24H40O4** | **H** | **Chenodeoxycholic acid;Deoxycholic acid;Hyodeoxycholic acid;Isoursodeoxycholic acid;Ursodeoxycholic acid** | **Bile Acids** | **neg** |
| **431.2766** | **474.30** | **0.39** | **C24H40O5** | **Na** | **(22R)-3alpha,7alpha,22-Trihydroxy-5beta-cholan-24-oic Acid or related compounds** | **Bile acids and derivatives** | **pos** |
| **407.2796** | **477.06** | **4.32** | **C24H40O5** | **H** | **1b,3a,12a-Trihydroxy-5b-cholanoic acid;Cholic acid;Hyocholic acid;Ursocholic acid** | **Bile Acids** | **neg** |
| **450.3209** | **457.86** | **1.11** | **C26H43NO5** | **H** | **Chenodeoxycholic acid glycine conjugate** | **Acyl Glycines;Bile Acids** | **pos** |
| **494.2848** | **476.10** | **0.99** | **C26H43NO5** | **Na_Na** | **3alpha,12alpha-Dihydroxy-5beta-cholan-24-oylglycine or related compounds** | **Acyl Glycines;Bile Acids** | **pos/neg** |
| **516.2936** | **476.70** | **1.30** | **C26H43NO5;** | **HCOONa** | **Chenodeoxycholic acid glycine conjugate** | **Acyl Glycines;Bile Acids** | **neg** |
| **464.3010** | **447.54** | **1.68** | **C26H43NO6** | **H** | **Glycocholic acid** | **Acyl Glycines;Bile Acids** | **neg** |
| **488.2986** | **449.16** | **0.63** | **C26H43NO6** | **Na** | **Glycocholic acid** | **Acyl Glycines;Bile Acids** | **pos** |
| 482.3475 | 453.96 | 4.73 | C27H44O6 | NH3 | 3alpha,6alpha,7alpha,12alpha-Tetrahydroxy-5beta-cholest-24-en-26-oic acid;3-Epiecdysone;Ecdysone;Ponasterone A | Bile acids and derivatives | pos |
| 468.3681 | 478.02 | 0.58 | C27H46O5 | NH3 | 3a,7a,12a-Trihydroxy-5b-cholestanoic acid or related compounds | Bile acids and derivatives | pos |
| 484.3633 | 459.72 | 0.06 | C27H46O6 | NH3 | 1beta,3alpha,7alpha,12alpha-Tetrahydroxy-5beta-cholestan-26-oic acid or related compounds | Bile acids and derivatives | pos |
| 686.3416 | 488.34 | 3.26 | C32H51NO12 | HCOOH | (3a,5b,7a,12a)-24-[(carboxymethyl)amino]-1,12-dihydroxy-24-oxocholan-3-yl-b-D-Glucopyranosiduronic acid | Bile Acids | neg |
| **Vitamins and derivatives** | | | | | | | |
| 393.2099 | 86.04 | 1.04 | C22H32O4S | H | (6RS)-22-oxo-23,24,25,26,27-pentanorvitamin D3 6,19-sulfur dioxide adduct / (6RS)-22-oxo-23,24,25,26,27-pentanorcholecalciferol 6,19-sulfur dioxide adduct | Vitamin D2 and derivatives | pos |
| 415.1918 | 443.04 | 3.78 | C22H34O4S | Na | (6RS)-22-hydroxy-23,24,25,26,27-pentanorvitamin D3 6,19-sulfur dioxide adduct / (6RS)-22-hydroxy-23,24,25,26,27-pentanorcholecalciferol 6,19-sulfur dioxide adduct | Vitamin D2 and derivatives | neg |
| 597.3108 | 561.06 | 1.19 | C37H48O3 | NaCl | 1alpha,25-dihydroxy-25,25-diphenyl-26,27-dinorvitamin D3 / 1alpha,25-dihydroxy-25,25-diphenyl-26,27-dinorcholecalciferol | Vitamin D2 and derivatives | neg |
| 469.3142 | 620.76 | 1.26 | C23H44O5 | HCOONa | 1,2-Didecanoylglycerol | Vitamin D2 and derivatives | pos |
| 501.2882 | 476.70 | 3.49 | C29H48O2 | KCl | (22alpha)-hydroxy-isofucosterol;"(24R,24'R)-Fucosterol epoxide or related compounds | Vitamin D2 and derivatives | neg |
| 453.2883 | 612.96 | 1.32 | C28H44O | NaCl | (5E)-isovitamin D2 / (5E)-isoergocalciferol or related compounds | Vitamin D2 and derivatives | neg |
| 422.3263 | 481.86 | 0.43 | C25H40O4 | NH3 | 11'-carboxy-gamma-chromanol or related compounds | Vitamin D2 and derivatives | pos |
| 452.3368 | 464.46 | 0.49 | C26H42O5 | NH3 | (24R)-1alpha,24,25-trihydroxy-22-oxavitamin D3 / (24R)-1alpha,24,25-trihydroxy-22-oxacholecalciferol or related compounds | Vitamin D2 and derivatives | pos |
| 460.2728 | 429.00 | 3.17 | C26H43NO | KCl | 25-azavitamin D3 / 25-azacholecalciferol;; | Vitamin D2 and derivatives | pos |
| 455.2678 | 583.50 | 4.70 | C27H42O2 | NaCl | (22E)-1alpha-hydroxy-22,23-didehydrovitamin D3 / (22E)-1alpha-hydroxy-22,23-didehydrocholecalciferol or related compounds | Vitamin D2 and derivatives | neg |
| 509.2575 | 390.90 | 3.28 | C26H43O4P | NaCl | 24-(dimethoxyphosphoryl)-25,26,27-trinorvitamin D3 / 24-(dimethoxyphosphoryl)-25,26,27-trinorcholecalciferol or related compounds | Vitamin D2 and derivatives | pos |
| 464.3367 | 463.56 | 0.67 | C27H42O5 | NH3 | (23R)-1alpha,23,25-trihydroxy-24-oxovitamin D3 / (23R)-1alpha,23,25-trihydroxy-24-oxocholecalciferol or related compounds | Vitamin D2 and derivatives | pos |
