



Mass spectrometric proteomic profiling of acute wound healing processes in the porcine model

by

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Abstract

Wounds are a common occurrence and generally heal well within a short time depending on whether tissue needs to be replaced or not. Although there is an understanding of the physiological processes taking place during wound healing, the changes taking place at the molecular level as well as the causes of chronic wounds are not well defined.

The purpose of this study was a proof of concept making use of complementary current mass spectrometric techniques to better define the spatial and kinetic changes occurring during healing of well-defined acute wounds across proteomic, metabolomic and lipidomic bases. Several wound tissue preparation techniques, analytical approaches, data conversion methodologies and statistical tools were assessed to best visualise, characterise and identify molecular features in the complex and continuously changing environment of the acute wound at six pre-selected time points over 16 days of healing. Different mass spectrometric approaches to analyse snap frozen histological sections of wound tissue collected from a porcine wound healing model allowed 'omic' analyses to provide a better understanding of the acute wound healing process. Unidentified features found at key time points were compared to provide a foundation to develop an acute wound healing model, which could be applied to non-healing chronic wounds.

An initial animal study involved full-skin thickness wounds being surgically created in the porcine model using strictly controlled wound formation and the subsequent healing thereof accurately investigated. Photographic and histological analyses of resected wound tissue were used to track the sequential yet overlapping phases of the acute wound healing pathway. The spatial resolution capabilities of matrix-assisted laser desorption ionisation imaging mass spectrometry (MALDI-IMS) was linked to histological staining and localised healthy reference tissue to successfully characterise changes in wound tissue. Optimised multivariate analyses and statistical analysis could statistically characterise the kinetic changes and revealed molecular features and key time points during acute wound healing from proteomic, metabolomic and lipidomic assay data collected individually from sequential tissue sections from the same wound.

Methods for extraction of soluble protein from histological wound tissue sections were compared and further analysed using hydrophilic interaction-based bead capture with in-solution tryptic digestion followed by classic proteomic liquid chromatography tandem mass spectrometry (ESI-LC-MS/MS) analysis. The extracted proteins were also assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis where protein mass fingerprints were visualised and major protein bands excised, followed by standard in-gel digestion and LC-ESI-MS/MS analysis. All mass spectral proteomic data was subject to bioinformatic analysis using open source packages.

A comparative analysis of in-solution versus on-tissue sample preparation was performed to determine optimal methodology for tissue protein extraction and identification. The optimised protocol was used to identify proteins across six time points and 16 days showing potential as predictive acute wound healing phase features.

A total of 18 proteins showed potential as acute wound healing phase markers through relative quantitative change and functional healing relation. Biological functions of these proteins included involvement in adaptive and innate immunity, inflammation, cellular adhesion and various biosynthetic processes, and may be used as validating features of acute wound healing in clinical settings or as new drug targets for healing therapies.

This study revealed the best time range and individual features that could be used to most accurately represent essential molecular environmental changes in the progression of healing of acute wounds across proteomic, metabolomic and lipidomic bases. These approaches may aid in comparative healing studies, such as those seeking to better define differences that can be linked to chronic wounds. Using a well-defined but reduced sample set of acute wound molecular environment data as a fixed comparable model, as well as potential markers of successful healing, may simplify the approach and aid in the identification of potential drug targets in non-healing chronic wounds.

Keywords: Acute wound healing, porcine model, MALDI-IMS, LC-MS/MS, proteomics, metabolomics, lipidomics, multivariate statistical analysis, in-solution analysis, on-tissue analysis, bioinformatics.

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“My definition of an expert in any field is a person who knows enough about what’s really going on to be scared.” – P.J. Plauger

Declaration

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Declaration

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Glossary of Abbreviations

°C	Degrees Celsius
µg/µl	Micrograms per microlitre
µl	Microlitre
µm	Micrometre
%	Percent
2D	Two-dimensional
3D	Three-dimensional
AD	Anderson-Darling
AGC	Automatic gain control
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAS	Central Animal Service
CHCA	α-cyano-4-hydroxycinnamic acid
CHD-FA	Carbohydrate-derived fulvic acid
CID	Collision-induced dissociation
CuSO ₄	Cupric sulfate
CV	Coefficient of variation
D Ave	Average difference
Da	Dalton
DDA	Data-dependent acquisition
DFUs	Diabetic foot ulcers
DHB	2,5-dihydroxybenzoic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESI	Electrospray ionisation
FDR	False discovery rate
FFPE	Formalin-fixed paraffin embedded
FGF	Fibroblast growth factor
FTICR	Fourier transform ion cyclotron resonance
g	Grams
<i>g</i>	Gravity
g/l	Grams per litre
GO	Gene Ontology
GP	Glycoprotein
GS/s	Giga samples per second

H&E	Haematoxylin and eosin
HCD	Higher-energy collisional dissociation
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
IFN	Interferon
IL	Interleukin
IMS	Imaging mass spectrometry
kDa	Kilodalton
kg	Kilograms
kHz	Kilohertz
kV	Kilovolts
l	Litre
LC-ESI-MS/MS	Liquid chromatography tandem mass spectrometry
LIT	Linear ion trap
M	Molar (moles per litre)
m	Metres
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption ionisation
mg	Milligrams
mg/ml	Milligrams per millilitre
mgf	Mascot generic format
ml	Millilitre
mM	Millimolar
MMPs	Matrix metalloproteinases
MPs	Membrane-bound proteins
MS	Mass spectrometry
ms	Milliseconds
$\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O}$	Disodium tartrate dihydrate
$\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$	Sodium carbonate decahydrate
NaHCO_3	Sodium bicarbonate
NaOH	Sodium hydroxide
NHLS	National Health Laboratory Service
nl	Nanolitre
nm	Nanometre
OCT	Optimal cutting temperature
PAS	Periodic Acid Schiff

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCs	Principal components
PDGF	Platelet-derived growth factor
pH	Negative logarithm of the hydrogen ion concentration
PKW	Kruskal-Wallis p-value
PR	Picrosirius red
PSMs	Peptide to spectrum matches
Q-TOF	Quadrupole time-of-flight
ROIs	Regions of interest
SA	Sinapinic acid
SC	Single contribution
SDS	Sodium dodecyl sulfate
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIS	Skin immune system
SWR	Standard working reagent
TC	Total contribution
TFA	Trifluoroacetic acid
TGF- β	Transforming growth factor-beta
TGX	Tris-Glycine eXtended™
TNF- α	Tumour necrosis factor-alpha
TOF	Time-of-flight
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UV	Ultraviolet
V	Volts
VEGF	Vascular endothelial growth factor
vs	Versus

Glossary of Terms for MALDI-IMS Analysis

Note: Descriptions of these terms were copied and adapted from:

“Bruker Daltonics: ClinProTools Version 2.2, User Manual” (November 2007).

Part #: 249619, First edition: June 2004. Printed in Germany.

- **2D Peak distribution plots**

2D peak distribution plots display the distribution of two selected peaks (features) in the spectra of loaded data classes. Data is shown on a two-dimensional plane with feature numbers and m/z values indicated on the x- and y-axes. The x- and y- axes show the feature intensity values with respect to the first and second most important features based on the sort criterion e.g. its p-value, difference average etc., respectively. All data points belonging to the same data class are displayed with the same coloured symbol e.g. red cross (class 1 spectra), green diamond (class 2 spectra) etc. 2D peak distribution plots do not show averaged peaks but peaks from all spectra. The ellipses represent the standard deviation of the class average of the peak intensities.

- **Influence plots**

The influence plot shows which influence a spectrum has on the current PCA model. Each data point in this plot represents a spectrum included in the PCA model. The influence plot is a diagnostic tool for the identification of outliers.

- **Loading plots**

During calculations for principal component analysis, the variables (features) obtain different loadings in dependence on their contribution to the explained variance of a principal component. The values for the loadings are between -1 and 1, where a value of '0' shows that the respective variable has no influence on the variability of the principal component. Within the loading plots each data point represents one peak and each plot contains as many points as spectra in the used dataset.

- **Principal component analysis (PCA) plots**

The PCA output represents the original data mapped into a new coordinate system which is defined by principal components (PCs). Within the PCA plots each data point represents one spectrum and each plot contains as many points as spectra in the used dataset. PCA plots show how the spectra are distributed in the corresponding sub-space determined by the selected PCs, and visualise the relationship between different spectra, e.g. whether different groups are separated from each other or which spectra may be outliers.

- **Pseudo-gels**

Pseudo-gels display all spectra of the loaded sample classes. The x-axis records the m/z value. The left y-axis displays the running spectrum number originating from subsequent spectra loading. The feature intensity is expressed by a colour code. The colour bar and the right y-axis indicate the relation between the colour a feature is displayed with and the feature intensity in arbitrary units.

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Chapter 1: Introduction

1.1 Literature Review

1.1.1 Introduction

A wound by definition occurs when the integrity and protective function of the skin is compromised with disruption that may extend from the epithelium into underlying tissues (*i.e.* connective tissue, nerves, muscle or bone) following injury caused by a number of possible factors including physical force, extreme temperatures, chemicals, surgery or disease (Epstein *et al.*, 1999, Leaper and Harding, 1998). Wounds are classified according to various criteria (Robson *et al.*, 2001) where healing time impacts on injury management and extent of wound repair. Wounds are clinically classified as acute or chronic dependent on the time frame of healing (Lazarus *et al.*, 1994, Velnar *et al.*, 2009).

Acute wounds are defined as those wounds that undergo self-repair in a controlled and timely manner by proceeding through the normal and predictable phases of wound healing being the haemostasis, inflammatory, proliferative and remodelling phases. Completion of this healing pathway then ultimately results in the restoration of both anatomical and functional parameters at the wound site. Chronic wounds, such as diabetic foot ulcers, do not progress through the controlled and timely phases of normal wound healing and do not heal in an orderly manner. This is the result of various factors that prolong one or more phases during wound healing, ultimately leading to poor anatomical and functional outcomes where wounds frequently relapse (Degreef, 1998, Robson *et al.*, 2001). Numerous studies have provided a substantial understanding of the healing process however, this complex process of tissue repair is yet to be completely defined (Demidova-Rice *et al.*, 2012, Guo and DiPietro, 2010, Landén *et al.*, 2016, Minutti *et al.*, 2017, Velnar *et al.*, 2009).

Mass spectrometry offers powerful molecular analysis of small samples with high sensitivity and accuracy, where unique precursor mass and fragmentation patterns can identify many different molecules simultaneously. Although most mass spectrometry techniques use homogenised tissue extracts resulting in averaged tissue molecular content, Matrix Assisted Laser Desorption Ionisation Imaging Mass Spectrometry (MALDI-IMS) allows for direct intact tissue analysis to provide added

spatial distribution data with potential for identification of thousands of molecules within a single sample. Combining mass spectrometry techniques allows for the tracking of physiological changes at a molecular level and provides an ideal basis for kinetic 'omic'-type analyses of complex processes such as wound healing (Norris and Caprioli, 2013, Schober *et al.*, 2011, Taverna *et al.*, 2014).

By better defining the acute wound healing process, suitable key kinetic features may be revealed that can be used as therapeutic targets and in turn be applied to non-healing, chronic wounds such as lower leg ulcers or diabetic foot ulcers (DFUs). These chronic wounds affect tens of millions of people globally each year causing significant morbidity and mortality (Ogurtsova *et al.*, 2017). No reliable therapy currently exists for the comprehensive treatment of chronic wounds and there is an urgent need to discover and identify new key therapeutic targets for such treatment (Eming *et al.*, 2010, Grey *et al.*, 2009b). Utilising current mass spectrometric techniques to characterise the kinetics of controlled acute wound healing holds promise for identifying such targets for improved treatment options and strategies in wound care.

1.1.2 The skin

The integument, comprising the skin in mammals, is the largest organ of the body with an estimated total weight of 5 kg in adult humans. There are at least five different resident cells types present in the skin's structure with various immune and circulatory system cells moving through the skin at any given time (Elias, 2007, Menon, 2002). The skin performs many critical functions to maintain homeostasis. The primary function of the skin is to form a barrier between the external environment and the inner organism, where it provides major physical, chemical, immunological, ultra violet radiation and free radical defences to prevent injury and/or infection from occurring (Menon, 2002, Summerfield *et al.*, 2015). A skin immune system or 'SIS' has been described, where resident immune and epithelial cells provide tolerance and immunity to various antigens (Di Meglio *et al.*, 2011, Heath and Carbone, 2013, Summerfield *et al.*, 2015). In addition to the physical protection that the skin provides, it also plays a major role in thermoregulation, absorption, excretion, transmission of stimuli and performs endocrine functions (e.g. vitamin D synthesis) (Elias, 2007, Menon, 2002, Proksch *et al.*, 2008).

Mammalian skin consists of three distinct layers being: the epidermis, dermis and hypodermis (also known as the subcutis or subcutaneous layer). The overlying, avascular epidermis is a stratified epithelium, which is comprised of five distinct organised layers made up from almost 95% keratinocytes. These epidermal layers begin at the outer, dead and dry *stratum corneum* followed by the *stratum lucidum*, the *stratum granulosum*, the *stratum spinosum* and the innermost *stratum basale*. The deepest layer is found at the dermal-epidermal junction resting on a basement membrane, where basal keratinocytes proliferate and migrate through the supra-basal layers toward the skin surface. This migration has a turnover time of approximately three weeks and ultimately results in the formation of fully differentiated corneocytes on the surface of the skin. The remaining 5% of the epidermal cell population includes melanocytes, Langerhans cells (dendritic cell subset of the epidermis) and Merkel cells (mechanoreceptors). Some morphological features of the epidermis, known as rete ridges protrude downwards into the underlying dermal layer, between what are referred to as the dermal papilla (Debeer *et al.*, 2013, Elias, 2007, Menon, 2002, Rittié, 2016).

The dermis, which forms the bulk of the skin, is highly vascular and contains various appendages including hair follicles, sweat glands, sebaceous glands, nerves, blood vessels and lymphatics. The dermis comprises mostly of dense extracellular matrix (ECM) of the skin, being made up of collagen, elastin and glycosaminoglycans, which are firmly attached to the basement membrane of the epidermis. The dominant cells are fibroblasts, but mast cells and infiltrating leukocytes are important for the function of the dermal layer. While the epidermis plays a vital role as a protective barrier, the dermis enables such a barrier by providing the required support in strength, elasticity and nourishment (Figure 1.1) (Debeer *et al.*, 2013, Elias, 2007, Menon, 2002, Proksch *et al.*, 2008, Summerfield *et al.*, 2015).

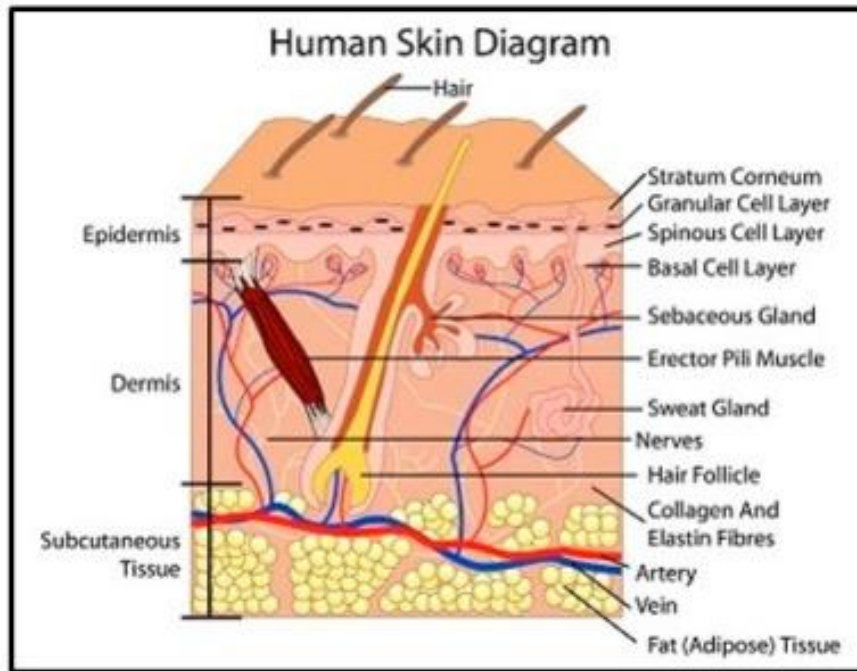


Figure 1.1: The structure of human skin. The skin consists of three main layers being the epidermis, dermis and hypodermis, which form an effective barrier between the organism and the outside environment. The skin primarily prevents injuries, infections and unregulated water loss (Xie *et al.*, 2015) (with permission).

1.1.3 Acute wound healing

Acute wounds are defined as those that have the ability to repair themselves by proceeding through the normal and predicted wound healing sequence, which ultimately results in both functional and anatomical restoration at the wound site. Acute wounds of first intention, without tissue loss, will usually heal in a range of 5 - 10 days or, wounds of second intention heal within 30 days depending on severity of tissue loss (Lazarus *et al.*, 1994, Velnar *et al.*, 2009). Wound healing is a complex, highly integrated and dynamic process involving co-ordinated interactions between diverse immunological and physiological systems, leading to predictable tissue repair. This process is achieved through four sequential yet overlapping phases namely: (i) haemostasis (also referred to as homeostasis); (ii) inflammation; (iii) proliferation and (iv) remodelling (Figure 1.2). For successful wound healing these four phases must occur in a synchronous and regulated manner within a controlled time frame (Demidova-Rice *et al.*, 2012, Gonzalez *et al.*, 2016, Guo and DiPietro, 2010).

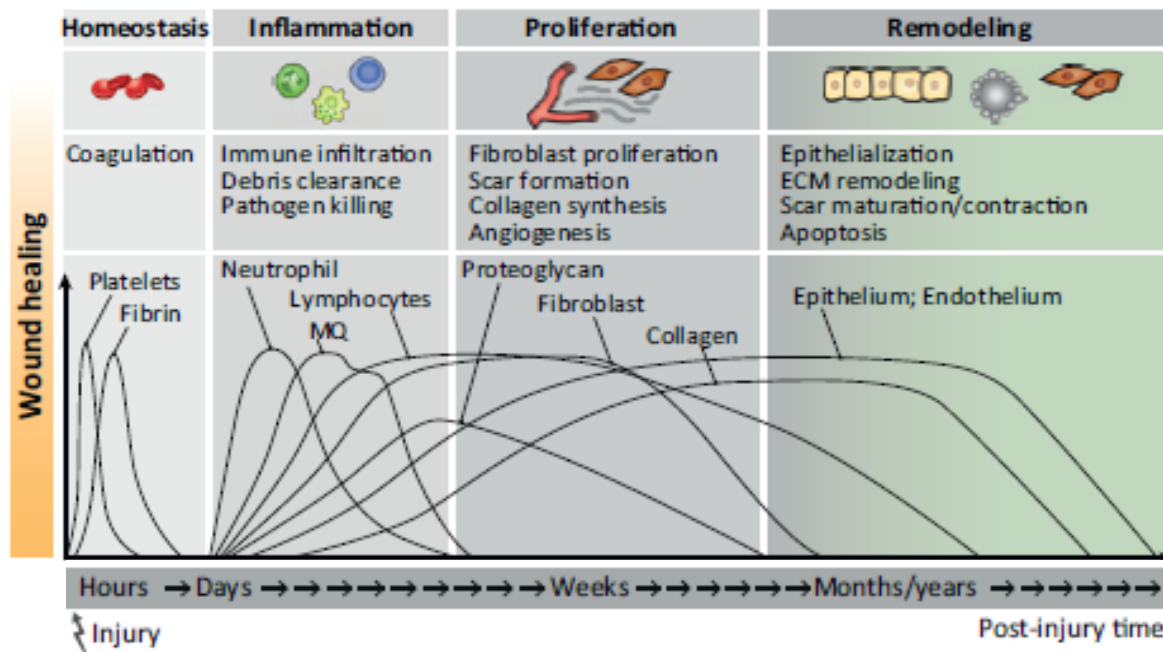


Figure 1.2: The four sequential yet overlapping phases of the classical acute wound healing model with the respective cells and mediators involved over time. Wound healing is typically described as the succession of four overlapping phases being haemostasis (also referred to as homeostasis), inflammation, proliferation and remodelling. For successful wound healing, these phases must occur in a synchronous and controlled manner, within an acceptable time frame. The main role of the haemostasis and inflammation phases is to stop bleeding via the formation of a fibrin clot, and to cleanse the wound from pathogens or foreign cells to allow successful rebuilding of the skin. Proliferation aims to repair the wound site by inducing granulation tissue formation and ECM deposition leading to re-epithelialisation; and the last phase, remodelling, normalises factors such as epidermal thickness, cellular content, extracellular matrix composition and blood vessel density to represent the pre-wound tissue as closely as possible (Shechter and Schwartz, 2013) (with permission).

1.1.3.1 Haemostasis and inflammation

Haemostasis and inflammation occur immediately following wound formation up to day 4 - 6. The haemostatic reaction is initiated when platelets adhere to damaged blood vessels and come into contact with exposed collagen and other extracellular components. This activates both the intrinsic and extrinsic pathways of the clotting cascade through the release of clotting factors from the platelets, which prevents excessive bleeding through the formation of a blood clot. Loss of blood is also decreased by vasoconstriction through the release of the potent vasoconstrictors

thromboxane A₂ and prostaglandin 2 α from the cell membranes of damaged wound site cells. The clot consists of platelets, fibrin, fibronectin, vitronectin and thrombospondin and trapped blood cells. In addition to haemostasis, the blood clot provides a scaffold for cells migrating into the wound site and concentrates the already present cytokines and growth factors. Platelets within the wound release several growth factors, cytokines and other important wound healing agents in low concentrations that initiate the localised inflammatory response and these include platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), fibroblast growth factor (FGF), epidermal growth factor (EGF) and insulin-like growth factors (Broughton *et al.*, 2006, Demidova-Rice *et al.*, 2012, Pool, 1977, Velnar *et al.*, 2009, Witte and Barbul, 1997).