| 511.2728 | 545.52 | 1.55 | C27H43F | NaCl_HCOONa | 3-Fluoro-9,10-secocholesta-5,7,10(19)-triene | Vitamin D3 and derivatives | neg |
| 485.2726 | 734.22 | 1.24 | C27H44O4S | Na | (6R)-25-hydroxyvitamin D3 6,19-sulfur dioxide adduct / (6R)-25-hydroxycholecalciferol 6,19-sulfur dioxide adduct or related compounds | Vitamin D2 and derivatives | neg |
| 466.3525 | 470.34 | 0.39 | C27H44O5 | NH3 | (23S,25R)-1alpha,23,25,26-tetrahydroxyvitamin D3 / (23S,25R)-1alpha,23,25,26-tetrahydroxycholecalciferol or related compounds | Vitamin D2 and derivatives | pos |
| 452.3735 | 518.04 | 0.11 | C27H46O4 | NH3 | (10R)-1alpha,19,25-trihydroxy-10,19-dihydrovitamin D3 / (10R)-1alpha,19,25-trihydroxy-10,19-dihydrocholecalciferol or related compounds | Vitamin D2 and derivatives | pos |
| 409.3103 | 780.12 | 3.35 | C28H42O2 | H | calicoferol D or related compounds | Vitamin D3 and derivatives | neg |
| 553.2653 | 575.70 | 4.57 | C28H45ClO3 | Na_HCOONa | 11alpha-(chloromethyl)-1alpha,25-dihydroxyvitamin D3 / 11alpha-(chloromethyl)-1alpha,25-dihydroxycholecalciferol;; | Vitamin D3 and derivatives | neg |
| 487.2770 | 575.70 | 4.10 | C28H46O2 | KCl | (22alpha)-hydroxy-campest-4-en-3-one or related compounds | Vitamin D3 and derivatives | neg |
| 499.2940 | 585.48 | 4.27 | C29H46O3 | NaCl | (22E)-1alpha,25-dihydroxy-26,27-dimethyl-22,23-didehydrovitamin D3 / (22E)-1alpha,25-dihydroxy-26,27-dimethyl-22,23-didehydrocholecalciferol or related compounds | Vitamin D2 and derivatives | neg |
| 492.3680 | 478.02 | 0.73 | C29H46O5 | NH3 | 18-acetoxy-1alpha,25-dihydroxyvitamin D3 / 18-acetoxy-1alpha,25-dihydroxycholecalciferol | Vitamin D2 and derivatives | pos |
| 497.3147 | 617.04 | 0.72 | C30H48O2 | NaCl | 26,27-Dihomo-1alpha-hydroxy-24-epivitamin D2;"26,27-Dihomo-1alpha-hydroxyvitamin D2";"4,4-Dimethyl-14a-formyl-5a-cholesta-8,24-dien-3b-ol";"4,4-dimethyl-14alpha-formyl-5alpha-cholesta-8,24-dien-3beta-ol" | Vitamin D2 and derivatives | neg |
| 479.3195 | 662.52 | 0.69 | C27H43FO3 | HCOOH | (10E)-19-fluoro-1alpha,25-dihydroxyvitamin D3 / (10E)-19-fluoro-1alpha,25-dihydroxycholecalciferol or related compounds | Vitamin D2 and derivatives | neg |
| 509.3297 | 662.52 | 4.59 | C32H46O5 | H | 11-(3-acetoxy-1-propynyl)-1alpha,25-dihydroxy-9,11-didehydrovitamin D3 / 11-(3-acetoxy-1-propynyl)-1alpha,25-dihydroxy-9,11-didehydrocholecalciferol | Vitamin D2 and derivatives | neg |
| 597.3098 | 398.76 | 1.64 | C34H50O4 | KCl | 1alpha-hydroxy-18-[m-(1-hydroxy-1-ethylpropyl)-benzyloxy]-23,24,25,26,27-pentanorvitamin D3 / 1alpha-hydroxy-18-[m-(1-hydroxy-1-ethylpropyl)-benzyloxy]-23,24,25,26,27-pentanorcholecalciferol | Vitamin D2 and derivatives | pos |
| **Prostaglandins** | | | | | | | |
| 349.1986 | 427.08 | 0.32 | C18H30O5 | Na | 2,3-dinor-6-keto-prostaglandin F1 a or related compounds | Prostanoids | pos |
| **365.1939** | **410.46** | **0.99** | **C18H30O6** | **Na** | **2,3-dinor, 6-keto-PGF1alpha;"2,3-Dinor-TXB2** | **Prostaglandins** | **pos** |
| **375.2142** | **446.22** | **4.34** | **C20H32O5** | **Na** | **13,14-Dihydro-15-keto-PGE2;20-Hydroxy-leukotriene B4;Prostaglandin E2;Thromboxane A2** | **Prostanoids** | **pos** |
| **373.1998** | **465.72** | **4.80** | **C20H32O5** | **Na** | **(13E)-11alpha-Hydroxy-9,15-dioxoprost-13-enoate or related compounds** | **Prostanoids** | **neg** |
| **391.2089** | **429.00** | **0.92** | **C20H32O6** | **Na** | **11-Dehydro-thromboxane B2** | **Prostanoids** | **pos** |
| **393.2247** | **425.28** | **0.13** | **C20H34O6** | **Na** | **10,11-dihydro-20-dihydroxy-LTB4;19(R)-hydroxy-PGE1 or related compounds** | **Prostaglandins** | **pos** |
| 341.2686 | 503.40 | 0.12 | C20H36O4 | H | 11-deoxy-PGF1a;11-deoxy-PGF1b;15S-HpEDE;PGE1 alcohol;PGF2alpha alcohol | Prostaglandins | pos |
| **353.2385** | **568.92** | **0.76** | **C20H40** | **KCl** | **8-Isoprostane** | **Prostanoids** | **neg** |
| **368.2793** | **459.72** | **0.71** | **C21H34O4** | **NH3** | **11-deoxy-11-methylene-PGD2 or related compounds** | **Prostaglandins** | **pos** |
| **351.2529** | **487.80** | **0.31** | **C21H34O4** | **H** | **3alpha,11beta,17alpha-Trihydroxy-5beta-pregnan-20-one or related compounds** | **Prostaglandins** | **pos** |
| 426.2052 | 581.94 | 1.13 | C23H33NO4 | K | 17-phenyl-trinor-PGF2alpha amide | Prostaglandins | pos |
| 517.2413 | 520.02 | 3.48 | C23H40O7 | Na_HCOONa | 1(3)-glyceryl-PGF2alpha;2-glyceryl-PGF2alpha | Prostaglandins | neg |
| 428.3008 | 429.00 | 0.37 | C23H41NO6 | H | PGF2alpha-dihydroxypropanylamine | Prostaglandins | pos |
| 554.2462 | 445.32 | 0.97 | C23H41NO6 | NaCl_HCOONa | PGF2alpha-dihydroxypropanylamine | Prostaglandins | pos |
| **Leukotrienes** | | | | | | | |
| **440.2449** | **438.66** | **1.32** | **C23H37NO5S** | **H** | **11-trans-LTE4;Leukotriene E4** | **Leukotrienes** | **pos** |
| **462.2284** | **440.52** | **0.19** | **C23H37NO5S** | **Na (Na)** | **(7E,9E,11Z,14Z)-(5S,6R)-6-(cystein-S-yl)-5-hydroxyicosa-7,9,11,14-tetraenoate";11-trans-LTE4;Leukotriene E4;** | **Leukotrienes** | **pos/neg** |
| 442.2621 | 446.22 | 0.18 | C23H39NO5S | H | Leukotriene E3 | **Leukotrienes** | pos/neg |
| 517.2351 | 429.72 | 2.20 | C25H40N2O6S | Na | 11-trans-LTD4;"14,15-LTD4";Leukotriene D4 | Leukotrienes | neg |

**Porphyrins**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 580.1019 | 468.66 | 0.90 | C25H27N3O6 | NaClx2 | Biotripyrrin-a;Biotripyrrin-b | Porphyrins | neg |
| 589.2993 | 585.48 | 1.63 | C33H40N4O6 | H | D-Urobilin | Porphyrins | pos |
| 569.3151 | 377.16 | 4.99 | C34H40N4O4 | H | Adouetine Y;Crenatine A;Protoporphyrinogen IX | Porphyrins | pos |
| 567.2999 | 551.40 | 4.00 | C34H40N4O4 | H | Adouetine Y;Crenatine A;Protoporphyrinogen IX | Porphyrins | neg |

**Isoprenoids**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 285.2210 | 448.14 | 0.95 | C20H28O | H | retinal; 11-cis-3,4-didehydroretinol or related compounds | isoprenoids | pos |
| 653.3361 | 379.14 | 2.77 | C40H50O4 | NaCl | 4-Keto-4'-hydroxyalloxanthin; 7,8-Didehydroastaxanthin | Isoprenoids | pos |
| 679.3389 | 417.72 | 2.49 | C40H52O4 | HCOOK | 3R,3'S-astaxanthin or related compounds | Isoprenoids | neg |
| 655.3396 | 411.78 | 2.37 | C40H52O5 | Na_Na | 4-Oxomytiloxanthin | Isoprenoids | neg |
| 657.3676 | 387.00 | 0.82 | C40H54O4 | NaCl | 4,4'-Dihydroxydiatoxanthin;4-Ketomyxol;7-Methylhypoxanthine;Halocynthiaxanthin;Mytiloxanthin | Isoprenoids | pos |
| **641.3521** | **571.62** | **1.93** | **C40H56O2** | **KCl** | **(3R,3'R,13-cis)-b,b-Carotene-3,3'-diol or related compounds** | isoprenoids | **neg** |
| **637.4225** | **657.84** | **3.09** | **C40H56O2** | **HCOONa** | **Lutein** | isoprenoids | **pos** |
| 643.3900 | 614.88 | 1.76 | C40H56O3 | NaCl | Antheraxanthin;Caloxanthin;Capsanthin;Cucurbitaxanthin A;Deepoxysalmoxanthin;Flavoxanthin;Loroxanthin/ 19-Hydroxylutein;"Lutein 5,6-epoxide";Micromonal;Myxol;; | Isoprenoids | pos |
| 623.3976 | 613.92 | 2.73 | C41H58O | NaCl | Anhydrorhodovibrin | isoprenoids | neg |
| 789.4253 | 572.34 | 0.10 | C46H68O6 | KCl | 1'-Hydroxy-gamma-carotene glucoside or related compounds | Isoprenoids | neg |

**Sulphates/Sulphones**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **321.0765** | **384.06** | **HCOOH** | **C10H16N2O5S** | **HCOOH** | **Biotin sulfone** | **Biotin and Derivatives** | **neg** |
| **413.1370** | **464.40** | **0.29** | **C19H28O5S** | **Na_Na** | **DHEA sulfate; Testosterone sulfate** | **Steroids and Steroid Derivatives** | **pos** |
| **367.1576** | **467.94** | **2.37** | **C19H28O5S** | **H** | **3beta-Hydroxyandrost-5-en-17-one 3-sulfate; Testosterone sulfate** | **Steroids and Steroid Derivatives** | **neg** |
| 735.3234 | 467.94 | | C19H28O5S | H | 3beta-Hydroxyandrost-5-en-17-one 3-sulfate; Testosterone sulfate | Steroids and Steroid Derivatives | neg |
| **425.1174** | **468.66** | **4.00** | **C19H28O5S** | **NaCl** | **DHEA sulfate; Testosterone sulfate** | **Steroids and Steroid Derivatives** | **neg** |
| **435.1454** | **471.30** | **1.13** | **C19H28O5S** | **HCOONa** | **DHEA sulfate; Testosterone sulfate** | **Steroids and Steroid Derivatives** | **neg** |
| **435.1451** | **503.46** | **1.70** | **C19H28O5S** | **HCOONa** | **(15S)-15-Hydroxy-5,8,11-cis-13-trans-eicosatetraenoate or related compounds** | **Steroids and Steroid Derivatives** | **neg** |