Once the bleeding is controlled, and inflammatory mediators accumulate, blood vessels dilate to allow easier access for inflammatory and repair-related cells such as neutrophils, lymphocytes, macrophages, endothelial cells and fibroblasts to the wound site. Neutrophils are the first cells to be attracted into the wound site by various chemoattractant agents including interleukin (IL)-8, TGF- β and tumour necrosis factor-alpha (TNF- α). Neutrophils and lymphocytes are responsible for clearing invading microbes and debris from the wound site through the release of proteolytic enzymes and reactive oxygen species. Activated macrophages (transformed from monocytes) play an important long-term role in wound healing, whereby they release cytokines which attract additional leukocytes to promote the inflammatory response in the early stages of wound healing. Later, these activated macrophages induce and clear apoptotic inflammatory cells while releasing collagenases for wound debridement. TNF and ILs stimulate fibroblasts to produce new collagen and promote angiogenesis, while TGFs stimulate keratinocytes. This promotes new tissue generation and marks the resolution of the inflammatory phase (Broughton *et al.*, 2006, Guo and DiPietro, 2010, Meszaros *et al.*, 2000, Mosser and Edwards, 2008, Witte and Barbul, 1997). Inflammation is crucial in wound healing as it prevents infection spreading, clears debris and induces the proliferation phase of healing, however prolonged or inappropriate inflammation can result in extensive tissue damage (Midwood *et al.*, 2004). Chronic wounds appear to maintain an active inflammatory process that hinders further wound repair through inappropriate levels of proteolytic activity.

1.1.3.2 Proliferation

The proliferative phase of wound healing generally follows and overlaps with a subsiding inflammatory phase. Proliferation consists mainly of epithelialisation, angiogenesis, granulation tissue formation and collagen deposition. This begins 3 - 4 days after wounding, lasting for approximately 14 days thereafter (Broughton *et al.*, 2006, Gonzalez *et al.*, 2016, Velnar *et al.*, 2009). Re-epithelialisation begins early in wound repair where it forms a protective barrier between the wound and the external environment, thus preventing fluid loss and bacterial invasion. This barrier is established when cells on the edge of the wound begin to proliferate by sending out projections due to stimulation by EGF and TGF- α released by platelets and macrophages (Lawrence and Diegelmann, 1994). Additionally, re-epithelialisation is initiated when keratinocyte growth factors (KGF) 1 and 2, and IL-6 are secreted by fibroblasts causing healthy, neighbouring keratinocytes to migrate into the wound area where they proliferate and differentiate to form the epidermis. Epithelial cells initially migrate over the newly formed granular tissue in a single layer of cells, where subsequent layers later form due to an increase in cell mitotic activity (Broughton *et al.*, 2006, Grotendorst *et al.*, 1989, Lawrence and Diegelmann, 1994, Smola *et al.*, 1993).

As cell proliferation and migration require oxygen, nutrients and metabolite exchange, an adequate blood supply to the wound bed is required implying that angiogenesis is imperative for the progression of successful wound healing. Growth factors produced by inflammatory as well as epidermal and dermal cells maintain cell proliferation, whereas proangiogenic factors released during haemostasis stimulate angiogenesis. Vascular endothelial growth factor (VEGF), FGF-2 and PDGF all induce angiogenesis through endothelial cell migration into the wound site where they proliferate to form new blood vessels. Angiogenesis is characterised by endothelial cell migration and capillary formation, occurring concurrently with the proliferation phase (Demidova-Rice *et al.*, 2012, Epstein *et al.*, 1999, Kiefer *et al.*, 2002).

Another characteristic of the proliferative phase is the formation of granulation tissue, which is characterised by the presence of high concentrations of proliferating fibroblasts within the wound site. Fibroblasts from the wound periphery are stimulated to migrate into the wound site and proliferate from the time of wound

formation. This is achieved through the actions of EGF, TGF- β and PDGF, which are released by inflammatory cells and platelets (Goldman, 2004, Witte and Barbul, 1997). While proliferating, fibroblasts synthesise ECM, including collagen III which is vital in successful wound healing as it provides integrity and strength to the new developing tissues while providing a foundation for ECM formation. Additionally, fibroblasts synthesise the matrix proteins hyaluronan, fibronectin as well as glycosaminoglycans, all of which complex with collagen and accumulate to form an abundant, water-retaining extracellular matrix scaffold that is important for support of cell migration and essential in wound repair. Fibroblasts already within the wound site transform into myofibroblasts in response to TGF- β 1 released by macrophages and are responsible for wound contraction. Myofibroblasts are mainly found in the periphery of the wound and help aid in the approximation of wound edges. This is achieved through pseudopodia, originating from actin bundles within the plasma membrane that actively extend and attach to ECM components such as collagen and fibronectin. Wound contraction occurs as these cellular extensions retract. Following this action, it appears that redundant myofibroblasts are removed from the wound site through apoptosis (Baum and Arpey, 2005, Broughton *et al.*, 2006, Gonzalez *et al.*, 2016, Pierce *et al.*, 1991, Ramasastry, 2005, Robson *et al.*, 2001, Velnar *et al.*, 2009).

1.1.3.3 Remodelling

The final phase of wound healing begins approximately eight days after wound formation and can last for years. This phase is mainly responsible for the development of new epithelium and scar tissue by way of fibrous collagen deposits in an organised network and replacement of collagen III with thicker, more fibrous collagen I. If matrix deposition at the wound site is sub-optimal (e.g. due to diet or disease) wound strength will be compromised, whereas excessive collagen deposition can result in keloid formation.

Initially in wound healing the extracellular matrix is thin and yielding allowing the unhindered movement of fibroblasts, neutrophils, lymphocytes and macrophages. The matrix consists mainly of fibrin and fibronectin becoming thicker as glycosaminoglycans, proteoglycans and thicker, stronger collagen fibres are deposited by fibroblasts (Kurkinen *et al.*, 1980, Witte and Barbul, 1997). In the initial

wound healing stages, collagen III and IV deposited in the wound bed are thinner fibres than the collagen present in healthy skin and are deposited in a disorganised arrangement. In uninjured skin, 80 – 90% of the collagen is collagen I and 10 – 20% collagen III. Granulation tissue comprises 30% of collagen III, which then decreases to $\pm 10\%$ in a mature scar (Ehrlich and Krummel, 1996). During the remodelling phase the thinner collagen fibres are progressively replaced with more robust fibrous collagen I, which is deposited in a well-organised, cross-linked manner providing higher tensile strength (Figure 1.3). This collagen replacement is dependent on the degradative activity of several matrix metalloproteinases or 'MMPs' more specifically known as collagenase and elastases that remodel the matrix. Several different MMPs exist, each being selective for different collagen types and influenced through different cytokines, whose activity is regulated through the changing concentrations of TGF- β , PDGF, IL-1 and EGF. Fibroblasts under the influence of TGF- β and IL-6, produce tissue inhibitors of metalloproteinase that suppress the activity of specific MMPs. In contrast, TNF- α stimulates fibroblast release of IL-6 (Henry and Garner, 2003, Janis and Attinger, 2006). For successful completion of healing of acute wounds, a delicate balance must be maintained between synthesis and degradation of the different collagen types, and extracellular matrix remodelling, which take place continuously and appear to reach equilibrium at approximately 21 days post-wounding. As wound healing progresses, new capillary growth subsides and blood flow to the wound area decreases, while apoptosis of excess fibroblasts and macrophages increases. Depending on the initial wound characteristics (*i.e.* location, depth, size, tissue loss, cleanliness), the end result of the wound healing process can range from tissue closely representing that of unwounded tissue, to a matured scar with a relatively high tensile strength (Broughton *et al.*, 2006, Clark, 1993, Gonzalez *et al.*, 2016, Rittié, 2016, Velnar *et al.*, 2009).

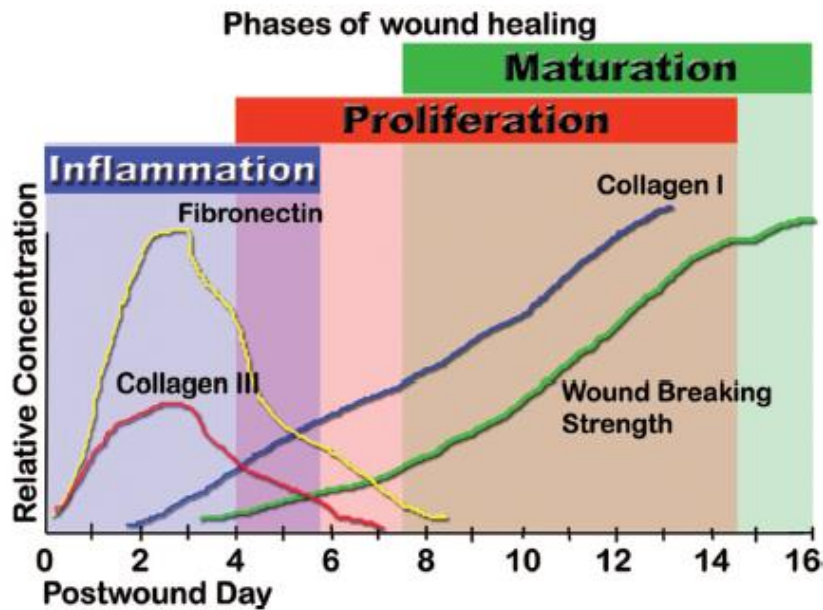


Figure 1.3: Extracellular matrix remodelling over time. Thinner collagen III along with fibronectin initially constitute the extracellular matrix, where thicker collagen I continuously increases and begins to only dominate during the proliferation phase, accompanied by an increased wound breaking strength (Janis and Attinger, 2006) (with permission).

It should be kept in mind that although dividing wound healing into ‘phases’ may aid in research and have descriptive purposes, it tends to undermine the fact that wound healing is continuous and that wounds are heterogeneous in their healing phase progression. The classic depiction of the four wound healing phases allows a better understanding of the progress of healing but these phases are not distinct from each other and overlap to varying degrees depending on the wound size and factors affecting the process. Furthermore, the nature of the wound plays a significant role in the dominant repair mechanism and time required for successful re-epithelialisation depending on factors such as wound depth, size, tissue loss, location and degree of sterility (Rittié, 2016).

Wound depth directly relates to the number of different skin layers and appendages affected. Typically, wounds are either described as being ‘partial- or full-skin thickness wounds. A partial-skin thickness wound is when the epidermis and possibly only the upper portion of the dermis are wounded, being sub-classified as either a ‘superficial’ or ‘deep’ partial-skin thickness wound respectively. Skin appendages in partial-skin thickness wounds, including hair follicles, sweat glands and sebaceous glands tend to mostly remain intact. Despite the degree of wounding

in a partial-skin thickness wound, re-epithelialisation is the primary method of healing. In full-skin thickness wounds, the dermis and sometimes even the deeper underlying tissues are damaged. Due to the extent of the wound, re-epithelialisation alone is insufficient for successful healing and thus co-depends on granulation tissue formation and deposition to fill the wound space before a new epithelial layer can be formed (Figure 1.4) (Rittié, 2016, Van Winkle Jr, 1968).

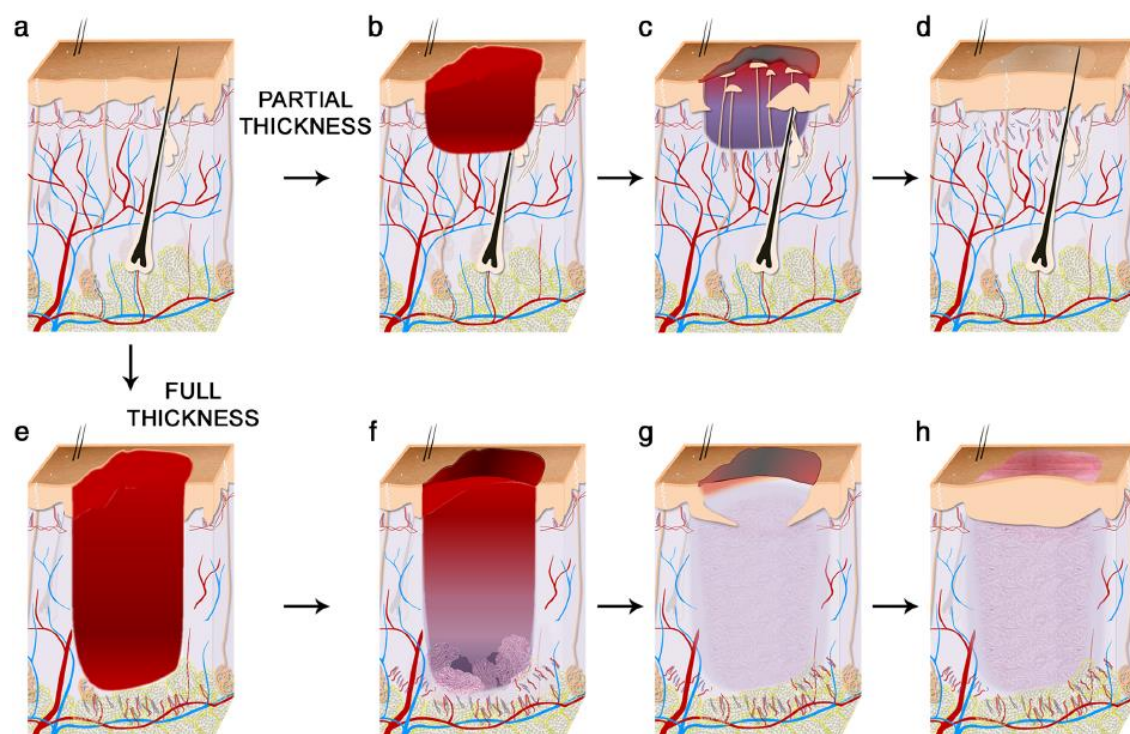


Figure 1.4: Partial-skin versus full-skin thickness wounds. Healthy human skin as depicted in (a) is highly vascularised with multiple appendages including hair follicles, eccrine sweat glands and sebaceous glands. (b-d) Partial-skin thickness wounds damage the epidermis and even the upper parts of the dermis. Such wounds heal mainly by re-epithelialisation with minimal scarring. (e-g) In contrast, full-skin thickness wounds damage the dermis (and even underlying tissues) and are unable to heal via re-epithelialisation alone. Formation and deposition of granulation tissue is required to fill the wound space before new epithelium can form on the wound surface. (h) Following the healing process, scarring is visible at the wound site, where skin structure remains altered with respect to appendages and vasculature (Rittié, 2016) (with permission).

1.1.4 Chronic wounds

Chronic wounds are defined as wounds that are unable to progress through the controlled, predictive phases of wound healing within the expected time frames, and thus do not heal in an orderly manner (Robson *et al.*, 2001, Szycher and Lee, 1992). The most common chronic wounds are leg ulcers, diabetic foot ulcers and pressure ulcers. Generally chronic wounds are wounds that do not heal within three months and tend to most often be 'trapped' in the inflammatory stage of wound healing. This results in an interruption of the wound healing process and prolongs the exposure to inflammatory mediators and enzymes, which may damage surrounding healthy tissue (Eming *et al.*, 2007). Some chronic wounds appear to progress to later phases of the healing sequence but without termination of the inflammatory phase. These wounds may take years to heal or may not heal at all. Chronic wounds result from impaired or potentially overactive wound healing processes that are responsible for patient morbidity and even mortality, while placing a significant financial burden on health care systems (Järbrink *et al.*, 2017). While the processes of acute, normal wound healing and the associated mediators are well described, the mechanisms leading to impaired wound healing and the identity of mediators involved are poorly defined. Despite treatment strategies involving synthetic skin covers, hyperbaric oxygen therapy, growth factors and stem cells, no reliable therapy currently exists for the comprehensive treatment of chronic wounds (Eming *et al.*, 2010, Fonder *et al.*, 2008, Grey *et al.*, 2009b, Johnston *et al.*, 2016, Pugliese *et al.*, 2018).

1.1.5 Wound healing models

In the search for new and innovative wound healing therapies, controlled healing models are indispensable with respect to product safety and efficacy assessment. Wound healing is a complex process that involves several different physiological systems and many processes that involve cellular responses from different immune and structural cells as well as varying concentrations of different inflammatory and growth promoting mediators. To allow controlled studies where specific outcomes are monitored, numerous *in vitro* and *in vivo* wound models have been developed to aid in the study of the healing process (Abergel *et al.*, 1987, Boyko *et al.*, 2017, Gottrup *et al.*, 2000, Sherratt and Murray, 1990). Selecting the most relevant wound healing model for a study is a critical decision where applicability, specific study

outcomes, variability, outcome measurements and approximation to human healing all need to be considered, while also taking into account the strengths and limitations of each model.

1.1.5.1 *In vitro* wound models

In vitro wound healing models refer to healing studies taking place outside of a living organism. In addition to the decreased cost, the main benefits of utilising *in vitro* techniques include the simplicity, speed and minimal ethical considerations offered. *In vitro* models allow for cellular components of wound healing to be isolated in different combinations and studied separately, away from the full complexity of the wound healing process. Most commonly the cellular functions of migration, proliferation, protein expression and wound contraction are focussed upon. This targeted approach may amplify specific healing factors and aid in selected result interpretation. While reducing wound healing complexity may be advantageous, it is also a limitation as results obtained from *in vitro* studies may not correlate to those performed *in vivo*, and thus need to be replicated and validated in a more complex environment to be considered significant when developing new therapies. It is therefore not uncommon for studies to use both *in vitro* and *in vivo* wound healing models to fully assess the repair process (Boyko *et al.*, 2017, Gottrup *et al.*, 2000).

There are a number of *in vitro* wound healing models available with varying complexity, the simplest of which is referred to as a monoculture where one cell type is cultured and studied. With respect to wound healing, fibroblasts (the predominant cell type in wound healing) along with keratinocytes, macrophages and endothelial cells are most commonly used in wound healing assays (Boyko *et al.*, 2017, Gottrup *et al.*, 2000, Walter *et al.*, 2010). Wounds are simulated in monocultures by mechanically removing cells and creating a scratch through a confluent cell monolayer. This well-developed assay, which is referred to as a 'scratch assay', is used to measure cell migration. The so-called 'wound' is healed when the remaining cells proliferate and migrate into the mechanically created cell free space. This assay is said to mimic cell migration rate during wound healing *in vivo* (Figure 1.5) (Liang *et al.*, 2007, Riahi *et al.*, 2012). Another common assay measuring cell migration of a monoculture is known as the Boyden chamber assay. Cells are incubated on one side of a two-chamber system where the chambers are separated by a microporous

membrane and cells can migrate through the membrane pores in response to a concentration gradient of a chemotactic compound being tested. The membrane is then fixed, stained and the number of migrated cells is determined on the receiver chamber side of the membrane (Chen, 2005).

Although these migration assays are well-developed and commonly used, the fact still remains that cells do not exist in monocultures within the skin. Cell-matrix attachment and cell-cell interactions that occur within the skin tend to alter cell behaviour. For this reason, three-dimensional *in vitro* wound healing models have been developed to better mimic the native wound healing environment (Chapman *et al.*, 2014, Sun *et al.*, 2006). Examples of this model include fibroblast-populated collagen lattices polymerised into a gel (Cukierman *et al.*, 2002, Kuhn *et al.*, 2000); and three-dimensional keratinocyte cultures which rely on the fact that when cultured to confluence, keratinocytes continue to proliferate into multiple layers and when the top layer of these cells is exposed to air, they terminally differentiate into a structure resembling stratified epidermis (Pruniéras *et al.*, 1983). To better represent the skin and the cell-cell interactions occurring within, healing model complexity may be increased to include co-cultures of different cells. Fibroblasts cultured with keratinocytes is a common example, whereby a layer of keratinocytes are seeded on top of a reconstituted collagen biomatrix supplemented with fibroblasts. The keratinocytes grow into multiple layers and are exposed to air for terminal differentiation, producing dermal- and epidermal-type layers (Dongari-Bagtzoglou and Kashleva, 2006, Vintermyr *et al.*, 2003) (Figure 1.5). *In vitro* models using multicell type cultures and 3D matrix allow for wound environments to be mimicked and the resulting reactions successfully studied. Such models, however, are still simple in comparison to the natural and highly complex process of *in vivo* wound healing, where *in vitro* results will not only need to be highly reproducible but must be replicated and validated *in vivo* to attain significance.

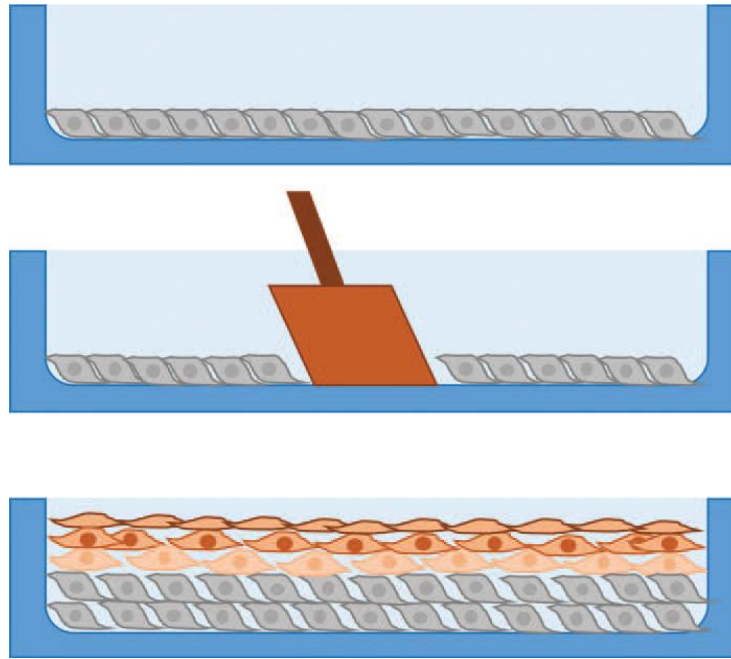


Figure 1.5: An illustration of different *in vitro* wound healing models. The simplest healing model is a monolayer (top), where a mechanical gap may be created as a ‘wound’ in the scratch assay (centre) or to more accurately simulate the skin, three-dimensional co-cultures may be used (bottom) (Boyko *et al.*, 2017) (with permission).

1.1.5.2 *In vivo* wound models

In vivo models refer to those that take place within living organisms *i.e.* animal models possessing natural or inherited characteristics that closely represent biological processes in humans. Wound healing studies in humans would be ideal, but ethically unacceptable. Even if possible, such studies would likely restrict experimentation to only small, clean wounds and also may prevent the use of untreated control wounds, significantly limiting the quality of the study data (Gottrup *et al.*, 2000, Sullivan *et al.*, 2001). Human studies are often also impractical as it is a challenging feat to obtain statistically relevant numbers of individuals with identical or similar, natural wounds for randomised trials. Accurate histological assessment may also be a limiting factor as multiple biopsies would need to be assessed throughout the healing process for a completely objective view and this is not normally possible with limited wound sizes.

Preclinical *in vivo* models make use of a variety of animal species each with unique benefits and limitations. The selection of an appropriate animal model is a critical

decision in any study and should be beneficial to the study outcome rather than the most familiar option. In addition to the specific study purpose, the choice is influenced by a number of different factors including budget, investigator experience, resources and model availability (Sullivan *et al.*, 2001). With respect to wound healing, the benefits and limitations of each model partly tend to correlate to specific dermal-epidermal characteristics of different animal species. All mammalian skin consists of three main layers being the epidermis, dermis and hypodermis (see Section 1.1.2). This however does not necessarily translate into similar wound healing physiology, as different mammals tend to display significant differences in the way that their wounds heal. Many animal species have successfully been used in wound healing studies including rodents, rabbits, pigs, horses and sheep. The murine model however, has been the most commonly used option (Lindblad, 2008).

1.1.5.3 The murine wound model

The murine wound healing model is extensively used in preclinical wound healing research. This is because rats and mice are easy to handle, obtained readily, relatively inexpensive and simply housed (Boyko *et al.*, 2017, Lindblad, 2008, Ren *et al.*, 2012). Both excisional and incisional wounds are frequently used in the murine model with variations being employed to address the specific study objectives. The most commonly used murine wound model is the excisional wound model where full-skin thickness wounds are created along the dorsum of the mouse. This model is used often because it is simple, reproducible and practical when dealing with large sample sizes. It also allows easy access to wounds for the application of test treatments as well as for photography and wound contraction measurements (Wong *et al.*, 2010). In addition, modifications to the excisional wound model have been incorporated to better mimic the repair mechanisms occurring in humans. One such modification is known as ‘wound splinting’ and involves the placement of silicone rings around the wound edges to promote healing through granulation and re-epithelialisation rather than through the typical rodent wound contraction mechanism (Galiano *et al.*, 2004, Ren *et al.*, 2012). Other murine wound models are listed below (Figure 1.6) (Wong *et al.*, 2010).

- The ischaemic skin flap model, where a full-skin thickness flap is created on the dorsum by cutting along three sides and lifting the segment from the basal

layers. An impermeable silicone membrane is inserted directly beneath the flap that is then sutured closed.

- The Ischemia-reperfusion model which is used to study pressure ulcers by using magnets to pinch the dorsal skin.
- The mechanical load model which involves mechanical weights being placed orthogonally onto a healing wound in order to induce wound fibrosis, which can then be used to mimic hypertrophic human scarring.

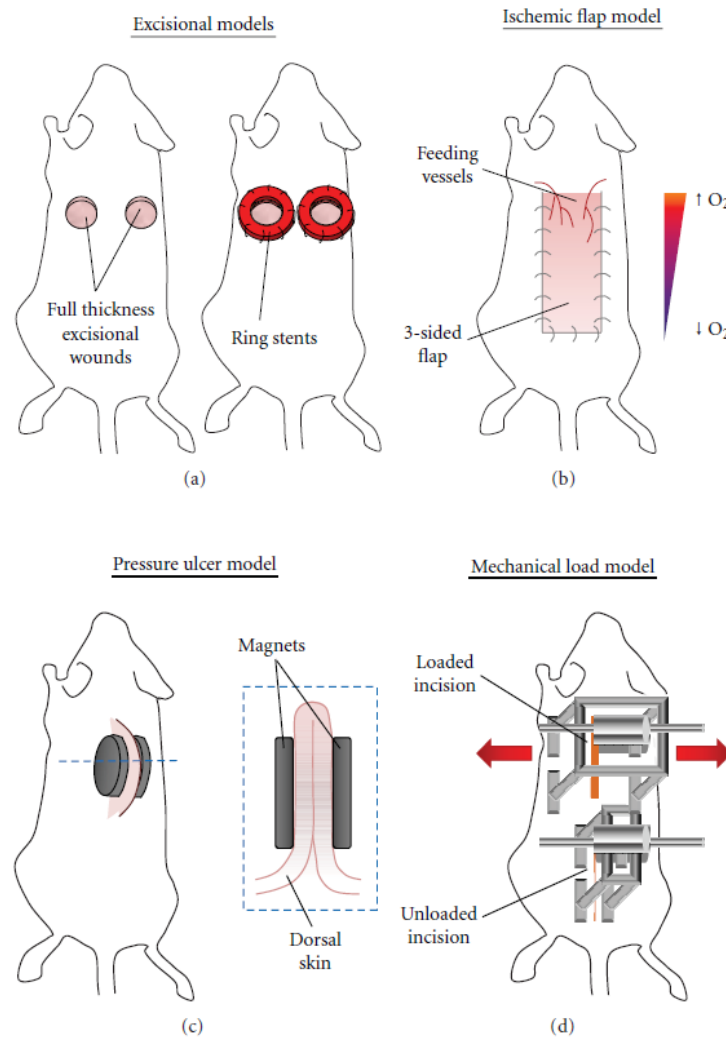


Figure 1.6: Overview of commonly used murine wound healing models. This schematic diagram highlights some of the most popular murine models used in cutaneous disease studies and wound healing. (a) The full-skin thickness excisional model is the most commonly used model, allowing numerous characteristics of healing to be assessed. Splinted wound models (right) are additionally used to prevent wound contracture and promote healing through re-epithelisation and granulation to better represent clinical wound healing. (b) The ischaemic flap model creates a healing environment with varying degrees of ischaemia where skin regions furthest from feeding blood vessels are the most ischaemic. (c) The pressure ulcer model is used to create an ischaemia-reperfusion injury which is thought to be involved in pressure ulcer pathophysiology. The injury is created when skin is positioned between two opposing magnets. (d) The mechanical loading of mouse incisions promotes the formation of wound fibrosis, which is similar to human hypertrophic scarring (Wong *et al.*, 2010) (with permission).

As advantageous as the murine wound healing model may be, the skin of small mammals differs anatomically and physiologically from humans, and their wounds heal in a significantly different way (Boyko *et al.*, 2017, Dunn *et al.*, 2013, Lindblad, 2008). One of the major anatomical differences between murine and human skin that influences wound healing, is that guinea pigs, rats and mice are classified as being 'loose-skinned' meaning that they have a thin layer of skeletal muscle lying between the dermis and subcutaneous fat, known as the panniculus carnosus. Due to this striated layer of subcutaneous muscle, murine excisional wounds heal primarily by contraction and not by new granulation tissue deposition and re-epithelialisation, as observed in humans (Dunn *et al.*, 2013, Gross, 1996, Wong *et al.*, 2010). The panniculus carnosus allows for skin to move independently of the deeper underlying muscle and thus skin quickly reacts by contraction following wound creation (Dunn *et al.*, 2013). As mentioned above, in an effort to overcome this difference, murine wound models have been modified to better replicate clinical healing by use of a splint (Figure 1.6 a) (Galiano *et al.*, 2004, Ren *et al.*, 2012). Although an improvement, this modification still does not mimic the human healing processes. Other significant anatomical and physiological differences between human and murine skin are that mouse skin is covered by a dense layer of hair that frequently cycles (approximately every 3 weeks) compared to the generally sparser distribution of human hair, where depending on location can remain intact for many years (Porter, 2003, van der Veen *et al.*, 1999). This difference affects re-epithelialisation, an important component of wound healing. Mice also lack skin apocrine sweat glands, dermal papillae as well as rete ridges in the epidermis, which are an anatomical skin feature often used to determine the extent of epidermal healing in humans (Wong *et al.*, 2010). Lastly it has been found that mouse skin is thinner (epidermis and dermis), softer and more flexible than that of humans (Aarabi *et al.*, 2007), which requires consideration in mechanical measurements of wound healing. Major differences between the skin of commonly used animal model species and humans can be seen in Table 1.1 (Summerfield *et al.*, 2015). In addition to the anatomical differences between rodent and human skin, the immune system differs in the response to wounding, with effects throughout the entire wound healing process (Dawson, 2011).

Table 1.1: Comparison of skin characteristics of different animal species used in wound healing studies (Summerfield *et al.*, 2015)

Criteria	Human	Mouse	Rat	Guinea pig	Pig
Skin attachment	Firmly attached	Loose	Loose	Loose	Firmly attached
Hair coat	Sparse	Dense*	Dense*	Sparse or dense	Sparse
Epidermis	Thick	Thin	Thin	Thick	Thick
Dermis	Thick	Thin	Thin	Thick	Thick
<i>Panniculus carnosus</i>	Absent	Present	Present	Present	Absent
Healing mechanism	Re-epithelialisation	Contraction	Contraction	Contraction	Re-epithelialisation

*Except some breeds

The murine model has contributed greatly to wound healing research and will continue to do so, being a practical, economical and least expensive option. However, such models are ultimately judged on their ability to predict outcomes in clinical settings.

Due to the anatomical similarity and the mechanisms by which wounds heal it has been suggested that the porcine healing model is the most accurate and reliable choice as it possesses many similarities to human skin with respect to general structure, thickness, pigmentation, hair follicle density, extracellular matrix composition and lipid composition (Summerfield *et al.*, 2015).

1.1.5.4 The porcine wound model

The porcine wound healing model is considered to be an excellent animal model for human studies. A fact supported by the more than 1500 publications on the pathophysiology of numerous wound types making use of the porcine wound healing model (Seaton *et al.*, 2015). This is because pig skin displays many similarities to that of human skin, not only anatomically, physiologically and genetically, but also in

the way that wounds heal (Meurens *et al.*, 2012, Meyer *et al.*, 1978, Montagna, 1964, Pomahac *et al.*, 2007, Seaton *et al.*, 2015, Sullivan *et al.*, 2001).

Genetically the porcine genome is comparable to that of humans both with respect to size and composition, where numerous homologies are evident in genome and protein sequences (Hart *et al.*, 2007, Meurens *et al.*, 2012). Physiologically pigs are markedly similar to humans as common functional features are found across multiple organs, including that of the integumentary system (Swindle *et al.*, 2012). The most relevant similarity is the fact that porcine wounds, like in humans, heal predominantly by deposition of granulation tissue and re-epithelialisation. Epidermal turnover time is also comparable between the two species, as well as the keratin protein types present and the lipid composition of the stratum corneum (Gray *et al.*, 1982, Weinstein, 1966). Porcine skin has been studied using various approaches, including histology and immunohistochemistry (Debeer *et al.*, 2013, Montagna, 1964), confocal Raman microspectroscopy (Tfaily *et al.*, 2012), infrared spectroscopy (Kong and Bhargava, 2011) and bright light and electron microscopy (Liu *et al.*, 2010). Porcine skin is very similar to that of human skin anatomically consisting of an epidermis, dermis and hypodermis that are layered in a similar manner. The thickness of the epidermis in human skin ranges from 50 – 120 μm and in pig skin from 30 – 140 μm (Hammond *et al.*, 2000, Meyer *et al.*, 1978). However, because skin thickness is highly dependent on location in both species, the dermal-epidermal thickness ratio is a more accurate measure. This ratio ranges from 10:1 to 13:1 in pigs which is very similar to that of humans (Vardaxis *et al.*, 1997). Unlike in the murine model, pigs' skin contains prominent rete ridges and dermal papillae correlating closely to what is seen in humans (Montagna, 1964, Morris and Hopewell, 1990). Histologically and with regards to protein and lipid composition of the different skin layers, the porcine epidermis, dermis and hypodermis are very similar to their human counterparts. Both the human and porcine epidermis consist of stratified, multi-layered, keratinising epithelium (Figure 1.7 A-B) (Debeer *et al.*, 2013). Additionally, there are multiple histological features of the porcine epidermis that closely match those of the human epidermis. Such features include: similar hair follicles with an inner and outer root sheath (Figure 1.7 A-B); a basal cell layer consisting of basophilic cylindrical cells that lie perpendicular to the dermal-epidermal junction; in the Malpighian layer (the stratum spinosum and stratum

basale combined) the keratinocytes appear more eosinophilic with a round nucleus; lying parallel to the surface of the skin are 1 - 2 layers of flattened keratinocytes; and the stratum corneum consists of flattened corneocytes (Debeer *et al.*, 2013, Kanitakis, 2002).

The porcine dermis further supports the comparable architecture between pig and human skin where histologically, the two species continue to show similarity. Staining of porcine collagen with haematoxylin-eosin-saffron (collagen appears yellow-orange) and Masson's trichrome (collagen appears blue-green) (Figures 1.7 A and D respectively) shows how collagen fibres are arranged in bundles as seen in human skin. Orcein stain visualises the presence of elastic fibres in the porcine dermis and in the walls of blood vessels (Figure 1.7 E). Although appearing more sparse and thinner than what is typically seen in humans, the pig has a relatively high elastic content when compared to other mammals (Debeer *et al.*, 2013, Marcarian and Calhoun, 1966). In addition, there is similarity in blood supply to the dermis between the pig and humans (Montagna, 1964, Vardaxis *et al.*, 1997); and the panniculus carnosus, found in small, loose-skinned mammals, is not present in either species. Lastly, although largely absent in pigs (being restricted to the carpus and nasolabial regions) eccrine sweat glands of the porcine skin show a similar structure to those found in humans (Fukui *et al.*, 2012).

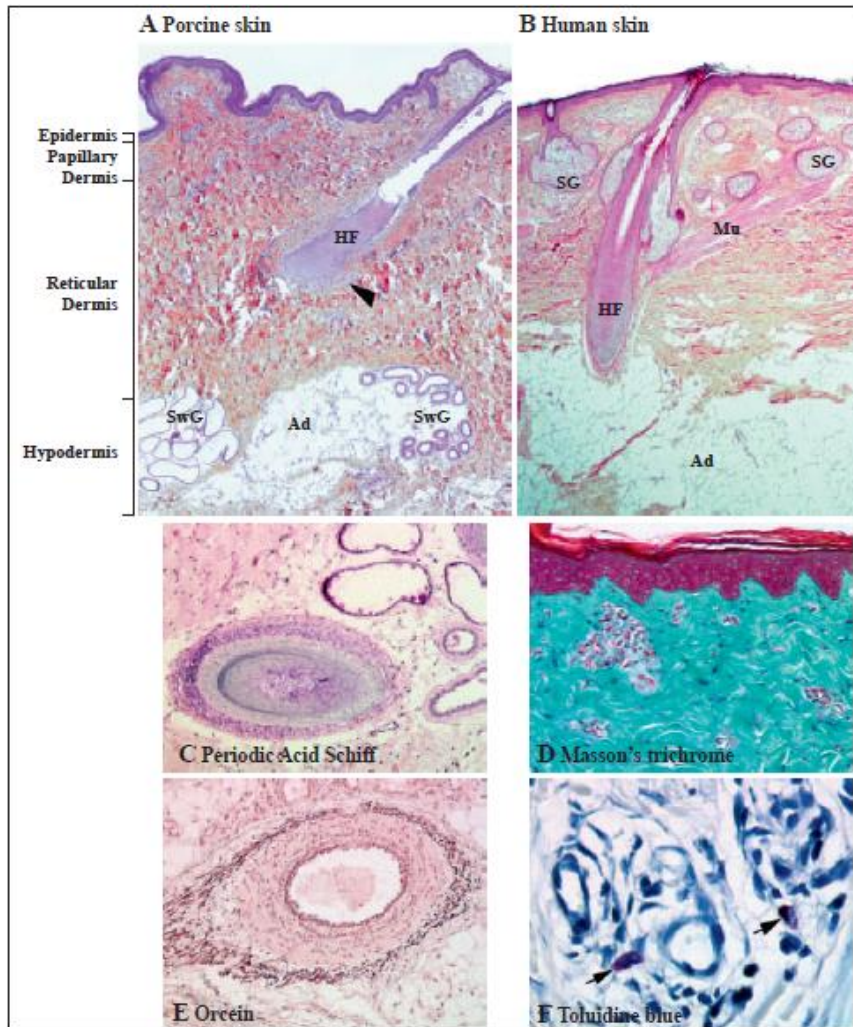


Figure 1.7: Histological analyses highlighting the similarities between porcine and human skin. (A-B) A comparative histological analysis of (A) porcine and (B) human skin using a haematoxylin-eosin-saffron staining technique. HF: hair follicle, Mu and arrowhead: arrector pili muscle, SwG: sweat gland, SG: sebaceous gland, Ad: adipose tissue. (C-F) Porcine skin stained with: (C) Periodic Acid Schiff (PAS) stain with glycogen, mucin and basement membranes in red/purple; (D) Masson's trichrome stain with collagen appearing blue/green and the epidermis red/purple; (E) Orcein: elastic fibres of blood vessel in dark brown; (F) Toluidine blue stain showing mast cells in purple (arrows) (Debeer *et al.*, 2013) (with permission).

Debeer *et al.* studied the immunohistochemical properties of porcine skin compared to those of human skin. This was done by determining whether antibodies used to detect cells in human skin showed immunoreactivity in porcine skin, and several such antibodies were found to react with both species. Porcine keratinocytes, Langerhans cells, melanocytes and Merkel cells were all successfully labelled to some extent using anti-human protein antibodies. With respect to cutaneous

vasculature and innervation, antibody reactions occurred in porcine blood, lymphatic endothelial cells, the smooth muscle of blood vessel walls, nerve endings and axons. Lastly, when looking at skin appendages immunoreactivity was seen in apocrine sweat glands and in the arrector pili muscle cells (Debeer *et al.*, 2013).

The porcine immune system shares striking similarities with humans where over 80% of porcine immune parameters are closely resembled (compared to less than 10% in mice) (Dawson, 2011). As in humans, pigs have a fully functioning innate and adaptive immune system where most of the proteins possess structural and functional similarities to the human immune system (Meurens *et al.*, 2012). Almost all of the identified human immune cell populations are present in the pig and in contrast to rodents, pigs and humans have high concentrations of neutrophils in their peripheral blood (Fairbairn *et al.*, 2011, Meurens *et al.*, 2012).

Just as in murine models (Figure 1.6), multiple porcine models have been developed and utilised in preclinical studies as a substitute for human skin in various fields of research. These include chronic, diabetic and infected wounds (Ansell *et al.*, 2012, Dai *et al.*, 2011, Roy *et al.*, 2009, Velandar *et al.*, 2008), burns and scarring (Cuttle *et al.*, 2006, Sheu *et al.*, 2014), transdermal penetration, topical drug delivery and toxicology (Laurent *et al.*, 2007, Yu *et al.*, 2015), as well as radiation and UV impact studies (Agay *et al.*, 2010, Smirnova *et al.*, 2014). Sullivan *et al.* evaluated 25 wound therapies making use of different animal healing models and compared the results to equivalent human studies. It was found that the porcine model was similar to the results of the human analyses in 78% of the studies as compared to the 53% and 57% for the small-mammal and *in vitro* studies respectively (Sullivan *et al.*, 2001). Obviously, no animal model can completely mimic wound healing in humans however, it is clear from years of research that the pig is an excellent preclinical model for wound healing studies.

In this study, the pig was chosen for the controlled acute wound healing model where full-skin thickness wounds were induced, following well planned, randomised but strictly controlled wound formation, and healing could be accurately investigated.

1.1.6 The basics of mass spectrometry

The mass spectrometer has been described as ‘the smallest weighing scale in the world ever used’ (Chughtai and Heeren, 2010, Siuzdak, 2006). Mass spectrometry (MS) is an extremely powerful tool, completely unique in its interdisciplinary applications and ability to analyse molecules both quantitatively (concentration) and qualitatively (molecular weight or molecular structure). MS is capable of diverse molecular analyses including that of large biomolecular complexes, individual biomolecules, small organic molecules and even single atoms and their isotopes. Since its invention in the early 20th century, MS has accelerated in development, with continual improvements in detectable mass ranges, sensitivity and resolution that have resulted in the impressive and superior analytical techniques of today (Chughtai and Heeren, 2010, El-Aneel *et al.*, 2009).

1.1.6.1 History

The first mass spectrometer was officially constructed in 1912 by Sir Joseph John Thomson, a professor of experimental physics at the University of Cambridge, who was just 28 years old at the time. Sir Thomson revealed the impressive capabilities of his novel discovery as soon as 1913 within the field of analytical chemistry (Griffiths, 2008, Thomson, 1913). Commercial mass spectrometry however, only came to light in the 1940s where it was used to diagnostically detect organic substances and fulfil demands in the oil industry. During this time physicists and industrial chemists also helped to ensure that MS became a firmly established technique. It is said that at that time nobody could really comprehend what occurred inside the instrument and thus its use was limited to quantitative analyses of known compounds (De Hoffman and Stroobant, 2007, Grayson, 2002, Griffiths, 2008). After gas chromatography was commercialised (James and Martin, 1952) and coupled to a MS (*i.e.* GC-MS) in 1957 (Holmes and Morrell, 1957), the interest in the technique expanded considerably, especially in the biochemistry sector. Analyses were however limited to volatile compounds with a low molecular weight range and the ionisation techniques used were harsh, meaning no complex biomolecules (e.g. proteins, complex carbohydrates and nucleotides) could be analysed as they would be destroyed and produce extremely complex fragmentation patterns. Additionally, protein analyses was not compatible with GC-MS as samples mixtures needed to be volatilised, and in being transmitted to the gas phase, proteins would undergo

significant denaturing and degradation (El-Aneed *et al.*, 2009). The introduction of soft ionisation techniques in the 1980s revolutionised the capabilities of mass spectrometry in the biological sciences, as it was now possible to analyse large, intact biomolecules. The two main techniques developed contributing to this revolution were electrospray ionisation (ESI) (Yamashita and Fenn, 1984) and matrix-assisted laser desorption ionisation (MALDI) (Karas and Hillenkamp, 1988, Tanaka *et al.*, 1988), a contribution that earned John Fenn and Koichi Tanaka a shared 2002 Nobel Prize in Chemistry (Griffiths, 2008). The development of ESI and MALDI made it possible to ionise not only smaller biomolecules such as drugs and metabolites, but also larger biomolecules including lipids, peptides and even whole intact proteins (Fenn *et al.*, 1989, Leinweber *et al.*, 2009).