| **367.1579** | **504.24** | **2.21** | **C19H28O5S** | **H** | **DHEA sulfate; Testosterone sulfate** | **Steroids and Steroid Derivatives** | **neg** |
| **367.1581** | **535.74** | **1.72** | **C19H28O5S** | **H** | **3beta-Hydroxyandrost-5-en-17-one 3-sulfate; Testosterone sulfate** | **Steroids and Steroid Derivatives** | **neg** |
| 383.1531 | 388.44 | 0.68 | C19H28O6S | H | 3b,16a-Dihydroxyandrostenone sulfate;;"(11Z)-8,18-ethanoretinal";14'-apo-beta-Carotenal;Desogestrel;; | Steroids and Steroid Derivatives | neg |
| 383.1531 | 420.60 | 0.60 | C19H28O6S | H | 3b,16a-Dihydroxyandrostenone sulfate | Steroids and Steroid Derivatives | neg |
| 383.1528 | 466.86 | 1.41 | C19H28O6S | H | 3b,16a-Dihydroxyandrostenone sulfate | Steroids and Steroid Derivatives | neg |
| **437.1605** | **442.08** | **2.31** | **C19H30O5S** | **HCOONa** | **Androsterone sulfate** | **Steroids and Steroid Derivatives** | **neg** |
| **369.1734** | **487.26** | **0.57** | **C19H30O5S** | **H** | **5a-Dihydrotestosterone sulfate;5alpha-dihydrotestosterone sulfate;Androsterone sulfate** | **Steroids and Steroid Derivatives** | **neg** |
| **437.1609** | **490.08** | **1.53** | **C19H30O5S** | **HCOONa** | **5a-Dihydrotestosterone sulfate;5alpha-dihydrotestosterone sulfate;Androsterone sulfate** | **Steroids and Steroid Derivatives** | **neg** |
| **369.1735** | **520.02** | **0.81** | **C19H30O5S** | **H** | **5a-Dihydrotestosterone sulfate;5alpha-dihydrotestosterone sulfate;Androsterone sulfate** | **Steroids and Steroid Derivatives** | **neg** |
| **369.1734** | **679.98** | **0.41** | **C19H30O5S** | **H** | **5a-Dihydrotestosterone sulfate;5alpha-dihydrotestosterone sulfate;Androsterone sulfate** | **Steroids and Steroid Derivatives** | **neg** |
| **445.1200** | **734.16** | **1.26** | **C19H30O5S** | **KCl** | **Androsterone sulfate** | **Steroids and Steroid Derivatives** | **pos** |
| 395.1893 | 509.04 | 0.68 | C21H32O5S | H | Pregnenolone sulfate | Steroids and Steroid Derivatives | neg |
| **512.2681** | **495.66** | **1.29** | **C26H43NO7S** | **H** | **Sulfoglycolithocholate;Sulfolithocholylglycine;;** | **Bile Acids** | **neg** |
| 506.2895 | 384.24 | 3.02 | C26H45NO5S | Na (HCOONa) | Lithocholyltaurine;Taurolithocholate | Bile Acids | pos/neg |
| **498.2893** | **444.18** | **3.23** | **C26H45NO6S** | **H** | **Tauroursodeoxycholic acid** | **Bile Acids** | **neg** |
| **522.2864** | **476.10** | **0.92** | **C26H45NO6S** | **Na** | **chenodeoxycholoyltaurine;Taurochenodeoxycholate;Taurodeoxycholic acid;Tauroursodeoxycholic acid** | **Bile acids and derivatives** | **pos/neg** |
| **498.2887** | **476.70** | **4.40** | **C26H45NO6S** | **H** | **Taurochenodesoxycholic acid** | **Bile Acids** | **neg** |
| **514.2834** | **444.18** | **1.93** | **C26H45NO7S** | **H** | **Taurocholic acid** | **Bile Acids** | **neg** |
| **552.2423** | **75.12** | **3.71** | **C26H45NO7S** | **K** | **Tauallocholic acid;Tauro-b-muricholic acid;Taurocholate;Taurohyocholate;Tauroursocholic acid;;Aldosterone 18-glucuronide** | **Bile Acids** | **neg** |
| 465.3037 | 648.36 | 0.99 | C27H46O4S | H | Cholesterol sulfate | Steroids and Steroid Derivatives | neg |
| 519.2527 | 484.80 | 4.80 | C27H46O5S | K | 26-hydroxycholesterol 3-sulfate | Steroids and Steroid Derivatives | neg |
| 481.2987 | 568.92 | 1.23 | C27H46O5S | H | 26-hydroxycholesterol 3-sulfate | Steroids and Steroid Derivatives | neg |

**Glucuronides**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **465.2494** | **573.78** | **2.36** | **C25H36O8** | **H** | **Testosterone glucuronide** | **Glucuronides** | **pos** |
| **465.2487** | **456.72** | **1.53** | **C25H38O8** | **H** | **Androsterone glucuronide** | **Glucuronides** | **neg** |
| 481.2436 | 423.24 | 0.46 | C25H38O9 | H | 11beta-hydroxyandrosterone-3glucuronide | Glucuronides | neg |
| 467.2646 | 443.16 | 0.86 | C25H40O8 | H | 17-hydroxyandrostane-3-glucuronide;18-acetoxy-PGF2alpha-11-acetate methyl ester;"3,17-Androstanediol glucuronide";3-alpha-androstanediol glucuronide | Glucuronides | neg |
| **554.2581** | **76.32** | **2.63** | **C27H36O11** | **NH3** | **Aldosterone 18-glucuronide** | **Glucuronides** | **pos** |