1.1.6.2 ESI vs MALDI MS

ESI-MS is a unique technique in many laboratories as it provides a sensitive, robust and reliable analysis both quantitatively and qualitatively. ESI is commonly coupled to high/ultra-performance liquid chromatography (HPLC/UPLC) for molecular separation before mass spectrometric analysis. With the addition of tandem mass spectrometry (MS/MS), as well as automated sample introduction, LC-ESI-MS/MS allows for rapid analysis and high throughput of sample sizes limited to as little as 50 µg of protein, even of complex biological samples (Ho *et al.*, 2003). Briefly, ESI works by using high voltage, direct current electrical energy to convert compounds in solution to ions in a gaseous phase for mass spectrometric analysis. This is done when the sample is dispersed as a fine spray of highly charged solvent droplets from the ESI source, after which the solvent is rapidly evaporated at atmospheric pressure, and the ions formed from the dissolved analytes are expelled from the droplets. This process takes place as sample is continuously passed through a capillary tube that is maintained at a higher voltage (approx. 3-6 kV) than the surrounding chamber, thus generating a mist of highly charged sample droplets. The sample ions are then accelerated down a pressure and potential gradient towards the mass analyser for analysis of molecular mass and ion intensity (El-Aneed *et al.*, 2009, Ho *et al.*, 2003). One advantage of the ESI source is the formation of multiply charged ions from the same analyte, which extends the mass range that can be analysed, and this is conducive to forming charged fragments in tandem mass spectrometry. Additionally, in the 1990s, nano-electrospray technology was

introduced to LC-MS (Emmett and Caprioli, 1994, Gale and Smith, 1993), whereby very low sample flow rates (nl/min compared to the conventional 2 - 20 μ l/min) are used to potentiate limited biological samples and improve analyte ionisation (El-Aneed *et al.*, 2009, Karas *et al.*, 2000).

MALDI is a leading soft ionisation technique that utilises added ionisable matrices and laser energy to ionise complex biological samples with minimal fragmentation and most often introduces a single charge to each ion formed (Hillenkamp *et al.*, 1991). With the MALDI technique, a sample is mixed with an appropriate matrix (dissolved in a suitable solvent), spotted onto a metal target plate and air-dried (alternatively a stream of nitrogen gas may be used) to allow co-crystallisation of the sample with the matrix. The result is a solid analyte sample, which is a notable difference when compared to the analyte solutions used in most other MS techniques. The fixed sample on the target plate is converted to ions that are desorbed directly from the solid sample in a chamber under vacuum. Energy from a laser (most commonly a nitrogen laser set at 337 nm) is used for ablation, desorption and subsequent ionisation of the sample. The matrix plays two critical roles: (i) absorbing energy from the laser and (ii) separating analyte molecules from one another to prevent the formation of clusters (El-Aneed *et al.*, 2009, Karas and Krüger, 2003, Schiller *et al.*, 2004). Selection of the correct matrix for the sample in question is critical for successful analysis and is based on its energy absorption properties at the lasers' wavelength, its ability to mix with the analyte homogeneously, and the nature of the analyte present *e.g.* peptides, proteins, metabolites, lipids, drugs or oligonucleotides. The three most commonly used matrices at present are 2,5-dihydroxybenzoic acid (DHB) (Strupat *et al.*, 1991), α -cyano-4-hydroxycinnamic acid (CHCA) (Beavis *et al.*, 1992) and 3,5-dimethoxy-4-hydroxycinnamic acid also known as sinapinic acid (SA) (Beavis *et al.*, 1989). There are multiple matrices available, each with advantages and disadvantages with respect to sample preparation and analyte class, that have been found to be useful in the application of various analyte types (Norris and Caprioli, 2013).

Samples are then exposed to the pulsing laser beam where the matrix absorbs the laser's energy and carries intact sample into the gaseous phase causing desorption of the analyte molecules. At this time charge exchange occurs between the charged matrix and the analyte, resulting in charged analyte ions. In this process, mainly

intact, single protonated ions are generated *i.e.* $[M+H]^+$ where M is the molecular weight of the sample analyte (as opposed to the multiply charged analytes often found in ESI-MS). This singular charge is one of the great advantages of MALDI-MS lending to simple data analysis (Pulfer and Murphy, 2003, Schiller *et al.*, 2004). MALDI is most often coupled to a time-of-flight (TOF) mass analyser where the ions are then accelerated across an electric field of a high voltage (approximately 20 kV), passed through a charged grid and then freely drift along the TOF tube. The time taken for the ions to reach the mass detector at the end of the tube (*i.e.* the drift time) is dependent on mass-to-charge ratio (m/z), where light ions travel faster, and accordingly ions of different mass are separated. The longer the free flight of ionised molecules (tube length typically ranges from 1-2 m in length) the better the mass resolution and accuracy (El-Aneed *et al.*, 2009, Schiller *et al.*, 2004).

Both ESI and MALDI mass spectrometry are highly sensitive techniques capable of detecting analyte concentrations down to the femtomolar range and are often used as complementary techniques (Banoub *et al.*, 2004, Banoub *et al.*, 2003, Schober *et al.*, 2011) for the same samples. There are however some major differences between the two techniques, one of which is the state in which sample is introduced to the ion source. While ESI requires solvated sample, MALDI is dependent on the sample being in a solid state. This aids in the explanation of why when used in combination with liquid chromatography (LC), ESI is capable of effective quantitative analyses (Cohen *et al.*, 2006). Despite the fact that there have been attempts to successfully utilise MALDI for quantitative analyses (Biroccio *et al.*, 2005, Grant *et al.*, 2003), the heterogeneous nature of the co-crystallised sample/matrix mixture poses a great challenge for this to be successfully completed. This is partly due to the fact that the absolute position of the laser beam on the sample has the ability to significantly influence the quality of the resulting spectra (El-Aneed *et al.*, 2009). MALDI has also been interfaced with LC, an idea partly motivated by the fact that in contrast to ESI, MALDI is robust with respect to the modest presence of salts and/or detergents. With ESI, contaminants in the sample can compete with analytes of interest as they adhere to the tubing through which sample is infused. MALDI however does not have this problem as analytes are separated from both the matrix and potential contaminants during ionisation (Breaux *et al.*, 2000, El-Aneed *et al.*, 2009, Fung *et al.*, 2004, Kallweit *et al.*, 1996). Another major difference between ESI

and MALDI is that ESI tends to produce multiply charged ions in biomolecule analysis, such as peptides and proteins, and as a result ESI theoretically has unlimited mass range as large proteins will have lower m/z values depending on the number of charges on the ions. MALDI however tends to produce singly charged entities, creating a great limitation in biomolecule size detection, but being of great advantage in the simplification of analyses of complex mixtures of proteins, carbohydrates or lipids (El-Aneed *et al.*, 2009). At low molecular mass there is interference in the mass spectra due to matrix adducts and from low mass sample components that dominate the spectra. Lastly, with the use of an additional technique known as MALDI Imaging Mass Spectrometry (MALDI-IMS) there is one major advantage that MALDI has over ESI, which is the ability to perform spatial resolution of region-specific molecular analytes directly from biological samples (Norris and Caprioli, 2013).

1.1.7 MALDI-IMS

The visualisation of complex biological systems has long been an important aspect in research, with new imaging technologies playing a key role in furthering critical knowledge and understanding. Biological tissue began being imaged and studied in the 17th century by way of light microscopy which ultimately revealed the cell as a vital component in biology, as reported by reviewing the history (Amos, 2000). Since then imaging has advanced extensively utilising a wide range of measurable physical phenomena. Today many imaging options are available in the field of biosciences including microscopy, radiography, ultrasonography and magnetic resonance imaging. MALDI-IMS has a number of advantages over such imaging techniques. Firstly, in IMS no labelling or structural modification of sample analytes is required for detection, which helps to avoid potential modifications (chemical and/or physical) to native molecules of interest. It also removes concern regarding the degree of accuracy of target-specific reagents and allows imaging and analysis of known or unknown compounds present in the sample without labelling. These unknown compounds may then be identified using tandem MS. Secondly, IMS has the ability to monitor thousands of molecules within a single experiment, which not only saves run time and cost, but is also crucial to analysing molecules of multiple pathways or components in systems biology. Although each imaging technique has its own

advantages and disadvantages and a unique ability to contribute to biological research, there is currently no tool comparable to that of MALDI-IMS with respect to the quality and quantity of spatial molecular imaging offered (Hendee *et al.*, 2003, Norris and Caprioli, 2013).

MALDI-IMS is a sensitive and robust imaging technique that allows for label-free spatial detection, relative concentration and location of peptides, proteins, lipids and small molecules such as drugs, sugars and metabolites within tissue sections. MALDI-IMS technology has the ability to make region-specific molecular measurements, and therefore allows for the rapid detection of molecules directly from complex, biological tissues. This allows for the collection of detailed data ranging from subcellular to multi-cellular levels and from organs to whole biological systems (Chughtai and Heeren, 2010, Cornett *et al.*, 2007, McDonnell and Heeren, 2007, Pacholski and Winograd, 1999, van Hove *et al.*, 2010).

The MALDI-IMS workflow although relatively straightforward, has critical steps in the sample preparation phases that need to be performed precisely to ensure the maintenance of sample integrity and spatial organisation, and to attain optimal imaging results. The MALDI-IMS process begins with the sectioning of fresh frozen sample tissue, ideally being snap-frozen and preserved as soon as possible after harvesting. Formalin-fixed paraffin embedded (FFPE) tissues pose a problem in mass spectrometric proteomic analyses due to protein cross-linking and therefore despite research efforts supporting these analyses, FFPE tissues are generally avoided (Gorzolka and Walch, 2014, Lemaire *et al.*, 2007, Metz *et al.*, 2004). Fresh frozen tissue is usually sectioned at 3 - 20 μm (most often at 10 - 12 μm) as most cellular content has been exposed by the blade at this thickness, and insignificant spectral difference is observed in thicker sections. Furthermore, if sections are cut too thick, they are prone to build-up charge on the surface during laser ablation, negatively influencing the quality of the resulting mass spectrum. Although thinner sections are more difficult to handle without tearing, they have been found to be ideal for proteomic analysis (Grove *et al.*, 2011, Sugiura *et al.*, 2006). Sectioned tissue is thaw mounted onto a specialised electrically conductive glass slide which is required to prevent the accumulation of surface electrostatic charges (also referred to as sample charging) as well as to support the potential that is required to accelerate sample ions into the source of the mass spectrometer. Indium-tin oxide (ITO) coated

glass slides are typically used for this purpose, and being transparent they have the added advantage of being used for histological staining and microscopic analysis post MS analysis (Chaurand *et al.*, 2004, Norris and Caprioli, 2013). A wash procedure may be performed on the tissue to remove potential chemical contaminants from the tissue surface in order to avoid downstream problems such as crystallisation and ion suppression that may compromise the sensitivity required to detect less abundant species. The exact procedure used is dependent on the class of target analytes, but typically consists of a fixation procedure involving increasing percentages of ethanol (Aerni *et al.*, 2006, Chaurand *et al.*, 2004, Seeley *et al.*, 2008). Depending on whether the specific analysis requires enzymatic digestion of proteins, on-tissue digestion may be done at this point, whereby trypsin is robotically spotted or sprayed onto the tissue surface and is incubated at 37 - 50°C (Casadonte and Caprioli, 2011, Groseclose *et al.*, 2007). A suitable matrix is then selected for optimal analyses as previously described. For high quality imaging analyses, it is imperative that the matrix is uniformly applied to the sample surface to avoid bias across tissue regions. Such homogeneity is usually accomplished by use of a robotic sprayer which applies matrix to the tissue surface in a controlled, repeatable and programmable manner, with minimal molecule migration. Matrix is typically applied in a 1:5000 ratio to ensure sufficient formation of matrix-analyte co-crystallisation (Chaurand *et al.*, 2005).

The sample is then loaded into the MALDI mass spectrometer for analysis as described in Section 1.1.7.2. Sample molecules are desorbed directly from discrete pixels of the tissue following laser irradiation in an ordered array across the sample surface. Each pixel of laser exposure leads to a combined full mass spectrum from all charged species ablated from that spot of the tissue sample. This process ultimately results in a large collection of mass spectra that can be spatially correlated to ion images of the complete imaged tissue surface (Figure 1.8) (Schwamborn and Caprioli, 2010b, Spraggins and Caprioli, 2011).

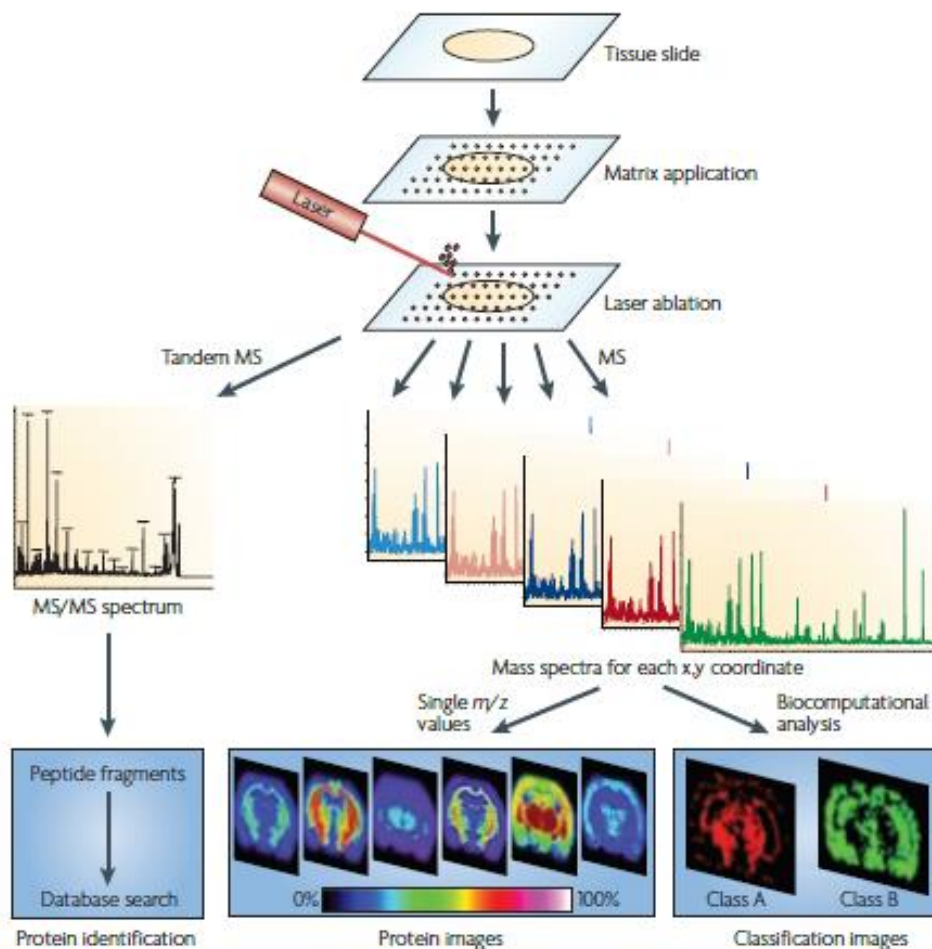


Figure 1.8: Typical workflow for MALDI-IMS. Sectioned fresh frozen tissue is thaw mounted onto a conductive ITO glass slide, after which ionisable matrix is evenly applied onto the tissue surface. Energy from the laser of the mass spectrometer is used to ionise and volatilise the matrix-bound sample which is then accelerated into the mass spectrometer for analysis. Ultimately, mass spectra are produced where each m/z value can be spatially correlated to the molecular ion images of the tissue sample (rainbow scale represents relative quantitative intensity) and in conjunction with statistical analysis, visualise selected mass images. With tandem mass spectrometry performed, the resultant peptide mass spectra may be searched against a database for protein identification (Schwamborn and Caprioli, 2010b) (with permission).

MALDI-IMS is a powerful and unique ‘omic’-based technique capable of analysing the spatial distribution of various molecules including peptides, proteins, lipids and metabolites across intact tissue sections, providing a large amount of data with minimal sample preparation time. This versatile imaging tool has become a leading method capable of analysing the spatial distribution of hundreds of intact molecular features directly from the tissue with minimal workup due to its high sensitivity,

tolerance for salts and other contaminants, minimal fragmentation and simple sample preparation (label-free detection). Data interpretation is relatively easy due to the majority of singly charged analytes allowing multiple compounds to be correlated with histological or clinical features. MALDI-IMS makes tracking physiological changes at a molecular level possible and therefore provides an ideal basis for kinetic 'omic' analyses, a typical example being the kinetic changes during acute wound healing (Chughtai and Heeren, 2010, Manzanares-Meza *et al.*, 2017).

1.1.8 Synopsis

Acute wound healing is a complex process characterised by a cascade of sequential yet overlapping events occurring within controlled time frames and although well-researched is yet to be completely defined. By using modern technology, it is hoped that key features can be revealed as potential indicators of progression of wound healing that can be identified for later use in non-healing chronic wounds, where the causes of this healing inefficiency remain unknown.

The work reported in this thesis set out as a proof of concept study to use current mass spectrometric techniques to define key kinetic changes occurring during acute wound healing across proteomic, metabolomic and lipidomic bases. This was done using MALDI-IMS of histological sections of resected wound tissue collected at pre-selected time intervals during the wound healing progression of full-skin thickness wounds (wounds healing by second intention) to determine spatial information in addition to revealing potential features to characterise the defined phases of acute wound healing.

One aspect that was unknown when setting out with this study was the capability of the MALDI-IMS technique to provide the type of information required and the ability to analyse the data collected, to directly identify specific features that could then be targeted using more traditional mass spectrometric techniques like ESI-MS/MS. If MALDI-IMS could provide direct molecular identities with spatial information, then the critical features within very small regions of the wound could be easily monitored compared to the homogenised type of sample analysis performed when doing ESI-based analysis.

By better understanding the kinetics of acute wound healing, and isolating regions of the wounds where critical events were taking place, key time points and features (molecules) of interest could be linked to spatial regions within the dynamically changing environment. This could provide a foundation for a potential acute wound healing model or template for key indicator features. The data collected from MALDI-IMS was to be matched to data collected from adjacent wound tissue sections analysed using ESI-based mass spectrometric methods that were to be optimised to enable semi-automated sample processing.

1.1.9 Collaborative study

It should be noted that this study was done in parallel with another wound healing study being performed on the porcine model, entitled “An investigation of wound dynamics using Carbohydrate Derived Fulvic Acid gel (CHD-FA) alone and Absorbatox®/CHD FA combination wound dressings”, that is under the supervision of Professor Cromarty. The collaborative aspect between the two projects involved inducing full-skin thickness wounds in the porcine model and the evaluation of three potential healing enhancement agents. The study was done to assess dressing materials to potentially enhance wound healing rates.

The collaborative study used the same resected control wound tissue as well as tissue from treated wounds, for histology and morphological change assessment.

This study used the untreated control wounds to perform a series of mass spectrometer-based ‘omics’ assays and histological staining to determine whether key features could be identified using closely controlled conditions of wounding and the healing processes.

This research study investigated changes that could be identified using MALDI-IMS in the proteome, metabolome and lipidome of the untreated control wounds during the wound healing process in a porcine full-skin thickness wound model. The full wound and at least 3 mm of the surrounding healthy tissue was resected at different pre-selected time points during a 16-day healing process. This was done to define the changes taking place within regions of interest within the wound tissue including the associated kinetics of the proteins, metabolites and lipids involved in the healing

process. It was expected that different features would dominate in different regions and during different phases of the healing process.

Although the basis of this thesis focuses mainly on the untreated, negative control wounds (with no active ingredients in the wound dressings), there are instances where experimentally treated wounds were also assessed using the MALDI-IMS technique for 'omic' analysis.

1.2 Scope of the Study

1.2.1 Study aim

To apply complementary mass spectrometric techniques to assess potential key feature changes of acute wound healing that can be used to characterise the different wound healing phases during full-skin thickness wound recovery.

1.2.2 Study objectives

- i. To perform an animal study using the porcine full-skin thickness wound model to create, visually monitor and excise acute wounds at six pre-selected sequential healing times over a 16-day recovery period
- ii. To histologically confirm wound healing progression using haematoxylin and eosin and Picrosirius red stains on FFPE sections of the resected tissue collected at six pre-selected sequential healing times over a 16-day recovery period
- iii. To perform mass spectral tissue imaging and tissue profiling across proteomic, metabolomic and lipidomic bases using MALDI-IMS on frozen histological sections taken from six pre-selected sequential healing times representing overlapping healing phases
- iv. To perform statistical analyses including principal component analysis of collected data to find correlations, visualise dominant patterns and reveal potential wound features occurring across all 'omic' bases over a 16-day recovery period

- v. To optimise in-solution sample preparation methods and compare this to in-gel and on-tissue digestion techniques for analysis of frozen sections of wound tissue
- vi. To perform in-solution digestion using an optimised workflow paired with LC-ESI-MS/MS (QTOF) and bioinformatics analysis on six pre-selected sequential healing times to identify unique phase specific features of the acute wound healing process

Chapter 2: Animal Study

2.1 Introduction

The porcine wound healing model is the most reliable animal model for healing studies as pig skin displays many similarities to human skin, both anatomically and physiologically, as well as in the way that wounds heal (Pomahac *et al.*, 2007, Seaton *et al.*, 2015, Sullivan *et al.*, 2001). In this study round, full-skin thickness wounds were created via punch biopsy along the dorsum of three healthy pigs. Wounds were photographed to scale and excised or treated and dressed according to a randomised schedule 3, 5, 8, 10, 12 and 16 days post-wound creation. Scaled measurements were used to determine the healing rate of the kinetic acute wounds over six time points. Wounds were immediately snap frozen in liquid nitrogen upon excision and stored at -80°C. Frozen whole wounds were cut in half using a hydraulic cutter. For histological analysis, each wound half was formalin-fixed and paraffin-embedded for microtome sectioning. Using haematoxylin and eosin (H&E) and Picrosirius red (PR) with bright-field and polarised light microscopy, the histological changes occurring in the porcine acute wound over 16 days were visualised.

2.2 Materials and Methods

2.2.1 Animals and housing

The study was performed with the Animal Ethics Committee approvals of the University of Pretoria (project number: H013-15) and the University of the Witwatersrand (clearance certificate number: 2015/07/30/C) (Appendix I) and carried out at the Central Animal Service (CAS) facility at the University of the Witwatersrand that has been approved for housing pigs, under the supervision of Professor D. Cromarty and M. Costello. Three young, female, large, white pigs of approximately 36 kg (36.1 kg, 36.0 kg and 36.65 kg upon arrival for pigs A, B and C respectively) were used. The pigs were housed singly in suitably sized animal pens with fresh straw bedding as well as a heat lamp as described in the facility's standard operating procedures. Each pig was in visual contact with the other pig/s and was provided enrichment objects (e.g. old tyres, raisins hidden beneath the straw bedding etc.).

The animals were fed standard pig feed twice daily with an *ad libitum* access to water. The animals were acclimatised together in the facility and exposed to regular human physical contact for one week prior to commencement of the study.