| 563.2459 | 455.88 | 0.64 | C29H38O11 | H | Eriocarpin | Glucuronides | pos |
| 613.3206 | 561.06 | 0.16 | C30H48O10 | HCOOH | "(3a,5b,7a)-23-carboxy-7-hydroxy-24-norcholan-3-yl-b-D-Glucopyranosiduronic acid";6alpha-Glucuronosylhyodeoxycholate;Deoxycholic acid 3-glucuronide | Glucuronides | neg |
| 609.3400 | 486.84 | 1.36 | C34H52O8 | Na | 25-hydroxyvitamin D2 25-(beta-glucuronide) / 25 hydroxyergocalciferol 25-(beta-glucuronide);25-hydroxyvitamin D2-25-glucuronide;25-hydroxyvitamine D2 25-(beta-glucuronide) | Glucuronides | neg |

**Sphingoid/Sphingolipid**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 226.2170 | 504.24 | 2.61 | C14H29NO | H | Halaminol A | Sphingoid bases | neg |
| 266.2093 | 513.24 | 0.94 | C14H29NO2 | Na | (4E,d14:1) sphingosine;2-amino-tetradecanoic acid | Sphingoid bases | pos |
| 310.2354 | 507.36 | 0.42 | C16H33NO3 | Na | Prosopinine | Sphingoid bases | pos |
| 368.2192 | 577.68 | 4.45 | C17H35NO2 | HCOOK | Anteiso (4E,14-methyl-d17:1) sphingosine";C17 Sphingosine;"iso (4E,15-methyl-d17:1) sphingosine" | Sphingoid bases | neg |
| 288.2898 | 458.88 | 0.45 | C17H37NO2 | H | C17 Sphinganine | Sphingoid bases | pos |
| **322.2719** | **474.30** | **0.78** | **C18H37NO2** | **Na** | **Sphinganine** | **Sphingoid bases** | **pos** |
| **322.2716** | **597.24** | **0.03** | **C18H37NO2** | **Na** | **Sphinganine** | **Sphingoid bases** | **pos** |
| **322.2719** | **474.30** | **0.78** | **C18H37NO2** | **Na** | **Sphinganine** | **Sphingoid bases** | **pos** |
| **322.2716** | **597.24** | **0.03** | **C18H37NO2** | **Na** | **Sphinganine** | **Sphingoid bases** | **pos** |
| 338.2665 | 450.12 | 0.21 | C18H37NO3 | Na | 4-hydroxysphingosine;6-hydroxysphingosine | Sphingoid bases | pos |
| 286.3106 | 476.10 | 0.42 | C18H39NO | H | Spisulosine | Sphingoid bases | pos |
| **302.3052** | **468.42** | **0.46** | **C18H39NO2** | **H** | **Sphinganine** | **Sphingoid bases** | **pos** |
| **302.3052** | **468.42** | **0.46** | **C18H39NO2** | **H** | **Sphinganine** | **Sphingoid bases** | **pos** |
| 318.3001 | 448.14 | 0.38 | C18H39NO3 | H | 4-hydroxysphinganine;Phytosphingosine | Sphingoid bases | pos |
| 314.3418 | 499.56 | 0.22 | C20H43NO | H | 2-amino-14,16-dimethyloctadecan-3-ol | Sphingoid bases | pos |
| 402.2855 | 410.46 | 1.14 | C21H39NO6 | H | Myriocin;Sphingofungin F | Sphingoid bases | pos |
| 438.3545 | 528.24 | 1.92 | C23H47NO2 | HCOONa | Choline stearate;Penazetidine A | Sphingoid bases | pos |
| 552.3096 | 648.06 | 4.18 | C24H47NO7 | Na_HCOONa | Galactosylsphingosine;Glucosylsphingosine | Sphingolipids | pos |
| 414.2999 | 476.10 | 4.66 | C26H36O3 | NH3 | Estradiol 17beta-cyclopentylpropionate;Estradiol cypionate (USP) | Sphingoid bases | pos |
| 563.3770 | 489.78 | 4.53 | C30H52N2O2 | Na_HCOONa | Leucettamol A | Sphingoid bases | pos |
| 534.3213 | 402.66 | 4.32 | C31H45NO5 | Na | Scyphostatin A | Sphingoid bases | pos |
| 560.5014 | 775.32 | 0.20 | C34H67NO3 | Na | N-Palmitoylsphingosine | Sphingolipids | pos |
| 562.5176 | 739.08 | 1.14 | C34H69NO3 | Na | Cer(d18:0/16:0) | Sphingolipids | pos |
| 722.5523 | 653.88 | 2.57 | C40H77NO8 | Na | Galactosylceramide (d18:1/16:0);GlcCer(d18:1/16:0);Glucosylceramide (d18:1/16:0) | Sphingolipids | pos |
| 722.5523 | 687.24 | 2.57 | C40H77NO8 | Na | Galactosylceramide (d18:1/16:0);GlcCer(d18:1/16:0);Glucosylceramide (d18:1/16:0) | Sphingolipids | pos |
| 722.5522 | 717.54 | 2.66 | C40H77NO8 | Na | Galactosylceramide (d18:1/16:0);GlcCer(d18:1/16:0);Glucosylceramide (d18:1/16:0) | Sphingolipids | pos |
| 722.5523 | 748.86 | 2.57 | C40H77NO8 | Na | Galactosylceramide (d18:1/16:0);GlcCer(d18:1/16:0);Glucosylceramide (d18:1/16:0) | Sphingolipids | pos |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 906.6986 | 739.08 | 2.01 | C50H95NO8 | HCOONa | Galactosylceramide (d18:1/26:1(17Z));Glucosylceramide (d18:1/26:1(17Z)) | Sphingolipids | pos |
| **Glycerolipids** | | | | | | | |
| 381.2979 | 691.08 | 0.97 | C21H42O4 | Na | MG(18:0/0:0/0:0) | Glycerolipids | pos |
| 396.3105 | 478.02 | 0.76 | C23H38O4 | NH3 | 1-Arachidonoylglycerol or related compounds | Glycerolipids | pos |
| 401.2661 | 573.78 | 0.57 | C23H38O4 | Na | 2-Arachidonoylglycerol or related compounds | Glycerolipids | pos |
| 430.3891 | 528.90 | 0.00 | C25H48O4 | NH3 | MG(0:0/22:1(13Z)/0:0);MG(22:1(13Z)/0:0/0:0) | Glycerolipids | pos |
| 471.3659 | 694.98 | 4.50 | C27H50O6 | H | Tricaprilin | Glycerolipids | pos |
| 599.4249 | 679.32 | 1.52 | C33H62O6 | Na_Na (NaCLx2) | TG(10:0/10:0/10:0)[iso] | Glycerolipids | pos/neg |
| **613.4246** | **656.88** | **1.09** | **C36H64O5** | **K** | **DG(15:0/18:3(6Z,9Z,12Z)/0:0) or related compounds** | **Glycerolipids** | **neg** |
| 669.4655 | 790.86 | 3.17 | C36H66O5 | Na_HCOONa | DG(15:0/18:2(9Z,12Z)/0:0) or related compounds | Glycerolipids | pos |
| 701.3729 | 569.76 | 4.65 | C37H62O5 | NaClx2 | DG(14:0/20:5(5Z,8Z,11Z,14Z,17Z)/0:0) or related compounds | Glycolipids | neg |
| 697.3989 | 582.54 | 1.23 | C40H64O5 | KCl | DG(17:2(9Z,12Z)/20:5(5Z,8Z,11Z,14Z,17Z)/0:0)[iso2] | Diacylglycerols | neg |
| 713.4774 | 464.94 | 1.37 | C40H70O5 | HCOOK | DG(15:0/22:4(7Z,10Z,13Z,16Z)/0:0) or related compounds | Glycolipids | neg |
| 723.5552 | 696.72 | 2.28 | C40H78O5 | HCOOK | DG(15:0/22:0/0:0);DG(16:0/21:0/0:0)[iso2];DG(17:0/20:0/0:0)[iso2];DG(18:0/19:0/0:0)[iso2];DG(22:0/15:0/0:0) | Glycerolipids | pos |
| 780.5947 | 700.86 | 1.59 | C42H81NO7 | HCOONa | DGTS(16:0/16:0) | Glycerolipids | pos |
| 780.5947 | 736.14 | 1.59 | C42H81NO7 | HCOONa | DGTS(16:0/16:0) | Glycerolipids | pos |
| 780.5950 | 767.46 | 1.28 | C42H81NO7 | HCOONa | DGTS(16:0/16:0) | Glycerolipids | pos |
| 795.5443 | 550.38 | 4.45 | C43H82O5 | NaClx2 | DG(16:0/24:1(15Z)/0:0) or related compounds | Glycerolipids | pos |
| 795.5430 | 636.36 | 2.84 | C43H82O5 | NaClx2 | DG(16:1(9Z)/24:0/0:0) or related compounds | Glycerolipids | pos |
| 795.5431 | 775.32 | 2.99 | C43H82O5 | NaClx2 | DG(18:0/22:1(13Z)/0:0) or related compounds | Glycerolipids | pos |
| 781.5978 | 769.44 | 3.33 | C46H80O5 | HCOONa | DG(21:0/22:5(7Z,10Z,13Z,16Z,19Z)/0:0)[iso2] | Diacylglycerols | pos |
| 791.5926 | 550.38 | 0.19 | C49H84O5 | K | DG(22:5(4Z,7Z,10Z,13Z,16Z)/24:1(15Z)/0:0) or related compounds | Glycerolipids | pos |
| 911.6267 | 630.54 | 2.72 | C54H92O6 | KCl | TG(16:1(9Z)/17:2(9Z,12Z)/18:3(9Z,12Z,15Z))[iso6];TG(17:2(9Z,12Z)/17:2(9Z,12Z)/17:2(9Z,12Z)) | Triacylglycerols | pos |
| 911.6267 | 710.70 | 2.72 | C54H92O6 | KCl | TG(16:1(9Z)/17:2(9Z,12Z)/18:3(9Z,12Z,15Z))[iso6];TG(17:2(9Z,12Z)/17:2(9Z,12Z)/17:2(9Z,12Z)) | Triacylglycerols | pos |
| 945.7081 | 775.32 | 0.73 | C56H102O6 | KCl | TG(16:0/17:0/20:3(8Z,11Z,14Z))[iso6] or related compounds | Triacylglycerols | pos |
| 963.7382 | 771.36 | 0.56 | C62H100O6 | Na | TG(17:0/20:4(5Z,8Z,11Z,14Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[iso6] or related compounds | Triacylglycerols | pos |
| **Phospholipids** | | | | | | | |
| 443.2405 | 448.14 | 0.02 | C19H39O9P | H | PG(13:0/0:0) | glycerophosphates | pos |
| 454.2930 | 751.80 | 0.48 | C21H44NO7P | H | 1-16:0-lysoPE;PC(13:0/0:0);PE(16:0/0:0) | glycerophosphates | pos |
| 531.2572 | 575.70 | 1.36 | C22H43O12P | H | PI(13:0/0:0) | glycerophosphates | pos |
| 516.2876 | 444.18 | 2.79 | C24H50NO6P | K | LysoPC(dm16:0);PC(O-16:1(11Z)/0:0) or related compounds | glycerophosphocholines | neg |
| 617.2943 | 557.28 | 0.16 | C25H49O12P | HCOOH | PI(16:0/0:0) | glycerophosphates | neg |
| 616.2625 | 189.54 | 0.47 | C27H44NO7P | Na_HCOONa | LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z));LysoPE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0) | glycerophosphoethanolamines | pos |
| 543.3197 | 586.44 | 1.36 | C27H46NO7P | NH3 | LysoPE(0:0/22:5(4Z,7Z,10Z,13Z,16Z)) or related compounds | glycerophosphoethanolamines | neg |
| 691.3055 | 566.94 | 2.10 | C27H53O12P | Na_HCOONa (HCOOH) | PI(18:0/0:0) | glycerophosphates | pos/neg |
| 557.3354 | 598.26 | 1.26 | C28H48NO7P | NH3 | LysoPC(20:5(5Z,8Z,11Z,14Z,17Z)) | glycerophosphocholines | neg |
| 625.3041 | 420.18 | 1.28 | C28H55O8P | KCl | PA(12:0/13:0) | glycerophosphates | pos |
| 710.3937 | 603.12 | 1.77 | C33H66NO8P | KCl | PE(16:0/12:0), PC(10:0/15:0)[U] or related compounds | glycerophosphoethanolamines | pos |
| 712.3601 | 408.24 | 0.48 | C34H62NO10P | K | PS(14:1(9Z)/14:1(9Z)) | glycerophosphoserines | neg |