2.2.2 Anaesthesia and analgesia

Prior to surgery, the animals were anaesthetised using injectable (deep IM) Dormicum (midazolam) (0.3 mg/kg) combined with AnaketV (ketamine) (11 mg/kg) and transferred to the theatre complex. Anaesthesia during surgery (for wound creation and excision) and all subsequent wound dressing changes was maintained using an inhalation agent, Isofor (isoflurane) introduced using a snout-adapted mask connected to an anaesthesia machine. Post-operatively, Temgesic (buprenorphine) (0.3-0.6 ml) was administered as an analgesic for pain management.

2.2.3 Acute wound generation and sample collection

This study was an intervention study where surgically induced wounds were monitored for 16 days post-wounding. Prof. Kennedy Erlwanger and Dr Kimberly Jardine, veterinarians at the CAS of the University of the Witwatersrand, performed the surgeries. Prior to the wound creation, the pigs' backs were shaved with an electrical clipper, scrubbed with soap and water and washed a second time with chlorhexidine. Twenty 8 mm round, full-skin thickness wounds were then created via punch biopsy ('Biopsy Punch', Kruuse, Langeskov, Denmark) (Shevchenko *et al.*, 2008) along the dorsum of the three healthy pigs with 10 wounds along each psoas muscle ridge (total of 60 wounds for all three pigs). Each pig acted as its own control. With respect to the collaborative healing study occurring (see Section 1.1.10), four different treatment groups were randomly allocated to five wounds each per pig (*i.e.* 12 wounds per treatment in total). The four treatment groups consisted of:

- i. Negative (untreated) control (a solution containing the thickening agent used in the CHD-FA based treatments)
- ii. 4.6% carbohydrate-derived fluvic acid (CHD-FA)
- iii. 4.6% CHD-FA + zinc chloride and
- iv. 4.6% CHD-FA + other organic compounds (proprietary ingredients: D-panthenol and citric acid)

A volume of 50 µl of each treatment was pipetted along the inner wound circumference, after which the wound was covered with medical grade gauze and OPSITE adhesive film. An elasticised protective, body-fitting sock spanning the length of the pigs' torso was used to ensure that all dressings remained in place.

As the localised concentrations of many structural molecules and metabolites can vary within the wound area during the healing phase, the collaborative study needed to compare wounds of equivalent healing phases. A randomised selection of the four different wound treatments (three different treatments and the control) were excised in either duplicate or triplicate (by random wound allocation) per time point by the veterinarian. Day 1 was deemed as wound creation and selected wounds were then subsequently excised on Days 3, 5, 8, 10, 12 and 16 according to a randomised schedule, during which the three different pigs were rotated (Figure 2.1). At each of these six time points all three pigs were subjected to general anaesthesia either for wound excision as mentioned (one pig per time point) or for dressing changes and retreatment (two remaining pigs). Wound excision was done using a surgical blade whereby the wound was excised within a canoe shaped biopsy that included the complete wound with at least 3 mm of surrounding healthy tissue in all dimensions. Once a wound was excised it was no longer used in the study and the biopsy area was sutured closed and covered with antiseptic ointment (F10 Germicidal Barrier Ointment, Johannesburg, South Africa) followed by medical grade gauze and OPSITE adhesive film (Figure 2.2). Immediately after excision, the removed wound tissue was snap frozen in liquid nitrogen and stored in pre-labelled plastic containers at -80°C.

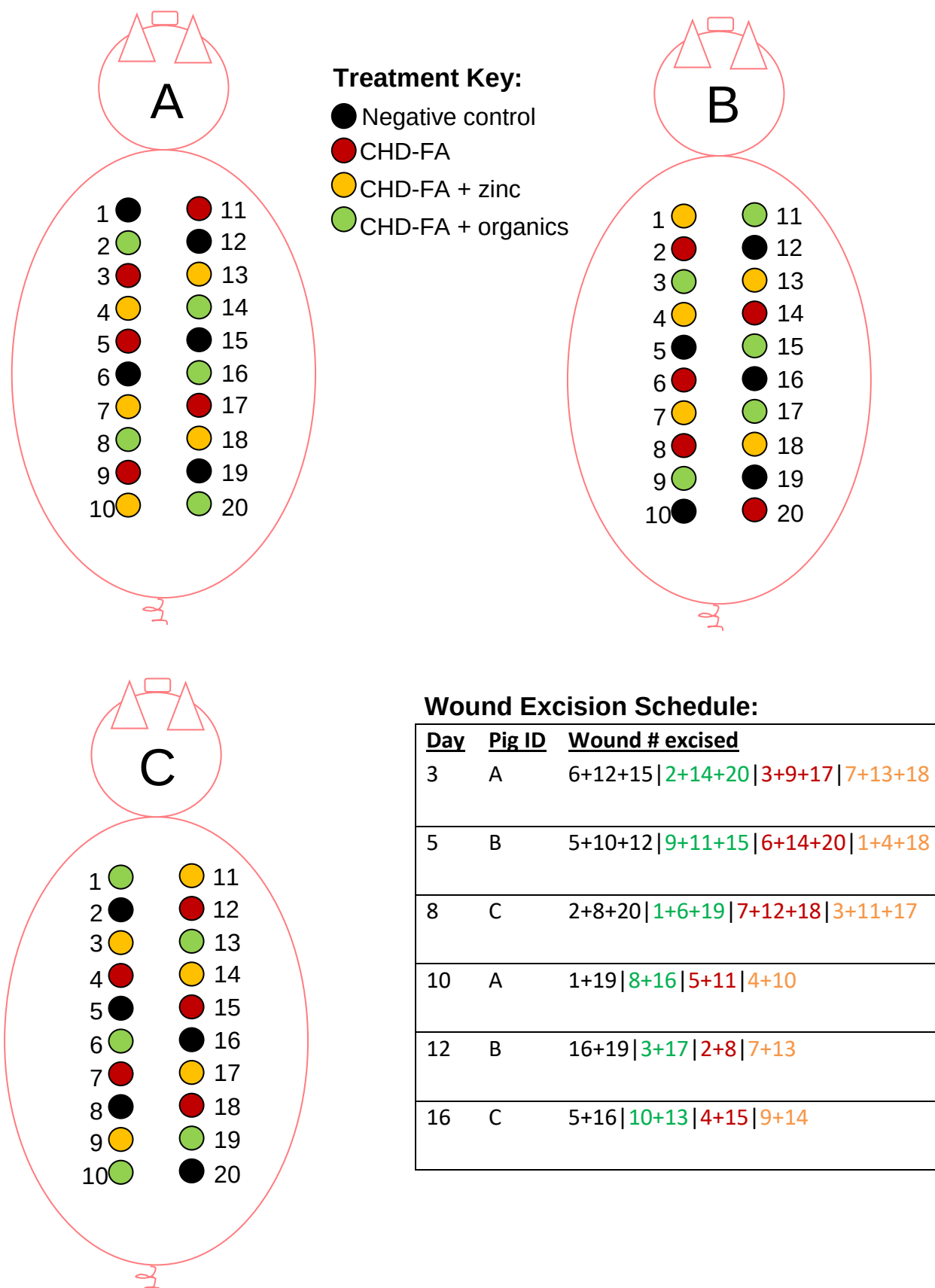


Figure 2.1: Wound designation and excision schedule. Wounds were created on Day 1 and were excised at six different time points over 16 days. Three pigs (A-C) were rotated according to a randomised wound excision schedule. At each time point wounds were either excised from one of the pigs or were retreated and redressed for the remaining two pigs.



Figure 2.2: Overview of animal study. (A) Pigs were acclimatised for one week prior to wound creation where they were housed together in a single pen. Pigs were placed under general anaesthesia (B) after which full-skin thickness wounds were created along the dorsum by punch biopsy, using a template (C). Each randomised wound treatment was pipetted along the wound circumference (D) at wound creation and was reapplied on Days 3, 5, 8, 10, 12 and 16. Wounds were excised in a canoe-shape using a surgical blade (E) and were immediately snap frozen upon excision. Once a wound was excised it was sutured closed (F) and excluded from the study. Excised and treated wounds were covered with gauze and OPSITE adhesive film (G) and a body-fitting sock was secured along the length of the pig's torso (H). For the duration of the study pigs were singly housed to minimise potential physical contact with the wounds (I).

2.2.4 Post-operative monitoring

Animals were monitored post-surgically for at least three hours or longer when the recovery appeared to be delayed. Twice daily monitoring that included food and water intake, general behaviour and signs of stress or pain was performed by trained animal care-givers who were familiar with the study. Wounds were inspected for signs of infection at each procedure when the dressings were removed and changed. At these times, wounds were also photographed under consistent lighting conditions against a scaled ruler for assessing the degree of closure and to record the status of the wound (Figure 2.3). Standard monitoring forms and weight charts were used to ensure that all information was recorded (Appendix II). The allocated veterinarian provided pain relief as required. No significant weight loss (a loss of 10% or more of original body weight), wound infections, allergic reactions or severe inflammatory responses occurred in response to the experimental treatment and thus no additional treatment, wound exclusion or animal withdrawal was required.

Following the completion of the experimental phase of the study, the pigs were euthanised.

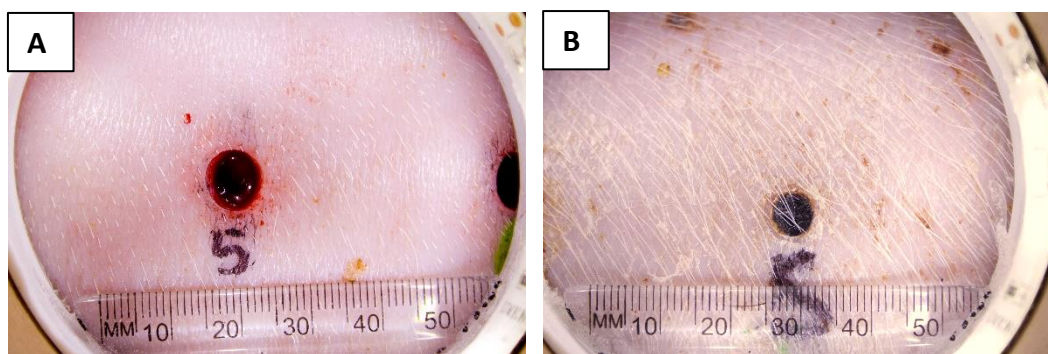


Figure 2.3: Wounds photographed to scale. Full-skin thickness wounds were photographed with a scaled ruler to allow for accurate measurement of wound contraction on Days 1 (A), 3, 5, 8, 10, 12 and 16 (B).

2.2.5 Wound sample snap freezing

Wound samples were snap frozen and stored at -80°C to preserve the samples in their original form and to avoid morphological distortions or damage due to autolysis, tissue drying or putrefaction. Samples were not preserved using a fixative as this causes protein cross-linking that interferes with proteomic analysis. At a later stage,

the whole fresh frozen wound samples were cut in half longitudinally (along the length of the canoe-shape from tip to tip) using a hydraulic cutter where one half of each excised wound was fixed in formaldehyde for histological analysis, while the other half was kept frozen at -80°C for future 'omic' analysis.

2.2.6 Wound healing rate

The scaled photographs of the wounds taken on Days 1, 3, 5, 8,10, 12 and 16 were assessed using ImageJ software (National Institutes of Health, Maryland, USA) (Schneider *et al.*, 2012). Wound surface area and perimeter were accurately measured and tracked over the 16-day study period. Based on the wound excision (and exclusion) schedule (Figure 2.1) only two untreated, control wounds remained on Day 16 that allowed for measurements over the complete study period. Wound healing rate was calculated as the relative percentage of wound surface area and perimeter at each one of the six time points compared with their respective Day 1 (wound creation) measurement *i.e.*

$$\text{Wound healing rate} = \frac{\text{Day N surface area or perimeter}}{\text{Day 1 surface area or perimeter}} \times 100\%$$

A wound healing rate percentage was calculated for each of the two control wounds based on surface area and perimeter over 16 days. The calculated percentages were then averaged for wound surface area and perimeter at each of the six time points.

2.2.7 Histological analysis

Whole, fresh, frozen wounds were cut in half using a hydraulic cutter where one half of each excised wound was fixed in 4% formaldehyde and 0.1 M phosphate buffered saline (PBS) for 48 hours, in preparation for paraffin wax embedding. Wounds were then washed three times in PBS (30 minutes each time) and dehydrated in increasing concentrations of ethanol (50% for 30 minutes, 70% and 90% for 60 minutes each and 100% overnight). The ethanol was cleared in a 1:1 xylene:ethanol solution for 30 minutes, followed by 100% xylene for 2 hours. The samples were then infiltrated with paraffin wax for 1 - 2 hours at 60°C, embedded in wax blocks and cooled to 4°C to allow for easy sectioning. Formalin-fixed paraffin-embedded (FFPE)

tissue was sectioned using a RM2255 microtome from Leica (Wetzlar, Germany) where sections were cut at 3 - 5 µm thick, depending on tissue quality and ease of cutting.

After mounting onto standard glass microscope slides, the FFPE tissue sections were prepared for staining. Samples were de-waxed in 100% xylene twice for 5 minutes each. Rehydration was done using decreasing concentrations of ethanol (100% for 2 minutes twice, 90% and 70% both for 1 minute each) and lastly distilled water (1 minute). Serial sections were stained with haematoxylin and eosin (H&E), and Picrosirius red (PR) where staining times had been optimised.

H&E is the most widely used histological stain and has been utilised for over a century. It was used in this study as it allows for the visualisation of general tissue structure as well as identification of inflammatory cells within wounds. Haematoxylin stains cell nuclei a blue colour, while eosin stains various cytoplasmic components a pink colour, allowing for the visualisation of cytoplasmic, nuclear and extracellular matrix components (Fischer *et al.*, 2008).

H&E staining was based on standard procedures (Fischer *et al.*, 2008) where samples were first stained in haematoxylin for 10 minutes, followed by Scott's Buffer (2 g potassium bicarbonate and 20 g magnesium sulphate in 100 ml distilled water) for 8 minutes and finally dipped in eosin and washed in distilled water for 1 minute. The tissue was then again dehydrated by dipping in increasing concentrations of ethanol (70%, 90% and 100% respectively) and mounted using a xylene-based mounting media and a glass coverslip.

The PR staining method used was based on previous protocols as described by Puchtler *et al.* and Junqueira *et al.* (Junqueira *et al.*, 1979, Puchtler *et al.*, 1973).

Briefly, nuclei were first stained in haematoxylin for 10 minutes and washed for 1 minute in distilled water. The tissue sections were then stained for 1 hour in PR, washed in two changes of acidified water (0.5% acetic acid) and air dried. Lastly, the tissue was dehydrated thrice in 100% ethanol and cleared in xylene. Samples were mounted using a xylene-based mounting media and a glass coverslip.

PR is used to stain collagen a red colour with a birefringence of yellow or green fibres appearing under polarised light (Dapson *et al.*, 2011). PR allows for the

differentiation of collagen fibre types by enhancing the natural birefringence of the different collagens. Under polarised light, thin type III collagen fibres exhibit a green colour, while the thicker type I collagen fibres appear a yellow-orange to orange-red colour. This difference in birefringent appearance is not only due to fibre thickness but also differential fibre deposition and aggregation. Differential collagen appearance is also used to determine the collagen density, where poorly packed fibres appear green in colour and tightly packed fibres appear an orange-red colour (Hirshberg *et al.*, 1999, Junqueira *et al.*, 1979, Rich and Whittaker, 2005).

All stained tissue sections were visualised using light microscopy (Nikon Optiphot transmitted light microscope and a Zeiss AXIO Imager M2). For polarisation microscopy, a polarising filter was placed between the light source and the sample.

2.3 Results and Discussion

Please take note: Due to the collaborative nature of the animal study (see Section 1.1.10), photographs, wound measurements and histological analysis were done on all 60 wounds across all treatment groups. For the purpose of this specific study and thesis the focus was mainly on the untreated control wounds and the molecular 'omic' analysis thereof. Selected photographic and histological results relevant to this study only will be shown.

2.3.1 Wound photography

The acute wounds created along the dorsum of each pig were photographed to scale on Day 1 (wound creation), Day 3, Day 5, Day 8, Day 10, Day 12 and Day 16. Progression of healing could clearly be seen from the general morphology of the wounds and recorded photographically, where wound contraction, a decrease in wound depth and progressive scabbing were all evident. From Day 1 through Day 3 and Day 5, the wounds were very similar in appearance. Fresh blood was present in the wound space upon all wound dressing changes on these days with a minimal reduction in wound circumference over the first five days. Day 8 exhibited a marked difference in wound appearance where a well-formed scab was seen over the complete area of the wound space. From Day 8 to the end of the study, Day 16, the scab and wound area decreased in size with the scab moving upwards, closer

toward the skin surface and appeared to be resting atop newly formed granular tissue. A sequence of one of the untreated, control wounds over the 16 days is shown in Figure 2.4.

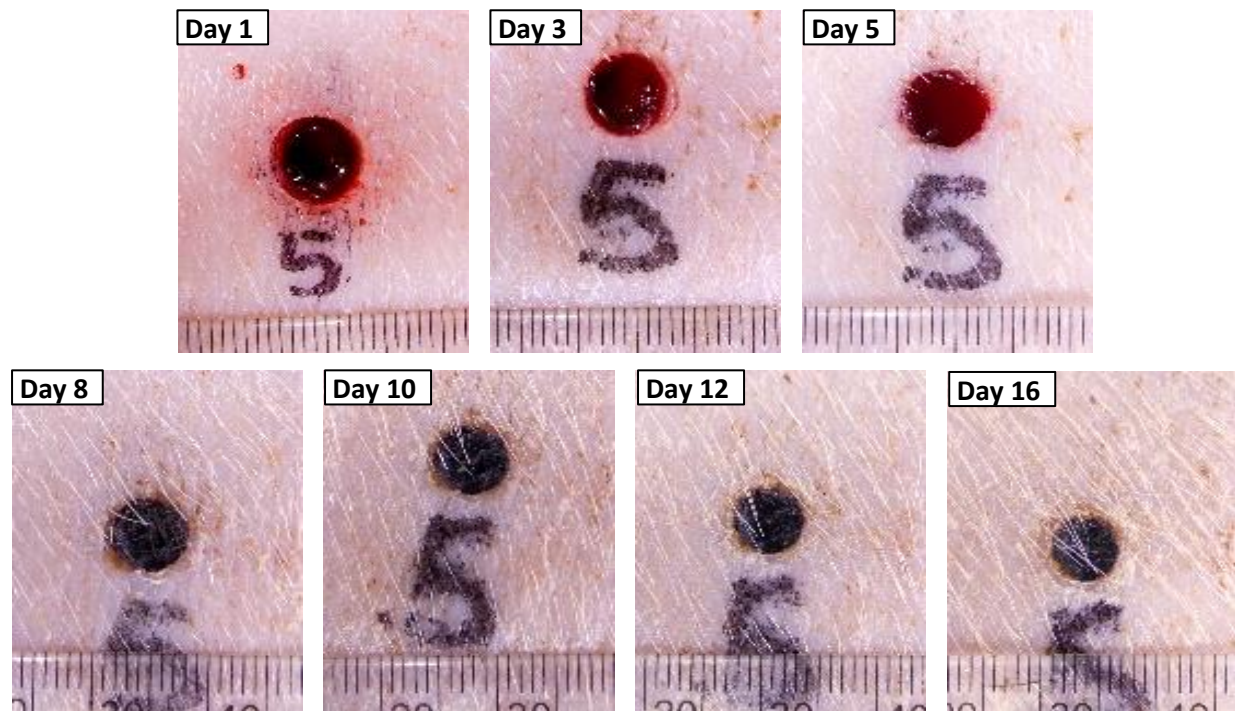


Figure 2.4: Scaled photographs tracking the progression of acute wound healing in the porcine model. Comparative photographs taken at constant focal length, lighting and with a scale bar to track one of the untreated, control full-skin thickness wounds at six time points over the 16-day study period. Slight contraction and reduction in wound depth can be seen from Day 1 to Day 5. On Day 8 the wound appears significantly different with the formation of a complete scab within the wound space and a progressive decrease in wound circumference through to Day 16.

2.3.2 Wound healing rate

ImageJ software was used to accurately measure the changing surface area and perimeter of acute wounds using scaled photographs taken on Days 1, 3, 5, 8, 10, 12 and 16. Due to pre-planned wound excision and exclusion, only two untreated, control wounds remained on Day 16 allowing for wound measurements over the whole study period. The surface area and perimeter of these acute wounds were measured and averaged in order to determine the healing rate over 16 days. The results are displayed in graph format in Figure 2.5. The size of the wounds decreased both in terms of surface area and perimeter over the study duration. From

Day 1 through to Day 16 the wounds seemed to heal predominantly by way of surface area reduction. The greatest reduction in wound size was seen from Day 5 to Day 8 for both surface area (reduction of 18%) and for perimeter (reduction of 15%). This supports the major visual change seen in the wounds in Figure 2.4. By Day 16 the wound perimeter was found to be 59% of its original size (healing rate of 41%), where the wound surface area was found to be 33% of its original wound size (healing rate of 67%). Although the round wound surface area was calculated using the formula πr^2 , which does not take wound depth into consideration, the wounds were photographed at such an angle that when using ImageJ, wound depth contributed to an increased radial measurement. *i.e.* the deeper the wound the greater the wound radius drawn from the wound centre to the wound edge. This supports the fact that wounds in the porcine model heal predominantly by new tissue deposition and re-epithelialisation (reduction in wound depth) as is seen in humans, rather than by wound contraction as is seen in the murine model (see Section 1.1.5.3).

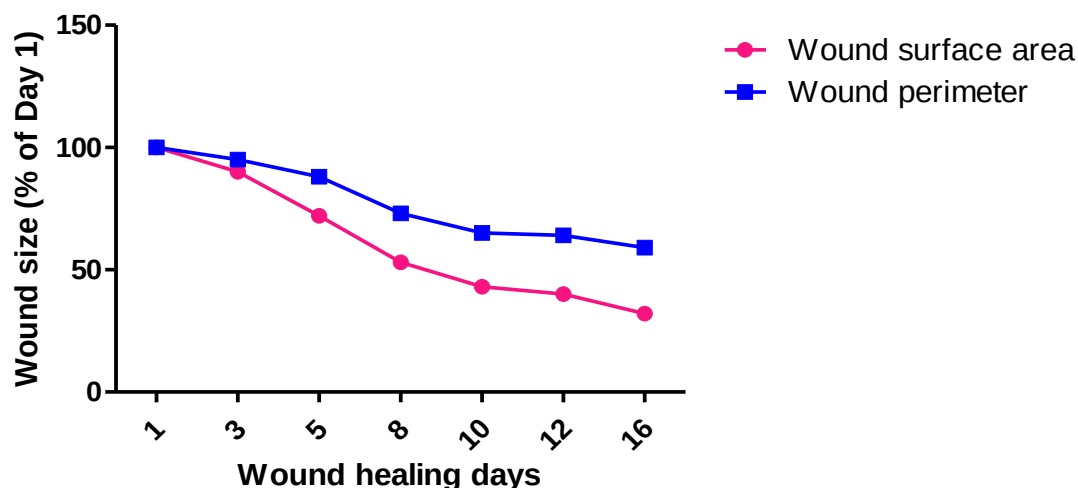


Figure 2.5: Wound healing rate expressed as a percentage of wound size on Day 1. A reduction in wound surface area and perimeter was seen from Day 3 with wound surface area decreasing at a higher healing rate than wound perimeter throughout the 16-day study. A maximum healing rate based on both surface area and perimeter was reached between Days 5 and 8.