| 745.3991 | 571.62 | 1.14 | C37H69O8P | KCl | 16:0-18:2-PA;*PA(16:0/18:2(9Z,12Z)); Phosphatidyl ethanol | glycerophosphates | neg |
| 752.4725 | 679.32 | 4.28 | C37H76NO7P | KCl | PE((0-16:0/16:0), PC(O-14:0/15:0) or related compounds | glycerophosphoethanolamines | pos |
| 752.5147 | 542.64 | 3.89 | C38H78NO8P | Na_Na | PS(O-16:0/O-16:0)[U] or related compounds | glycerophosphoserines | pos |
| 729.3889 | 567.90 | 1.00 | C39H65O8P | K | PA(18:3(9Z,12Z,15Z)/18:3(9Z,12Z,15Z)) or related compounds | glycerophosphates | neg |
| 765.3679 | 463.14 | 3.71 | C39H65O8P | KCl | 18:3-18:3-PA or related compounds | glycerophosphates | neg |
| 733.5494 | 587.46 | 0.50 | C39H74NO8P | NH3 | PE(14:0/20:2(11Z,14Z)) or related compounds | glycerophosphoethanolamines | pos |
| 760.5789 | 648.12 | 4.89 | C39H82NO6P | HCOONa | PE-NMe2(O-16:0/O-16:0) | glycerophosphoethanolamines | pos |
| 790.4674 | 682.08 | 4.78 | C40H70NO8P | HCOONa | PC(14:1(9Z)/18:4(6Z,9Z,12Z,15Z));*PC(18:4(6Z,9Z,12Z,15Z)/14:1(9Z))*,*PE(15:0/20:5(5Z,8Z,11Z,14Z,17Z))*,*PE(20:5(5Z,8Z,11Z,14Z,17Z)/15:0) | glycerophosphocholines | neg |
| 796.4550 | 426.06 | 3.06 | C40H72NO10P | K | 16:0-18:3-PS | glycerophosphoserines | pos |
| 842.4243 | 550.44 | 4.96 | C40H74NO8P | NaClx2 | PC(14:0/18:3(6Z,9Z,12Z)); PE(15:0/20:3(5Z,8Z,11Z)) or related compounds | glycerophosphocholines | neg |
| 909.4085 | 551.34 | 4.39 | C40H75O13P | NaClx2 | PI(17:0/14:1(9Z)) | glycerophosphoinositols | neg |
| 753.4823 | 681.30 | 1.05 | C40H75O8P | K | PA(18:2(9Z,12Z)/19:0), 1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycero-3-phospho-(1'-sn-glycerol) | glycerophosphates | pos |
| 774.5945 | 683.22 | 4.92 | C40H84NO6P | HCOONa | PC(O-12:0/O-20:0)[U];PC(O-14:0/O-18:0)[U];PC(O-16:0/O-16:0);PC(O-20:0/O-12:0)[U] | glycerophosphocholines | pos |
| 738.5050 | 775.32 | 0.81 | C41H72NO8P | H | PE(14:0/22:5(4Z,7Z,10Z,13Z,16Z)) or related compounds | glycerophosphoethanolamines | pos |
| 743.5705 | 741.00 | 1.02 | C41H76NO7P | NH3 | PE(18:2(9Z,12Z)/dm18:1(11Z)) or related compounds | glycerophosphoethanolamines | pos |
| 796.5443 | 542.64 | 0.58 | C41H78NO7P | HCOONa | PE(18:1(11Z)/dm18:1(11Z)) or related compounds | glycerophosphoethanolamines | pos |
| 816.6057 | 701.88 | 3.93 | C42H86NO7P | HCOONa | PC(18:0/O-16:0) or related compounds | glycerophosphocholines | pos |
| 838.4536 | 476.94 | 3.05 | C43H76NO8P | KCl | 20:2-18:3-PE or related compounds | glycerophosphoethanolamines | neg |
| 789.6122 | 675.42 | 0.73 | C43H82NO8P | NH3 | PC(15:0/20:2(11Z,14Z)) or related compounds | glycerophosphoethanolamines | pos |
| 839.5638 | 474.30 | 0.64 | C43H83O13P | H | PI(16:0/18:0);PI(18:0/16:0) | glycerophosphoinositols | pos |
| 820.5991 | 585.48 | 4.16 | C43H88NO7P | NaCl | PC(O-16:0/19:0) or related compounds | glycerophosphoethanolamines | pos |
| 830.6205 | 634.44 | 4.78 | C43H88NO7P | HCOONa | PE(O-16:0/22:0) or related compounds | glycerophosphoethanolamines | pos |
| 830.6209 | 737.10 | 4.41 | C43H88NO7P | HCOONa | PE(O-18:0/20:0) or related compounds | glycerophosphoethanolamines | pos |
| 817.4409 | 571.86 | 3.56 | C44H71O9P | Na_Na | 1-(6-[5]-ladderane-hexanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycero-3-phospho-(1'-sn-glycerol) | glycerophosphoglycerols | neg |
| 833.4513 | 573.18 | 1.82 | C44H73O9P | NaCl | 1-(6-[3]-ladderane-hexanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycero-3-phospho-(1'-sn-glycerol) | glycerophosphoglycerols | neg |
| 834.6154 | 612.84 | 0.74 | C44H90NO7P | NaCl | PC(O-14:0/22:0);PC(O-16:0/20:0);PC(O-18:0/18:0);PC(O-20:0/16:0) | glycerophosphocholines | pos |
| 844.6361 | 699.90 | 4.87 | C44H90NO7P | HCOONa | PC(O-14:0/22:0);PC(O-16:0/20:0);PC(O-18:0/18:0);PC(O-20:0/16:0) | glycerophosphocholines | pos |
| 868.5003 | 477.06 | 3.15 | C45H82NO8P | KCl | PC(15:0/22:4(7Z,10Z,13Z,16Z)) or related compounds | glycerophosphoethanolamines | neg |
| 846.6154 | 657.84 | 4.80 | C45H90NO7P | NaCl | PE(22:0/dm18:0);PE(24:0/dm16:0);, | glycerophosphoethanolamines | pos |
| 809.6154 | 699.90 | 1.57 | C46H82NO7P | NH3 | PC(20:4(5Z,8Z,11Z,14Z)/dm18:1(11Z)) or related compounds | glycerophosphocholines | pos |