2.3.3 Wound excision

Wounds were excised by the veterinarian by hand using a surgical blade at the six excision time points as mentioned above. The veterinarian cut around the wounds in a canoe-shape in such a manner that healthy tissue surrounded the wound upon excision (Figure 2.6). This approach, although successful, resulted in unavoidable variability in tissue excision size with variation in the amount of healthy tissue surrounding the wound. It was also found that the anatomical region in which the wound was excised from the dorsum played a role in the depth of wound excision. Wounds in the far upper and lower regions of the dorsum, which rested on less muscle tissue than those found in the mid-region, had less healthy tissue at their base once excised. It must be noted however that all variability occurred in the amount of surrounding healthy tissue only and thus did not affect the actual wound tissue. The unavoidable resultant variation in wound tissue:healthy tissue ratio did have some impact on the study which is addressed in Chapter 6.

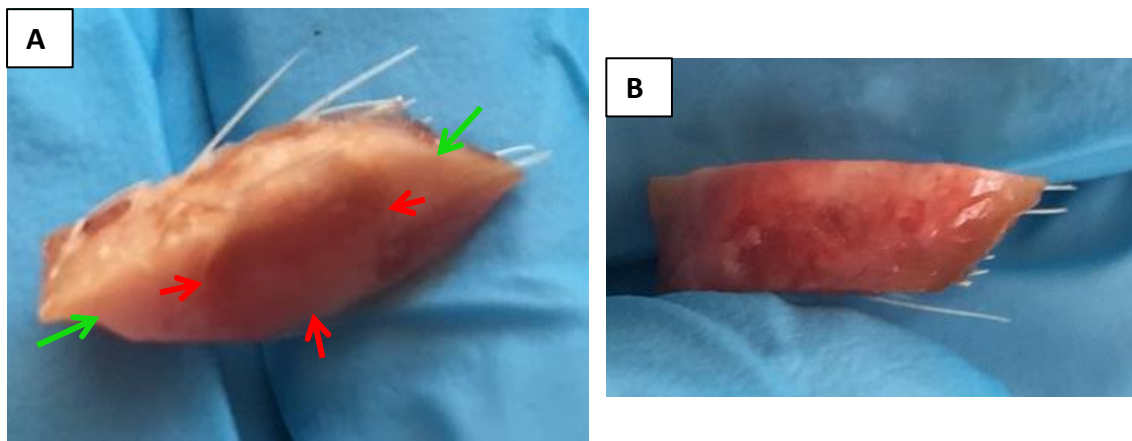


Figure 2.6: Excised wound surrounded by healthy tissue. Complete wounds with surrounding healthy tissue were excised in a canoe-shape using a surgical blade and cut in half longitudinally through the wound area. (A) A view from the epidermal and sagittal side of a divided wound excised on Day 16. The wound (red arrows) is surrounded by healthy tissue (green arrows). (B) A view of the healthy tissue from under the base of the excised wound.

2.3.4 Histological analysis

Acute wound healing is a kinetic process where wounds excised on Days 3, 5, 8, 10, 12 and 16 move predictably through the different phases of healing. This could

clearly be seen histologically by the varying presence of immune cells and changes in collagen deposition within the wound bed as visualised through the H&E and PR stained sections from each time point. Clear differences in morphology as well as reduction in wound depth and overall size could be seen as healing progressed, using both H&E and PR staining. Photographs of the actual wound tissue used for histological analysis both upon wound creation and just prior to wound excision, are shown in Figure 2.7 with kinetic histological observations shown in Figure 2.8. Briefly, on Day 3 a dense population of inflammatory cells were visualised along the border of the wound edge by H&E, indicative of the inflammatory phase of wound healing. No epidermal layer was present at the wound site at this time due to its complete removal by full-skin thickness punch biopsy (Figure 2.8 A). PR staining showed the wound to be a clear gaping space, void of collagen or any new tissue deposition, that had penetrated completely through the dermis into the subcutaneous fat of the hypodermis (Figure 2.8 B). Under bright and polarised light, the clean edge of the wound could be seen. The immediate non-wounded, healthy surrounding tissue consisted of thick, well-organised collagen bundles appearing red under bright-light (Figure 2.8 C). This was confirmed using polarised light where the surrounding tissue consisted mainly of thick yellow-orange type I collagen with thinner green type III collagen dispersed in between, representing the typical appearance of intact skin (Figure 2.8 D). At Day 5 a dense inflammatory cell infiltrate could be seen outlining both the wound edges and wound bed, showing an evolved inflammatory phase (Figure 2.8 E). A clear difference could be seen between the cellular makeup of the tissue immediately surrounding the wound site and more distant unaffected, healthy tissue. Minor inflammatory cell infiltration and cellular oedema were visualised in the tissue proximal to the wound site, which would be expected in acute wound healing (Figure 2.8 F). A lack of collagen in the wound space was confirmed by both bright-light and polarisation microscopy (Figure 2.8 G-H). By Day 8 the wound space had been covered with a large scab atop newly forming tissue (Figure 2.8 I). A new and almost completely formed epidermis was present, complete with rete ridges extending into freshly deposited granulation tissue. It was evident that significant proliferation had occurred over the three days of wound healing from Day 5 to Day 8, with an increasing presence of fibroblasts and keratinocytes seen throughout the forming dermal and epidermal layers. Inflammatory cells were still present in the newly deposited granulation tissue (Figure

2.8 J). This tissue more closely represented the immediate surrounding healthy tissue with respect to density, compared to three days prior, indicating progressive healing (Figure 2.8 F). The red colour of PR staining confirmed the presence of newly formed collagen filling the wound space. Using polarised light revealed that this collagen was predominantly sparse, unorganised collagen type III (appearing green in colour), which is to be expected for initial collagen deposition as takes place during wound healing (Figures 2.8 K-L). By Day 12 the epidermis had almost completely healed with the formation of rete ridges and a complete dermal-epidermal border present. Basement membranes (points of attachment of the epidermis to the dermis) were intact in the wound space with inflammatory cell infiltrates still present in the dermis. Cellular oedema in the keratinocytes of the epidermal layer and in tissue of the upper dermis was observed (Figure 2.8 M-N). While collagen within the wound space still appeared sparse and predominantly green when compared to the organised, thicker type I collagen in the surrounding healthy tissue, progression in deposition was evident (Figure 2.8 O). Further collagen type III deposition had occurred and appeared denser than on Day 8 (Figure 2.8 L). This was in addition to the increasing appearance of thicker, type I collagen (orange in colour) in more organised bundles, suggesting the beginning phases of remodelling (Figure 2.8 P).

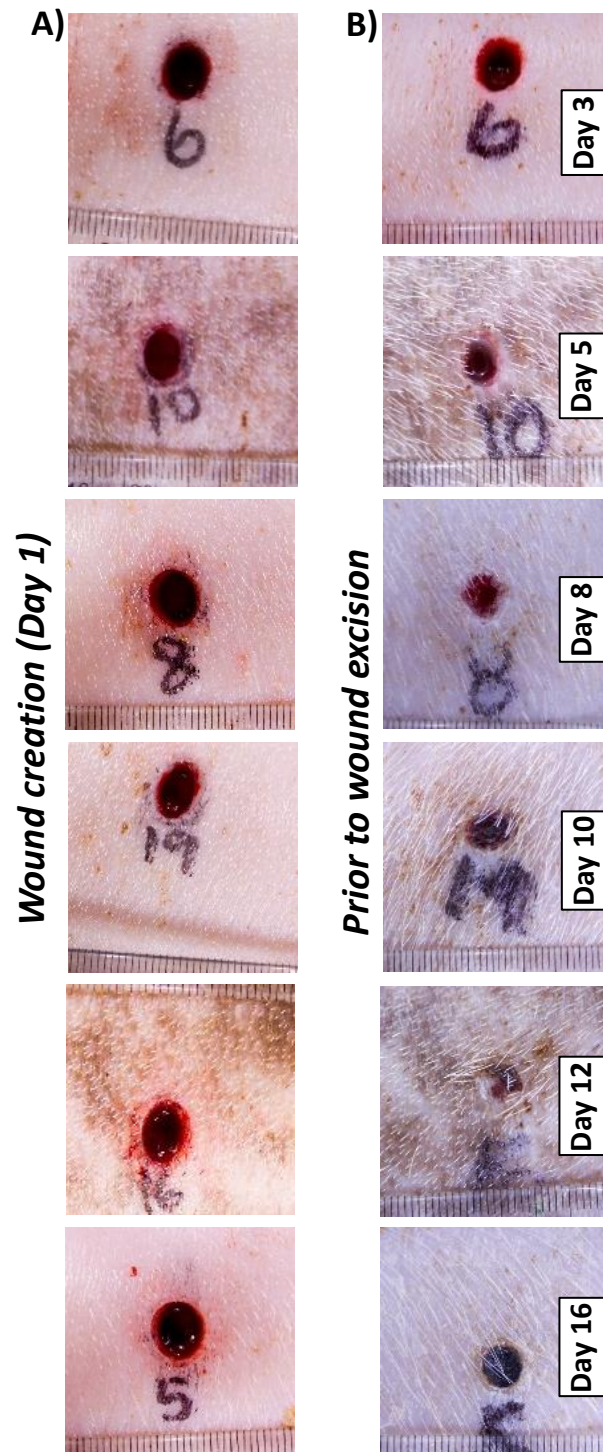


Figure 2.7: Scaled photographs of the wounds that were removed for histological analysis. (A) All wounds were created and photographed to scale on Day 1. (B) Wounds were later photographed just prior to each excision time point. The excised wound tissue was used for kinetic histological analyses across 16 days using H&E and PR staining.

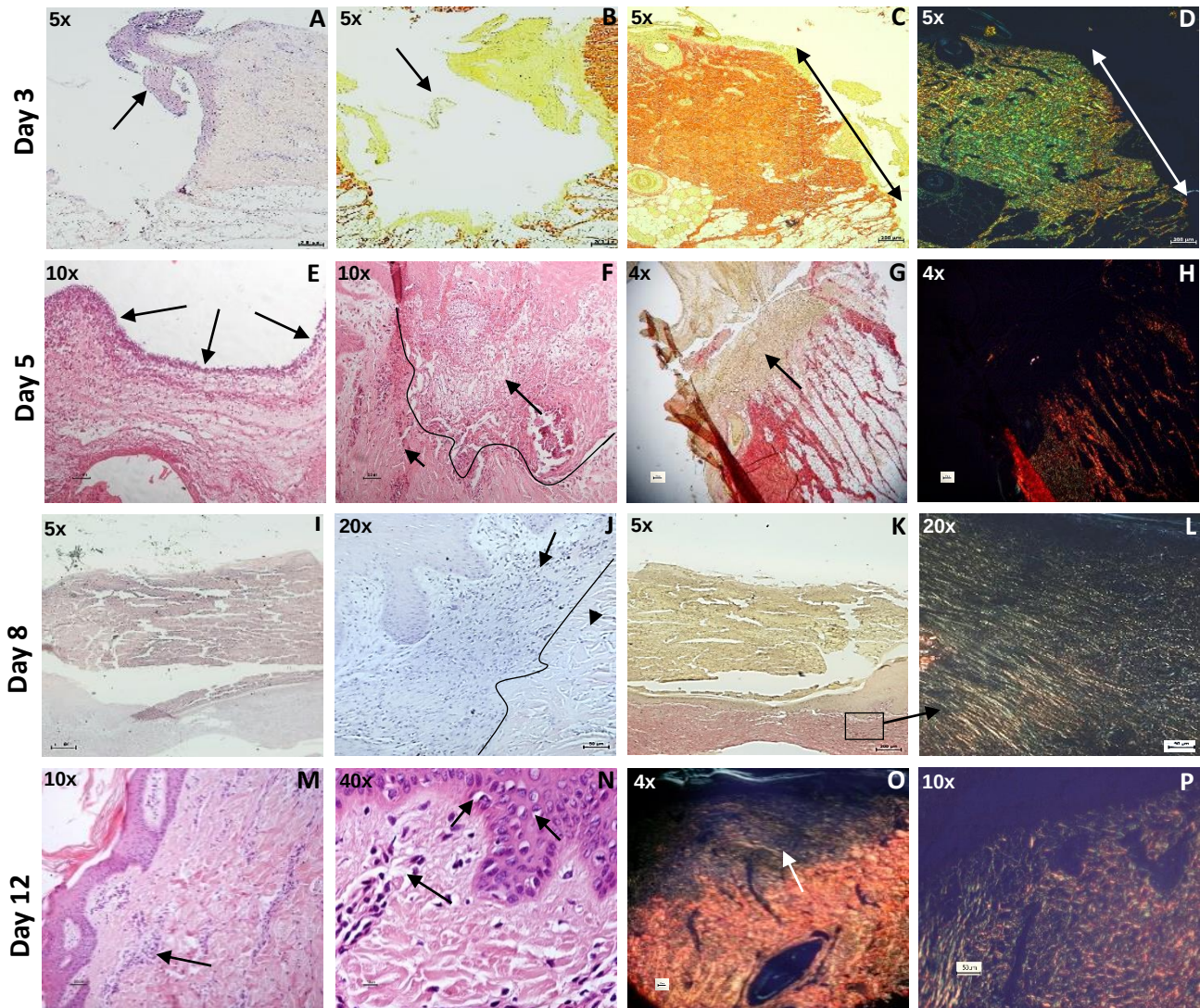


Figure 2.8: Kinetic histological analysis of acute porcine wounds. Wound tissue was stained with H&E or PR to visualise the progression of acute wound healing. (A-D) Day 3: (A) inflammatory cells have begun bordering the wound edge (arrow); (B) the wound space has penetrated into the hypodermis (arrow) with a clean wound edge (double arrows) visible under bright (C) and polarised light (D). (E-H) Day 5: (E) inflammatory cell infiltrate outlining the wound (arrows); (F) clear differences were visualised in the tissue immediately surrounding the wound (arrow) versus distal healthy tissue (arrowhead); (G-H) no collagen present in the wound space under bright or polarised light (arrow). (I-L) Day 8: (I) scab covers the wound area with (J) a newly formed epidermal layer present along with granulation tissue containing fibroblasts and inflammatory cells (arrow), appearing different to the surrounding tissue (arrowhead). (K) PR confirms new collagen deposition in the wound space and (L) polarised light reveals this as sparse, green stained type III collagen. (M-P) Day 12: (M) restored epidermis with rete ridges; presence of inflammatory cells (arrow) that when magnified (N) show cellular oedema in dermis and epidermis (arrows). (O) polarised light shows a filled wound site with predominantly green collagen (arrow) however (P) type I (orange-red) collagen fibres are being deposited in an organised manner, more closely representing healthy tissue. (A, E, F, I, J, M and N stained with H&E; B, C, D, G, H, K, L, O and P stained with PR.)

In this study, using both H&E as well as PR staining under bright-field and polarised light microscopy, the histological changes occurring over 16 days of wound healing could clearly be seen. Such changes included the presence and degree of inflammatory cells at the wound site, cellular oedema, structural markers and varying collagen deposition within the wound bed. Despite these successful visualisations, histology is limiting in both a qualitative and quantitative sense with respect to the molecular components of kinetic wound healing (Manzanares-Meza *et al.*, 2017). Minor quantitative changes may not be visualised, where certain molecular components will not be detected at all *i.e.* staining can only visualise specific parameters. Multiple stains may need to be used to obtain a more complete histological assessment of healing, which is not only labour intensive and time consuming but at times impractical and even impossible with limited samples available. With the rapid development of mass spectrometric technologies, especially that of imaging mass spectrometry (IMS), the amount and quality of data potentially available on acute wound healing is extensive and far surpasses that of classic microscopy techniques on many levels. MALDI-IMS allows for the direct analysis of endogenous wound healing-associated molecules within their native tissue environment. Together with the advantage of spatial information, which allows for isolation of molecules of the wound from those of surrounding healthy tissue, this unique imaging technique provides vital insight into the complex and continuously changing molecular makeup of acute wound healing (Cornett *et al.*, 2007, McDonnell and Heeren, 2007, Pacholski and Winograd, 1999, Taverna *et al.*, 2014).

Chapter 3: Proteomic MALDI Imaging Mass Spectrometry

3.1 Introduction

Matrix-assisted laser desorption ionisation imaging mass spectrometry (MALDI-IMS) is a relatively new, sensitive and robust imaging technique that allows for label-free detection with relative quantitation and spatial location of peptides, proteins, lipids and small molecules (such as drugs, sugars and metabolites) within histological tissue sections. MALDI-IMS makes tracking spatial variation at a molecular level possible and therefore provides an ideal basis for kinetic 'omic' analyses of the acute wound healing process (Manzanares-Meza *et al.*, 2017, Norris and Caprioli, 2013).

These 'omic' type analyses do however generate large, multidimensional datasets visualising the physical distribution of thousands of mass spectra with relatively high spatial resolution, which may, when dealing with multiple samples, overwhelm the analyses, thereby masking the ability of the technique. The complexity of spatial 'omic' information provided by the analysis of biological tissues is no exception and reduction of data to a manageable volume while retaining pertinent information is required. Principal component analysis (PCA) is a statistical technique capable of this data reduction as it has the ability to reduce data dimensionality while ensuring that critical information is not lost. In doing so, variations in related data is enhanced and strong patterns are easily visualised. PCA has proven itself to be a very useful tool in MALDI imaging analyses (Casadonte *et al.*, 2019, Deininger *et al.*, 2008, McCombie *et al.*, 2005, Taverna *et al.*, 2016, Trim *et al.*, 2008).

Chapters 3 and 4 demonstrate the capability of MALDI-IMS coupled with histology, optimised multivariate and feature statistical analysis to track the kinetic changes and to reveal features of interest observed during controlled acute wound healing experiments in the porcine model. This involved combined proteomic (Chapter 3), metabolomic and lipidomic (both in Chapter 4) assays from sequential histological sections taken from the same wound tissue. MALDI-IMS is advantageous in performing wound healing research where the spatiotemporal distribution of molecules using this technique is beneficial for accurate wound tissue analysis.

3.2 Materials and Methods

3.2.1 Wound tissue preparation

Fresh snap frozen wound tissue collected from the animal study (Section 2.2.5) required sectioning in preparation for MALDI-IMS analysis. For this cryosectioning, the frozen tissue was secured to the chuck of the cryotome using an industry standard polymer known as Tissue-Tek OCT (optimal cutting temperature) compound (Sakura, Tokyo, Japan). When fresh frozen tissues are sectioned during standard histopathology applications, they are completely embedded in OCT compound to provide the required sample rigidity and optimum cutting consistency for easy and successful sectioning, as fresh frozen tissue is more difficult to section cleanly and smoothly than FFPE tissues. For MALDI-IMS analysis however, precaution must be taken to ensure that no OCT compound residue is on the cut surface of the tissue. This is because OCT polymers ionise easily during the MALDI process and due to their relative abundance can cause significant ion suppression and contamination (Schwartz *et al.*, 2003, Seeley and Caprioli, 2008). To minimise interference of OCT polymers with analyte signals, only a small amount of the OCT was used to secure the base of the wound biopsy tissue to the cryotome chuck, leaving a safe distance between the surface of the tissue to be cut and the layer of OCT (Figure 3.1). Once the wound biopsy tissue was correctly orientated on the chuck, the entire unit including the chuck, tissue and OCT media were snap frozen in liquid nitrogen in preparation for sectioning.

The frozen wound tissue was sectioned at 12 μm thickness using a Cryotome E cryostat (Thermo Scientific, Massachusetts, USA) at the Department of Anatomical Pathology of the National Health Laboratory Service (NHLS) at University of Pretoria. Sections were thaw-mounted onto pre-cooled (-20°C) indium tin oxide (ITO)-coated glass slides (Bruker Daltonics, Bremen, Germany) in preparation for MALDI tissue imaging. Three sequential sections of the same tissue sample were placed onto one ITO slide at least 1 cm apart (Figure 3.2.) for independent proteomic, metabolomic or lipidomic analyses (see Chapter 4). Sample slides were stored at -80°C until MALDI analysis could be performed. A fourth adjacent section was placed onto a standard microscope slide for haematoxylin and eosin (H&E) staining according to standard

histology protocols (Section 2.2.7) (Fischer *et al.*, 2008). The H&E stained sections were then scanned at a low resolution using a Canon MB2040 flatbed scanner.

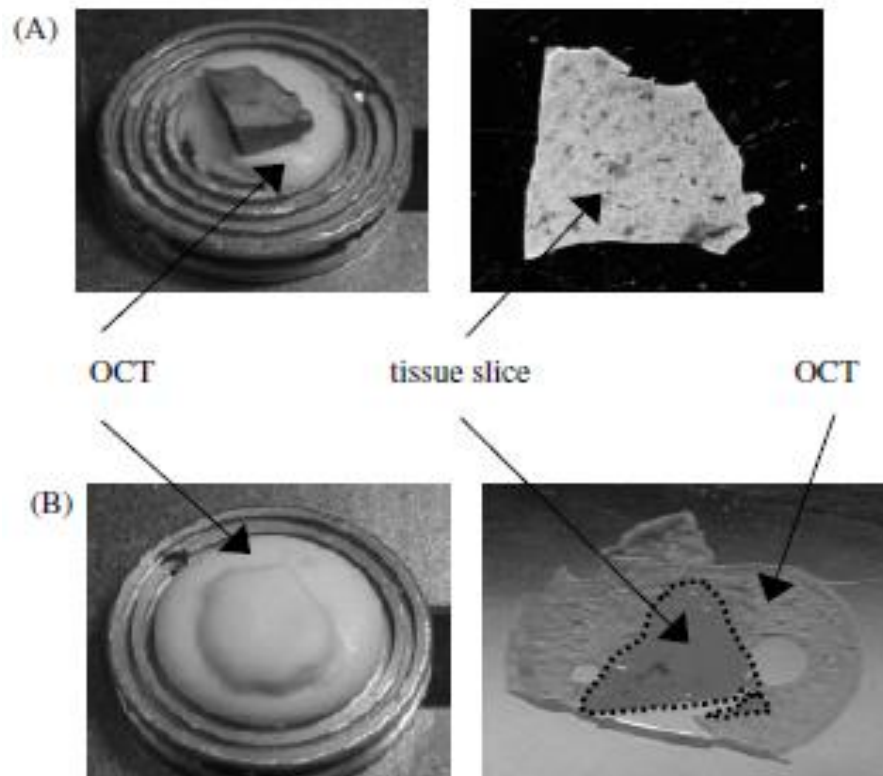


Figure 3.1: OCT placement for MALDI analysis. (A) For optimal MALDI analysis OCT is applied only to the base of the tissue securing it to the chuck and ensuring no contact is made with the cutting surface. This is opposed to (B) conventional methods whereby tissue is embedded in OCT, resulting in a section surrounded by media (Schwartz *et al.*, 2003) (with permission).

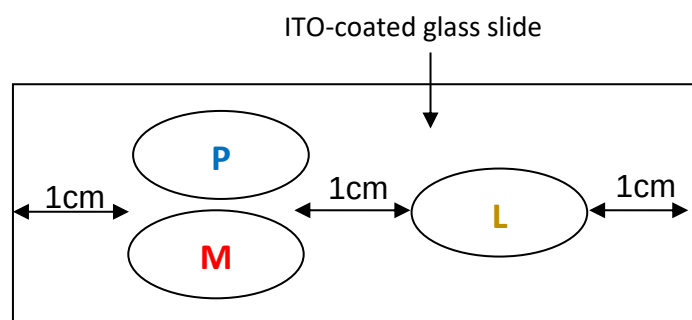


Figure 3.2: Sample setup on ITO slide. Three adjacent sections from the wound tissue were thaw mounted onto a single ITO slide to allow for triple ‘omic’ analyses. P: proteomic; M: metabolomic; and L: lipidomic.

3.2.2 MALDI-IMS analysis

Tissue sections were removed from the -80°C freezer and desiccated in a vacuum for 30 minutes to allow the samples to dry and equilibrate to room temperature. The slides were then marked with dots of liquid white-out fluid to provide a spatial reference of the sections and were optically scanned using a HP Deskjet F4583 scanner.

Biological samples are complex, containing many different chemical species, some of which are highly abundant and can interfere or mask the measurement of less abundant features such as peptides and proteins. Interference from highly abundant small molecules may cause poor sample/matrix co-crystallisation, ion suppression or gas-phase adduct formation during MALDI analysis (Norris and Caprioli, 2013). Therefore in preparation for proteomic MALDI-IMS, tissue sections were gently washed in a series of ethanol/water solutions, a common washing procedure used to remove interfering small molecules such as salts and lipids (Aerni *et al.*, 2006, Börnsen, 2000, Chughtai and Heeren, 2010, Goodwin *et al.*, 2008, Schwartz *et al.*, 2003), as well as to fix the sample proteins without crosslinking. The tissue was first washed twice by dipping in 70% ethanol and then once in 100% ethanol for one minute each. Samples were then again desiccated before matrix deposition.

Selected ionisable matrix was applied to specific tissue sections using the ImagePrep Station (Bruker Daltonics, Bremen, Germany) where pre-installed standard protocols were followed (Table 3.1). The matrix solution used for the protein MALDI-IMS analysis was prepared with sinapinic acid (SA) at 10 g/l in 60%

acetonitrile with 0.2% trifluoroacetic acid (TFA) (all purchased from Sigma-Aldrich, St Louis, USA). Following matrix deposition onto the selected sample section, the prepared slides were loaded into an MTP slide adapter II (Bruker Daltonics, Bremen, Germany). MALDI-IMS analysis was performed using an UltrafleXtreme MALDI TOF/TOF system (Bruker Daltonics, Bremen, Germany) accumulating 200-400 laser shots per pixel at 1 kilohertz (kHz) laser frequency, 200 μ m spatial resolution with data acquisition controlled through FlexControl 3.4 software (Bruker Daltonics, Bremen, Germany). To yield optimal results, data for MALDI-IMS protein analysis was acquired in linear positive mode with the following parameters:

- Laser power: 84%
- Laser repetition rate: 1 kHz
- Detector gain: 74 kV
- m/z measurement range: 0–30 000 Da
- Matrix suppression cut-off: 1500 Da
- Acquisition rate: 0.5 GS/s

FlexImaging 3.0 software (Bruker Daltonics, Bremen, Germany) was used for the visualisation, interpretation and analysis of the resulting MALDI image data.

Table 3.1: ImagePrep protocol used for sinapinic acid application

SA	Matrix thickness (fixed number of cycles OR sensor control (V) + number of cycles	Nebulise spray power, modulation and spray time (fixed time (sec.) or sensor control (V))	Incubation time (sec.)	Complete dry time
Phase 1: initialisation	15 cycles	25%, 30% and 2.2 s	15 s	50 s
Phase 2: 1 cycle	0.1 V in 5 and 10 cycles	25%, 30% and 0.05 V	30 s	Sensor control
Phase 3: 2 cycles	0.1 V in 6 and 18 cycles	25%, 30% and 0.1 V	30 s	Sensor control (every 2 th cycle)
Phase 4: 4 cycles	0.3 V in 12 and 40 cycles	25%, 30% and 0.2 V	30 s	Sensor control (every 4 th cycle)
Phase 5: 4 cycles	0.35 V in 12 and 64 cycles	25%, 30% and 0.3 V	30 s	Sensor control (every 4 th cycle)

3.2.3 Data analysis

3.2.3.1 Sample imaging and processing

Following MALDI-IMS, regions of interest (ROIs) were identified from the histological analyses and image co-registration manually performed (Figure 3.3). FlexImaging 3.0 software (Bruker Daltonics, Bremen, Germany) was used to construct and visualise wound tissue ROIs using associated mass spectra. The ROIs were defined to represent the wound bed, two wound edges and a region from the surrounding healthy tissue, which were registered manually, and the mass data saved as separate cumulative mass spectra files for each individual wound. Image analysis was performed by FlexImaging within the ROIs. The data from these ROIs were also exported into ClinProTools software (Bruker Daltonics, Bremen, Germany) for further inter-sample analyses and statistical evaluation. This data export was done using ClinProtSpectralImport XML Generator (Bruker Daltonics, Bremen, Germany) software, which generates a single condensed file in which spectra from multiple MALDI imaging runs are consolidated *i.e.* data from the six different wound healing time points over the 16-day study were compared.

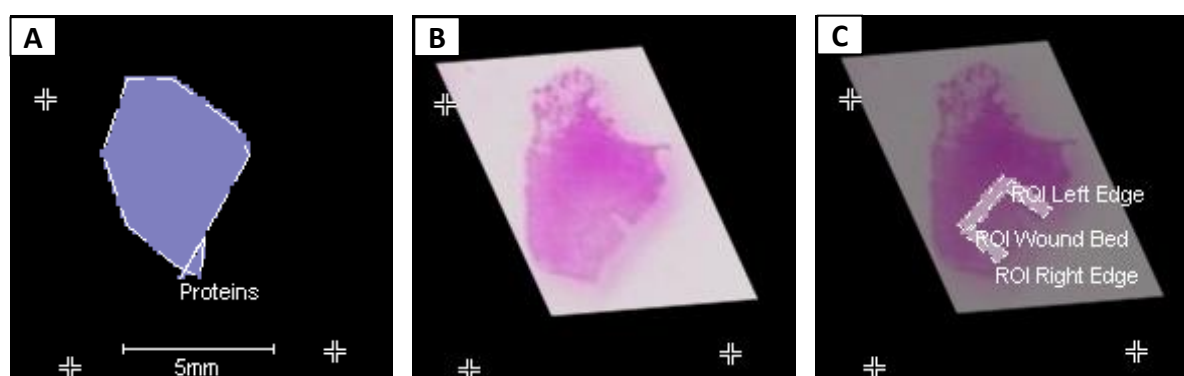


Figure 3.3: Example of co-registered imaging with H&E. (A) Wound spaces already filled with granulation tissue can be difficult to distinguish from surrounding healthy tissue in MALDI sample templates. (B) H&E-stained serial sections are co-registered to the sample image so that (C) wound regions of interest (ROIs) may be accurately selected based on histological analysis.

3.2.3.2 Statistical settings

ClinProTools 3.0 software (Bruker Daltonics, Bremen, Germany) was used for all statistical analyses of the MALDI imaging data. For analysis of protein data, only spectra in the mass range from 1500 – 30 000 Da was assessed due to matrix ion suppression and imaging mass limits. Mass spectra intensities were normalised to their own total ion count (TIC) in order to minimise differences in feature intensity across spectra from different time points. Feature calculations were performed on the total averaged spectrum created from all individual spectra within a ROI showing a signal-to-noise ratio of greater than three and utilised for peak picking and integration. The averaged integrations of each mass within a ROI were used to obtain the abundance of specific features for further analyses. All spectra were subjected to a baseline correction using the Top Hat Baseline algorithm within the ClinProTools 3.0 software, with a 10.0% minimal baseline width.

3.2.3.3 Principal component analysis

Resultant mass spectra were analysed and visualised using multiple statistical techniques, interpretations and plots. This included principal component analysis (PCA) to determine sample region variance and to track the kinetic changes at different time points in acute wounds over 16 days of healing by comparing the wound edge and wound bed ROIs individually to a fixed region of adjacent healthy tissue. Because PCA identifies absolute variance in data, intense mass signals tend to have a larger influence (larger intensity signals show a larger absolute variation than small signals) and therefore scaling is required to counter this effect. Unit variance scaling was used whereby the intensity of each feature was divided by the standard deviation for that feature in the dataset. This data scaling technique resulted in equal influence of each feature in the PCA. Both two-dimensional (2D) and three-dimensional (3D) PCA plots displaying the effect of the three most dominant principal components (PCs) contributing to the explained variance within the dataset were generated. These three PCs show an accumulative variance of at least 70% (Figure 3.4). The PCA plots show the three PCs in all possible combinations with the axes recorded in arbitrary units (arb. u.).

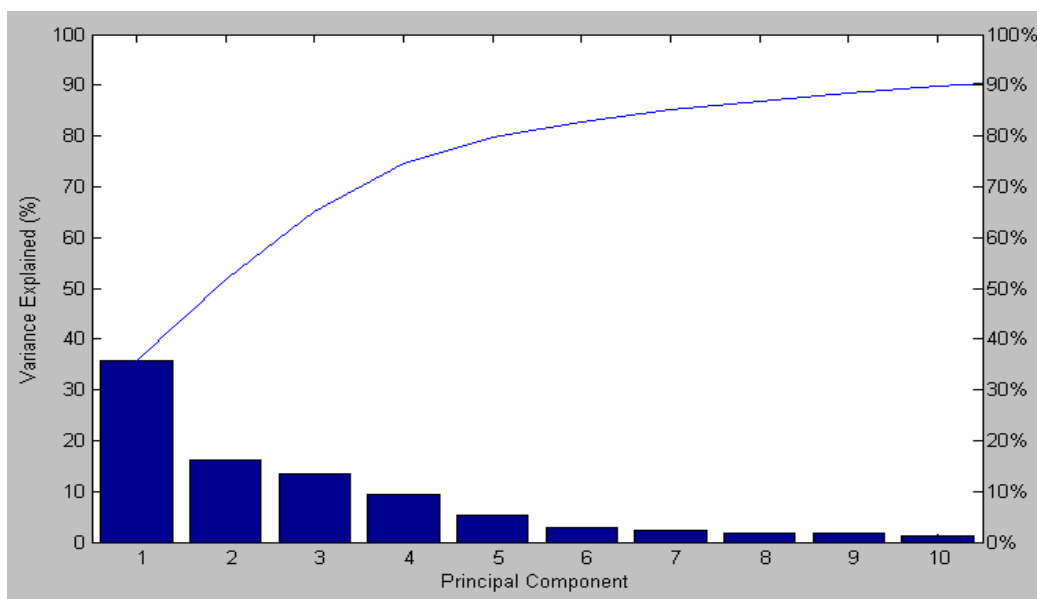


Figure 3.4: Variance plot. A typical example of a variance plot indicating how principal components (PCs) can account for the variance within a dataset. PCs are ranked based on the variance introduced by each PC beginning with PC 1. Typically, the first three PCs are responsible for at least 70% explained variance in the dataset. The plot displays as many PCs as are required to explain at least 95% of the variance within the dataset, however ClinProTools software limits this number to 10. The line graph curve shows the accumulated variance after including the next PC.

3.2.3.4 Loading plots

Loading plots, which were directly coupled to each PCA plot were generated. Loading plots show how principal components are related to the original features, while revealing the m/z ratio of the data points where outliers can easily be identified. The further that the individual points are away from the central data cloud, the greater the variance within that dataset. The axes of the loading plots record the loading values between -1 and 1.

3.2.3.5 Influence plots

Influence plots show the extent of the influence a specific feature has within a PCA model. Using data identified from the PCA calculations, influence plots were generated as a tool to identify data resulting in the greatest variance within that PCA model. The vertical axis (residual) is a measure of how far away a specific feature is from the model space, while the horizontal axis (leverage) is a measure of how far

away a feature is from the centre of the model. Outliers with extreme deviation have a high leverage and thus a strong influence within the PCA model.

3.2.3.6 Peak statistics

Peak statistic reports were generated across all spectra of all seven samples (healthy tissue and wound tissue from Days 3, 5, 8, 10, 12 and 16) analysed in ClinProTools. The reports encompass data for all features including average feature intensity, standard deviation of intensity, coefficient of variation, the difference between the maximal and minimal average feature intensity for all seven samples (average difference ~D Ave), the p-values for the Student's t-test, ANOVA, Wilcoxon and Kruskal-Wallis tests, as well as the p-value for the Anderson-Darling test (for normal distribution assessment). In addition to the peak statistic report, the data could be visualised in 2D peak distribution plots.

3.3 Results and Discussion

3.3.1 Regions of interest

Regions of interest (ROIs) were identified on the IMS images produced during MALDI-IMS analysis of the wound sections, based on adjacent H&E stained histological sections and co-registration with each of the sample images as references (Figure 3.5 B). Four ROIs were registered for each sample, these being the wound bed, the left and right wound edges, and a region from the surrounding 'healthy' (*i.e.* non-wounded) tissue (Figure 3.5 C). Figure 3.5 shows the gross morphology of the wounds on the selected biopsy days in Row A with the H&E stained histological section in Row B, from which the ROIs were manually identified per wound, and Row C shows the MALDI-IMS imaging area with the registered ROIs overlaid onto the image. The mass data for analysis using the various software programs was the averaged spectral intensity data collected from each of the individual ROIs indicated. The ROI definition and registration had to be performed manually for each sample and checked to confirm the correct areas from the MALDI-IMS image were used for further analysis.

The ROIs from the different samples of wound sections obtained at different time points over 16 days were used to assess the spatial changes of protein and peptide

features within the wound bed and the wound edges, compared to apparently healthy tissue. Each ROI was created as an individual composite mass spectral sample and could be analysed independently of one another. This allowed for the mass spectra from each region of interest from each wound collected on Days 3, 5, 8, 10, 12 and 16 to be visually tracked during the wound healing sequence. The comparative data shown as averaged mass spectral intensities per ROI for each time point, identified by different colours, is displayed on a common scale in Figure 3.6. Very low intensities were observed for proteins of mass greater than m/z 15000.

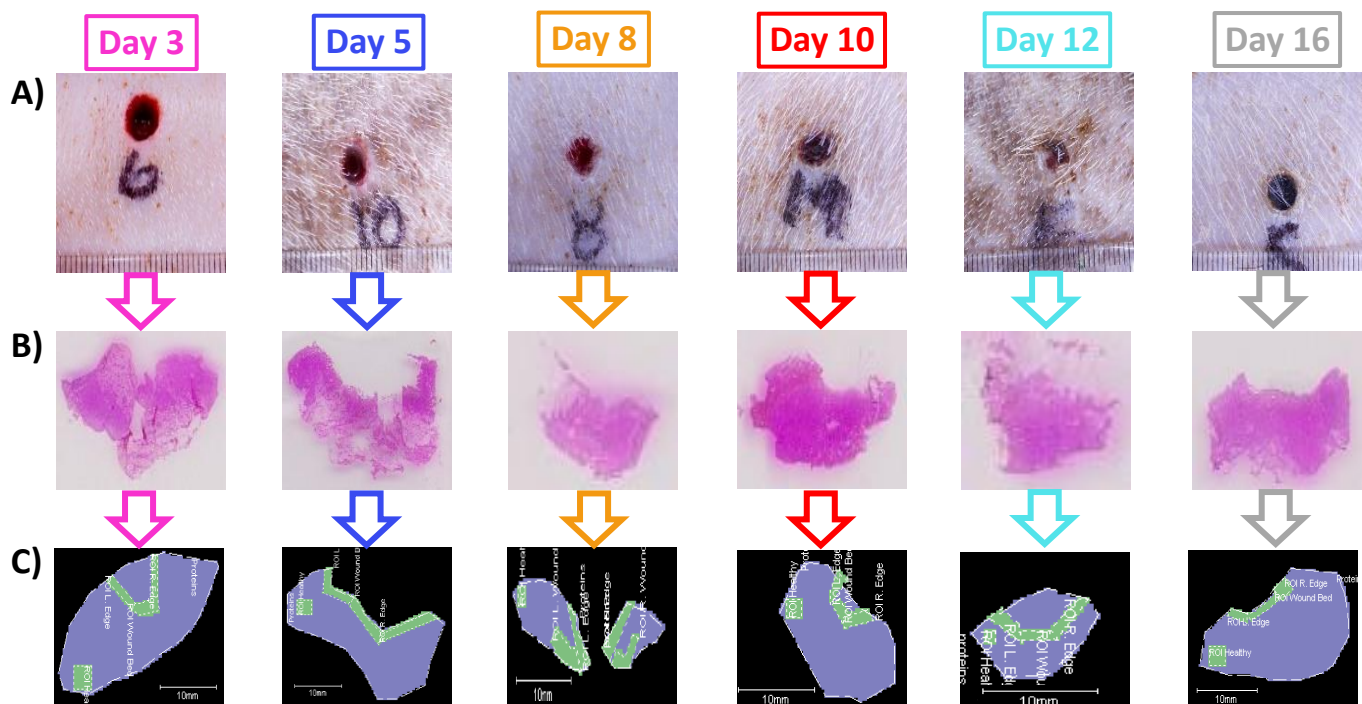


Figure 3.5: Wound regions of interest. (A) Wounds from Days 3, 5, 8, 10, 12 and 16 post-wounding were photographed *in situ* prior to excision and snap freezing. (B) Wound biopsies were cryosectioned and a sequential section stained with H&E for histological analysis from which ROIs were manually identified for co-registration on MALDI-IMS scan templates. (C) Corresponding wound areas were registered as ROIs (shown in green) on MALDI-IMS sample images for analysis.

Different wound section orientation on the slides required manual validation of the different ROIs. The grey areas are the areas scanned during MALDI-IMS.

ROIs: wound bed, wound edges and local healthy tissue (green rectangle).

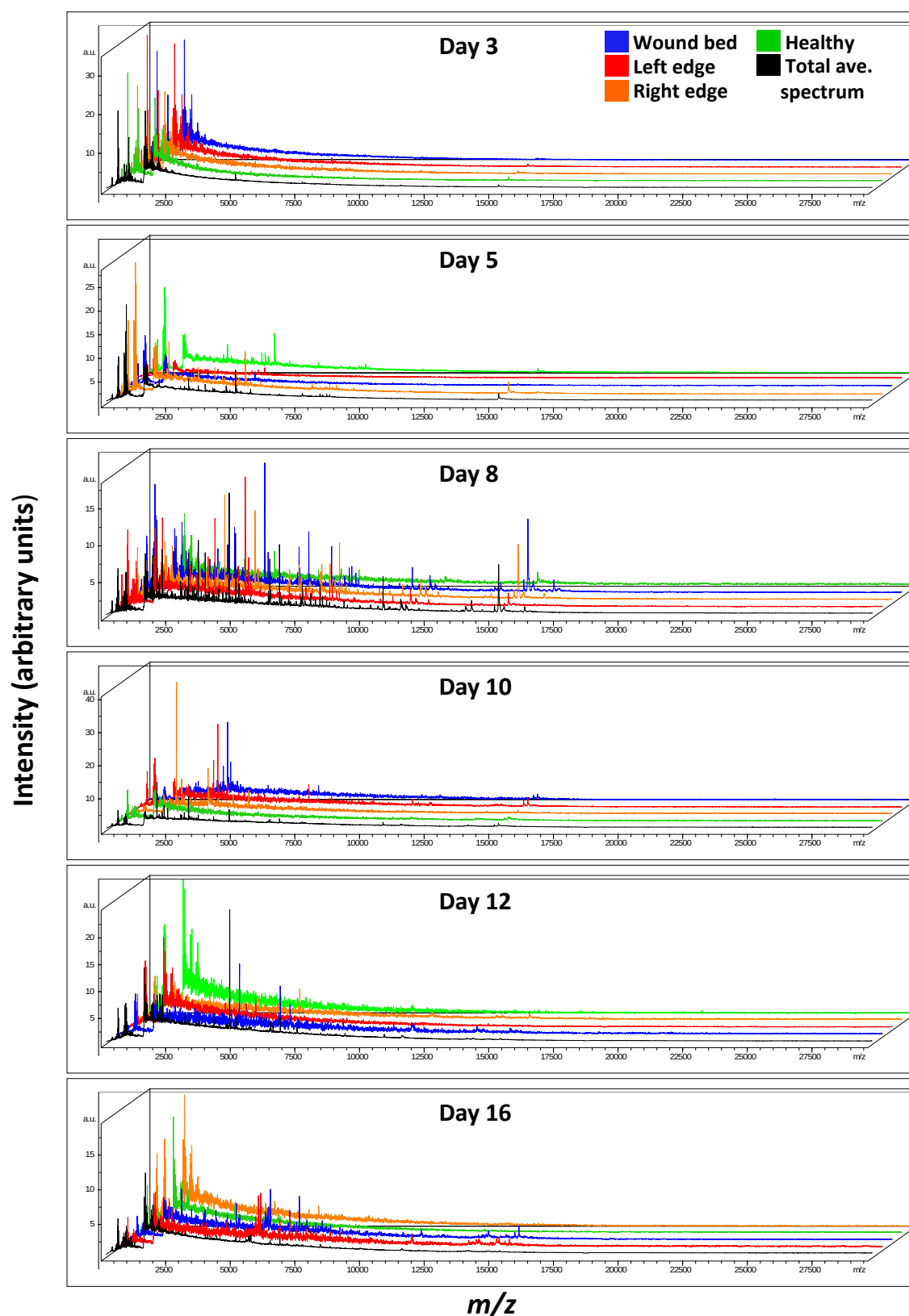


Figure 3.6: Summary of the wound proteome intensities over 16 days. Average peptide/protein mass spectra of the wound bed, left and right wound edges, healthy tissue and total average spectrum at Days 3, 5, 8, 10, 12 and 16 post-wounding. Quantitative feature differences are evident across the six time points including total absence of specific features. The y-axis scale is automatically normalised to optimally fit each dataset.