| 809.6156 | 748.86 | 1.35 | C46H82NO7P | NH3 | PC(20:4(5Z,8Z,11Z,14Z)/dm18:1(11Z)) or related compounds | glycerophosphocholines | pos |
| 905.5129 | 492.72 | 1.10 | C47H81O13P | Na | PI(16:0/22:5(4Z,7Z,10Z,13Z,16Z)) or related compounds | glycerophosphoinositols | neg |
| 908.7015 | 739.08 | 4.28 | C48H100NO6P | Na_HCOONa | 1,2-diphytanyl-sn-glycero-3-phosphocholine;PC(O-20:0/O-20:0) | glycerophosphocholines | pos |
| 848.6309 | 567.00 | 4.61 | C48H92NO6P | K | 1-(2E,6E-phytadienyl)-2-(2E,6E-phytadienyl)-sn-glycero-3-phosphocholine | glycerophosphocholines | pos |
| 848.6313 | 644.16 | 2.29 | C48H92NO6P | K | 1-(2E,6E-phytadienyl)-2-(2E,6E-phytadienyl)-sn-glycero-3-phosphocholine | glycerophosphocholines | pos |
| 899.6350 | 476.10 | 0.23 | C48H93O11P | Na | 1,2-ditetradecanoyl-sn-glycero-3-phospho-(2'-lyso-3'-tetradecanoyl -1'-sn -glycerol) | glycerophosphoglycerols | pos |
| 921.6165 | 476.10 | 0.17 | C48H93O11P | Na_Na | 1,2-ditetradecanoyl-sn-glycero-3-phospho-(2'-lyso-3'-tetradecanoyl -1'-sn -glycerol) | glycerophosphoglycerols | pos/neg |
| 888.6621 | 659.76 | 4.29 | C48H96NO7P | NaCl | PC(22:0/dm18:0); PC(24:0/dm16:0) | glycerophosphocholines | pos |
| 904.6939 | 769.38 | 4.70 | C49H100NO7P | NaCl | PC(O-19:0/22:0) | glycerophosphoethanolamines | pos |
| 866.6684 | 805.50 | 4.63 | C50H94NO8P | H | PC(18:2(9Z,12Z)/24:1(15Z)) or related compounds | glycerophosphocholines | neg |
| 980.6476 | 803.46 | 4.07 | C50H98NO7P | NaCl_HCOONa | PC(24:0/dm18:1(11Z)) or related compounds | glycerophosphocholines | neg |
| 964.7150 | 602.16 | 2.02 | C54H104NO8P | K | PC(22:1(13Z)/24:1(15Z)) or related compounds | glycerophosphocholines | pos |
| **Miscellaneous** | | | | | | | |
| **151.0478** | **385.80** | **0.13** | **C5H8N2O2** | **Na** | **5,6-Dihydrothymine** | **Pyrimidines and Derivatives** | **pos** |
| 167.0198 | 73.98 | 2.51 | C6H8N2O | Na_Na | Methylimidazole acetaldehyde;N-Propanoylimidazole | Aldehydes Imidazoles | neg |
| 255.9781 | 66.54 | 3.52 | C7H8NO5P | K | 3-[Phosphonomethyl]Pyridine-2-Carboxylic Acid | Pyridoxals and Derivatives | pos |
| 853.5855 | 538.68 | 1.96 | C54H82O4 | NaCl | Ubiquinone-9 | Quinones and Derivatives | pos |
| 853.5854 | 636.36 | 2.10 | C54H82O4 | NaCl | Ubiquinone-9 | Quinones and Derivatives | pos |
| 853.5857 | 712.62 | 1.75 | C54H82O4 | NaCl | Ubiquinone-9 | Quinones and Derivatives | pos |
| 627.3831 | 611.94 | 3.63 | C39H58O4 | K | Coenzyme Q6;ubiquinone-6 | Ubiquinones | neg |
| 511.2729 | 540.72 | 2.01 | C30H44O2 | KCl | 2-Phytyl-1,4-naphthoquinone;demethylphylloquinone | Naphthoquinones | pos/neg |
| 310.9307 | 65.58 | 1.80 | C3H7O7P; | NaCl_HCOONa | 2-Phosphoglyceric acid;3-Phospho-D-glycerate;D-Glycerate 2-phosphate | ;Acyl Phosphates | neg |
| 765.3972 | 443.04 | 4.49 | C40H68O7P2 | Na_Na | all-trans-Octaprenyl diphosphate;octaprenyl diphosphate; Prephytoene diphosphate | Acyl Phosphates | neg |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 308.0255 | 634.44 | 3.67 | C6H12N3O8P | Na | 2-(Formamido)-N1-(5-phospho-D-ribosyl)acetamidine | Sugar Phosphates | [redacted] |
| 687.3727 | 582.54 | 0.04 | C40H60O2 | NaCl×2 | 5,6-dihydro-5,6-dihydroxy-y,y-Carotene or related compounds | Alcohols and Polyols | [redacted] |
| 611.4239 | 530.82 | 2.32 | C40H60O2 | K | Dihydroparasiloxanthin/ 7,8,7',8'-Tetrahydrozeaxanthin or related compounds | Alcohols and Polyols | [redacted] |
| 611.4236 | 788.94 | 1.82 | C40H60O2 | K | OH-Demethylspheroidene or related compounds | Alcohols and Polyols | [redacted] |
| 368.9881 | 444.48 | 2.22 | C14H6O8 | HCOONa | Ellagic acid | Polyphenols | [redacted] |
| 293.1755 | 486.84 | 0.96 | C16H24O2 | HCOOH | 2E,6Z,8Z,12E-hexadecatetraenoic acid or related compounds | Polyphenols | [redacted] |
| 488.2680 | 475.26 | 0.08 | C18H37N9O4 | Na_Na | dynorphin A (6-8) | Solanidines and alkaloid derivatives | [redacted] |
| 416.3524 | 408.48 | 0.24 | C27H45NO2 | H | 22-iso-teinemine or related compounds | Solanidines and alkaloid derivatives | [redacted] |
| 581.4545 | 633.30 | 3.08 | C36H64O4 | Na | 2-methylbacteriohopane-32,33,34,35-tetrol | Hopanoids | [redacted] |
| 728.5527 | 646.14 | 2.81 | C40H73N3O4 | HCOONa | N-ornithinyl-35-aminobacteriohopane-32,33,34-triol | Hopanoids | [redacted] |