Complete wound proteome profiles were generated at each of the six wound excision time points over 16 days of healing (Figure 3.6). The acquired imaging mass spectra revealed that most peptides and small proteins were detected between m/z 1500-17000, despite the mass range extending to m/z 30000. Quantitative differences as well as the absence or presence of ions can clearly be visualised between the wound bed, wound edges and healthy surrounding tissue within and between excision time points. The total average spectrum (shown in black) represents the average mass spectrum of the whole tissue section analysed by MALDI-IMS *i.e.* not just the ROIs but the whole grey area in Figure 3.5.

On Day 3 the different ROI spectra closely represented one another with no major feature differences observed. Day 5 showed that a feature around m/z 5200 was found within the healthy tissue as well as the right wound edge but was not seen in the left edge or the wound bed. A feature at m/z 15300 was also found to be exclusive to the healthy tissue and right edge, however more prominently so at the wound edge. The mass spectra across all regions showed an increase in detected features, especially in the m/z 5000 – 9000 range, with an increase in intensity of the features at m/z 15300, when comparing Day 5 to Day 3.

Day 8 showed a significant increase in mass intensities in the average spectra of all ROIs compared to both earlier time points. Multiple features were observed in the wound regions which were either absent or at a low intensity in the healthy tissue. These included features at around m/z 5200, which had increased in intensity compared to the Day 5 time point. Other features prominently associated with the wound regions included multiple spectra in the m/z 7000 – 9500 range and those at m/z 11 500 and m/z 15300. The features around m/z 15300 appeared to have consistently increased in intensity from Days 3 to 5 to 8. By Day 10, however these features had once again decreased substantially compared to Day 8. The overall mass spectra intensities at Day 10 seemed to have decreased, however features highly associated with the wound region as compared to healthy tissue still remained around m/z 2500 and 7000.

On Day 12 the intensity of the mass spectra had decreased with fewer major features being observed across all ROIs. At m/z 6000 a feature was seen which was exclusive to the wound bed and right wound edge. A prominent feature observed

only in the wound bed ROI was seen at m/z 4250, however this feature was found at a higher intensity in the total average spectrum and therefore this small protein could not be exclusively associated with the wound bed and could not be regarded as having any significant association with the wound healing process. By Day 16, features of m/z 5000 and 6250, as well as m/z 11250 and 15300 were observed to be exclusive to the wound bed ROI and both the wound edge ROIs.

In addition to the mass spectra of each individual ROI being plotted, average mass profiles of combined ROIs were generated at each time point for the duration of the study. Average mass profiles show the average spectra for selected ROIs, in this case being the whole wound region *i.e.* the wound bed and both wound edges. These average profiles offer cleaner data and allow straight-forward assessment for tracking the kinetic changes in the average mass spectra of acute wound healing over 16 days at each of the six wound excision time points (Figure 3.7).

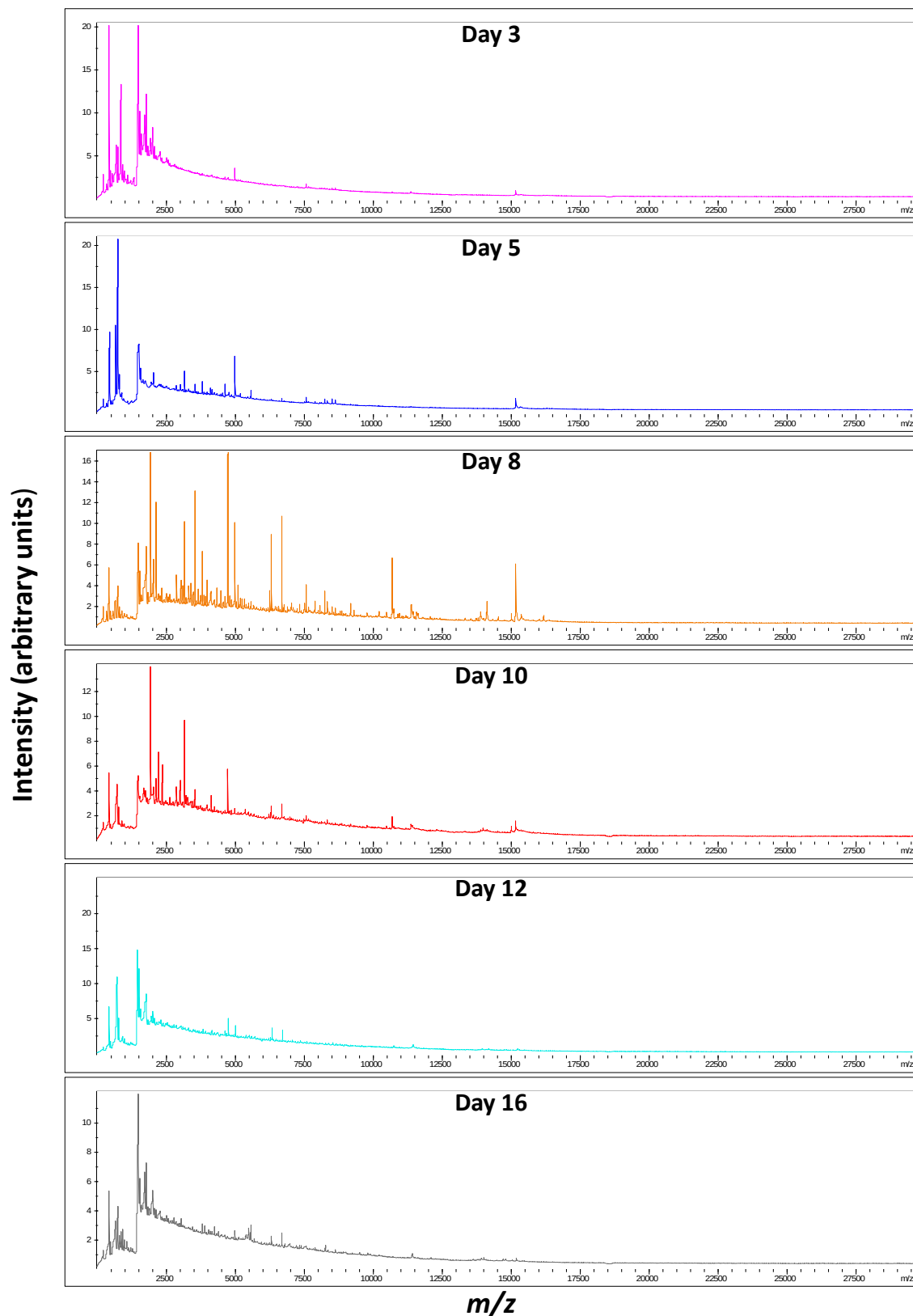


Figure 3.7: Average proteomic mass profiles of the kinetic acute wound. Average mass profiles are shown tracking the proteomic kinetics of the whole wound region on Days 3, 5, 8, 10, 12 and 16. Continuous changes in the wound proteome are evident across the six time points. Masses below 1500 Da are disregarded due to matrix effects. The y-axis scale is automatically normalised to optimally fit each dataset.

By visualising the average mass profile of whole wound regions (wound bed and both wound edge ROIs combined) across 16 days (Figure 3.7), tracking the continuously changing (kinetic) proteome of acute wound healing was made possible. The average mass profiles clearly showed a changing wound proteome from Days 3, 5, 8, 10, 12 and 16. From Day 3 to Day 5 an increase in detected mass spectra was seen, with a significant increase in both feature number and relative feature intensity occurring at Day 8. By Day 10 the number of features and the relative intensity decreased to more closely represent the average mass spectra seen on Day 5, with further decreases seen on Days 12 and 16. Mass profiling clearly indicated that the most intense proteomic activity within the healing acute wound occurred on Day 8. A result further supported and tracked by wound region data presented in Figure 3.6.

Visualising the mass spectra of the kinetic proteome of acute wound healing at six different time points provided the ability to track molecular changes occurring within the different wound ROIs over time. Such variations were tracked through relative quantitative changes (wound tissue versus healthy, non-wounded tissue), through intra-wound region comparisons (edges versus bed) and through wound ROI profiling by relative quantitation, absence and/or presence of peptides and proteins over time. The ability to visualise the proteomic changes occurring in a wound during acute healing over 16 days within a composite image provided a unique molecular overview of large datasets. Such an image may be used to isolate time points of interest for further analysis or as a characterisation tool or model for assessing the kinetic changes of acute wound healing in the porcine model.

3.3.2 Principal component analysis

MALDI-TOF technology is a unique and modern technique that has biological applications and therefore has been applied in diverse biomarker discovery studies including those involving depression (Lo *et al.*, 2009), ophthalmology (Grey *et al.*, 2009a) and various cancers (Balluff *et al.*, 2011, Rauser *et al.*, 2010, Resson *et al.*, 2008). MALDI makes tracking physiological changes at a molecular level possible and provides an ideal basis for kinetic 'omic' analyses and potential biomarker discovery of the acute wound healing phases (Manzanares-Meza *et al.*, 2017, Norris and Caprioli, 2013).

This type of MS analysis does however tend to generate large, multidimensional datasets visualising the spatial distribution of thousands of mass spectra which may, when dealing with multiple samples, overwhelm the analyses and reduce the ability of the technique. The complexity and wealth of spatial 'omic' information provided in biological tissue analyses is of no exception, where data requires reduction to an informative yet manageable volume. Principal component analysis (PCA) is a statistical technique capable of this as it has the ability to reduce data dimensionality while ensuring that no critical information is lost despite not having exact identities of the individual compounds being analysed. In applying PCA to complex data, greater variation in the data is identified and patterns are easily visualised in large datasets. PCA has been proven to be a highly useful tool in MALDI imaging analyses (Deininger *et al.*, 2008, McCombie *et al.*, 2005, Trim *et al.*, 2008). Such data reduction is vital in contributing to a meaningful and convenient acute healing model for clinical use.

3.3.2.1 Healthy tissue

In this study PCA was performed separately on the wound bed ROI and wound edge ROIs of samples collected on Days 3, 5, 8, 10, 12 and 16 where the wound tissue was compared to a consistent ROI of healthy tissue. The region of healthy tissue used across all the analyses was isolated from the tissue sample taken on Day 3. The reasoning behind choosing this specific tissue section was based on the fact that this sample had the largest amount of healthy tissue surrounding the wound upon excision, and therefore the farthest distance and potentially least interference from the wound tissue (Figure 3.5 C). The fact that three separate pigs were used across the 16-day analysis required that no significant inter-pig differences existed in the unwounded, healthy tissue. Therefore, PCA was performed on each healthy tissue ROI at each of the six time points in the study across pigs A, B and C (Figure 3.8).

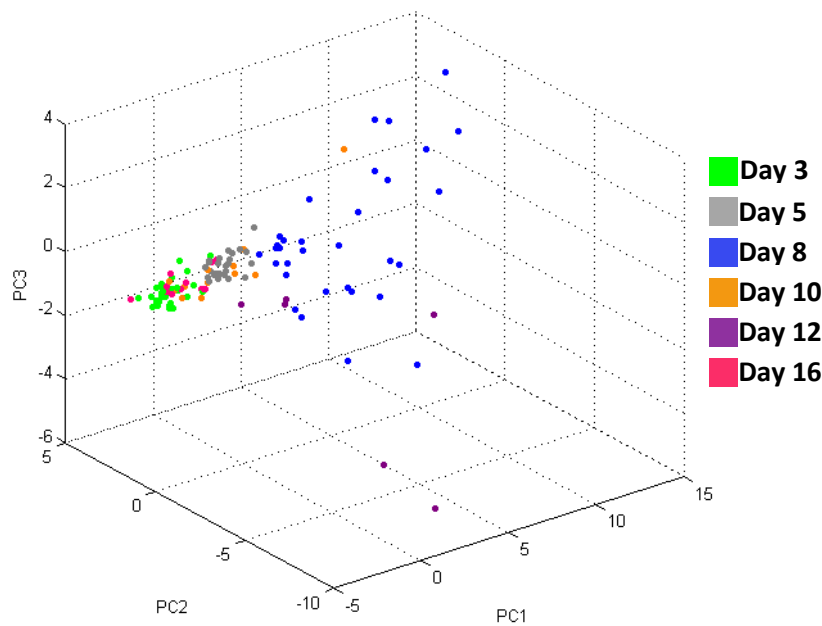


Figure 3.8: 3D PCA plot of healthy tissue regions of interest. ROIs of non-wounded, apparently healthy tissue were selected in tissue samples excised on Days 3, 5, 8, 10, 12 and 16. With the exception of Day 8, all healthy tissue features overlap and appear similar. The healthy tissue ROI from Day 8 appears significantly different in comparison to the other time points, with a high variance.

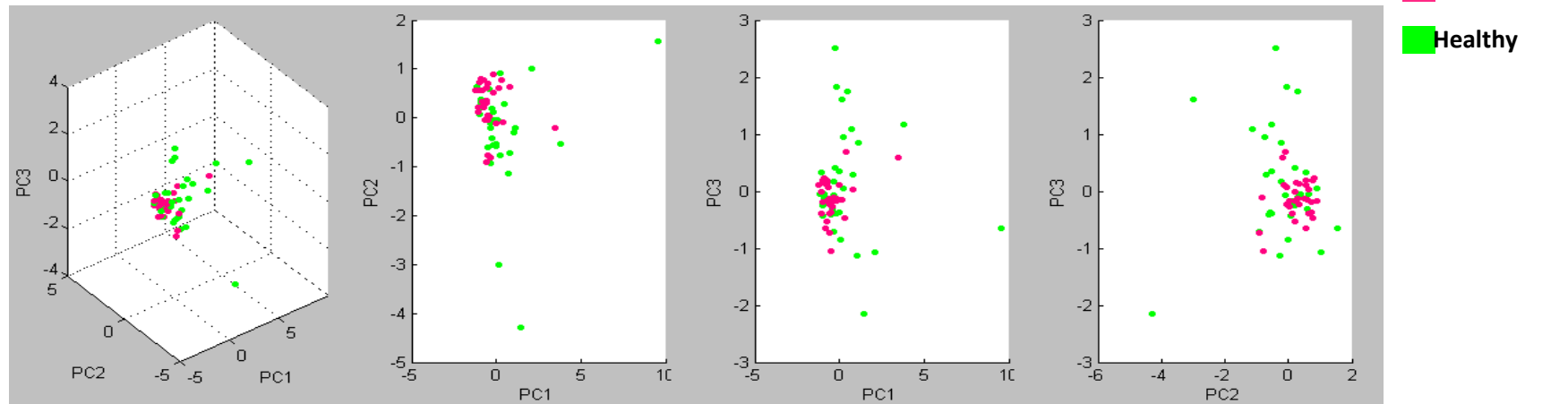
The PCA plot of average feature intensities showed a clear overlap between all the healthy tissues of Days 3, 5, 10, 12 and 16 proving significant similarity and comparability. Day 8 however showed very little correlation, if any, to all the other healthy tissues, being significantly different. Day 8 also showed the highest variability within the dataset compared to the remaining well-clustered healthy tissues. The reason for the major differences seen in the healthy tissue from Day 8 can be attributed to the fact that the wound sample excised on Day 8 was significantly smaller and had very little surrounding non-wounded healthy tissue when compared to the other samples (as can be seen in Figure 3.5 C). Therefore, even though the selected region of healthy tissue was taken on the excised tissue edge, this was probably not far enough from the wound to be considered as uninfluenced by the nearby wounded tissue. As was seen with the histological analysis in Chapter 2 (Figure 2.8 J) immediate surrounding tissue of the wound showed oedema and the presence of inflammatory cells, and thus was influenced by the healing process.

The tissue sample from Day 8 was taken from Pig C, as was the sample from Day 16 (Figure 2.1), where Day 16 shows significant overlap and correlation with the remaining healthy tissue samples in the PCA plot (Figure 3.8). This indicates that it was likely the close location of the healthy sample region of the Day 8 tissue to the actual wound causing variability rather than the animal, Pig C, itself. The overlap and comparable variation between the datasets from Days 3, 5, 10, 12 and 16 prove negligible inter-pig variation between Pigs A, B and C and therefore the healthy tissue ROI from the sample taken at Day 3 was used as a reference for all further comparisons.

3.3.2.2 Kinetic changes in acute wounds

PCA was performed on acute wound healing kinetics in order to highlight the continuously changing wound proteome over 16 days of healing in a manageable manner, which allowed tracking of molecular changes. This was done by comparing the wound bed and the wound edges (left and right combined) independently to a set region of healthy tissue (as described above) on Days 3, 5, 8, 10, 12 and 16. This was done to determine how the different regions within the healing wounds compared to non-wounded, healthy tissue throughout the study, and to reveal time points that may be considered as being the most representative of or even as the 'height' of healing activity. Plotting the PCA results as 3D and 2D graphs could easily visualise the changes effectively to show the times during the healing sequence when the greatest variation could be seen. These plots are shown for the averaged wound data for the six time points in Figures 3.9 and 3.10.

A) Day 3



Day 5

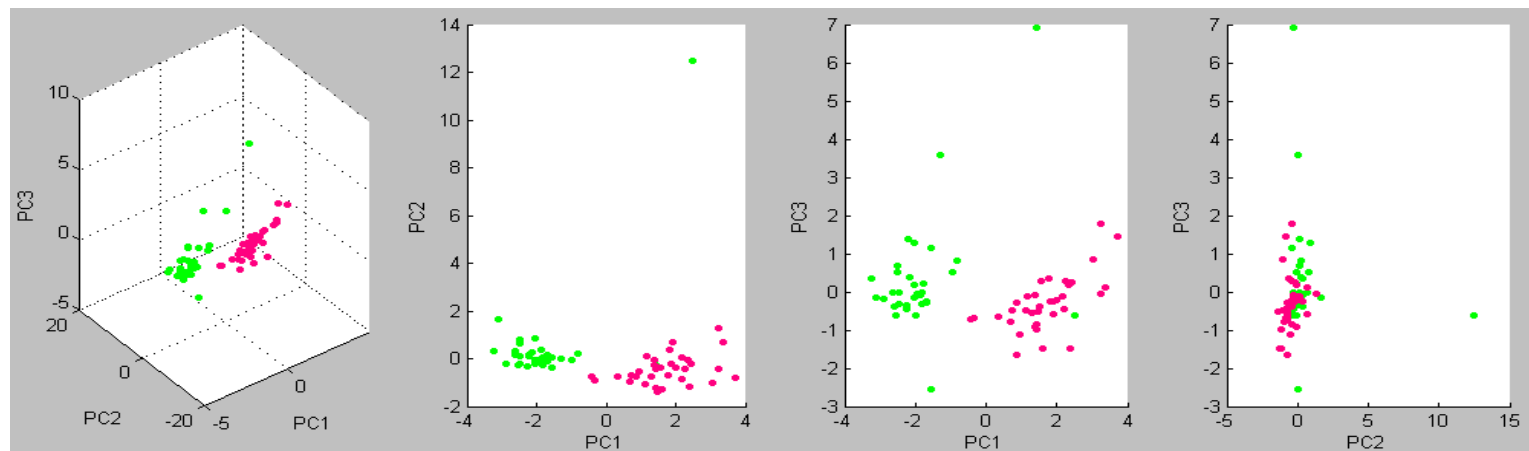
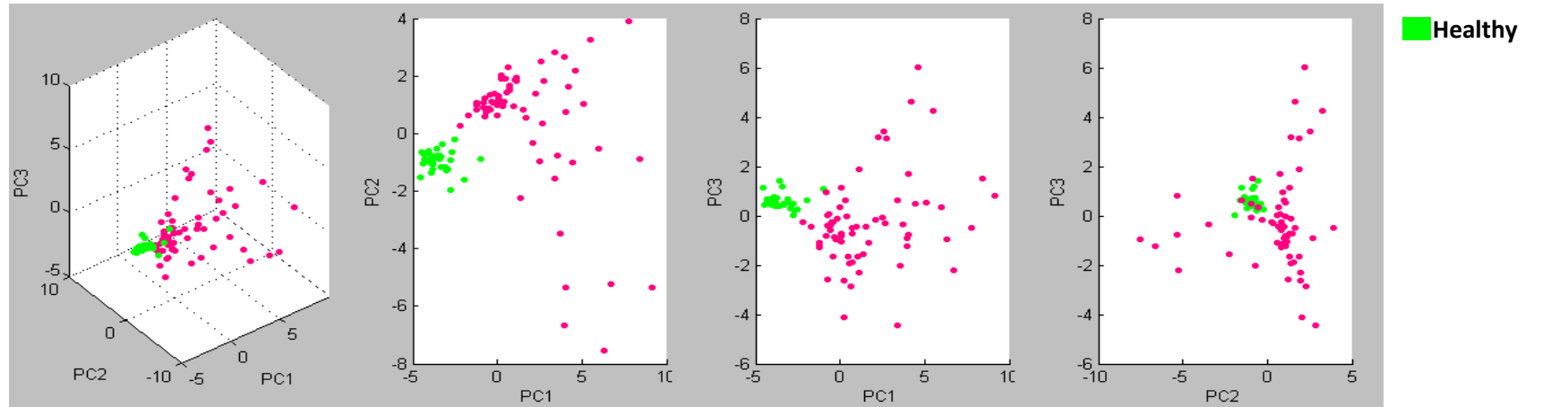


Figure 3.9 A: PCA plots of the wound bed versus healthy tissue proteome on Days 3 and 5. 3D and grouped equivalent 2D PCA plots were visualised for the wound bed ROI (pink) versus healthy tissue ROI (green).

B) Day 8



Day 10

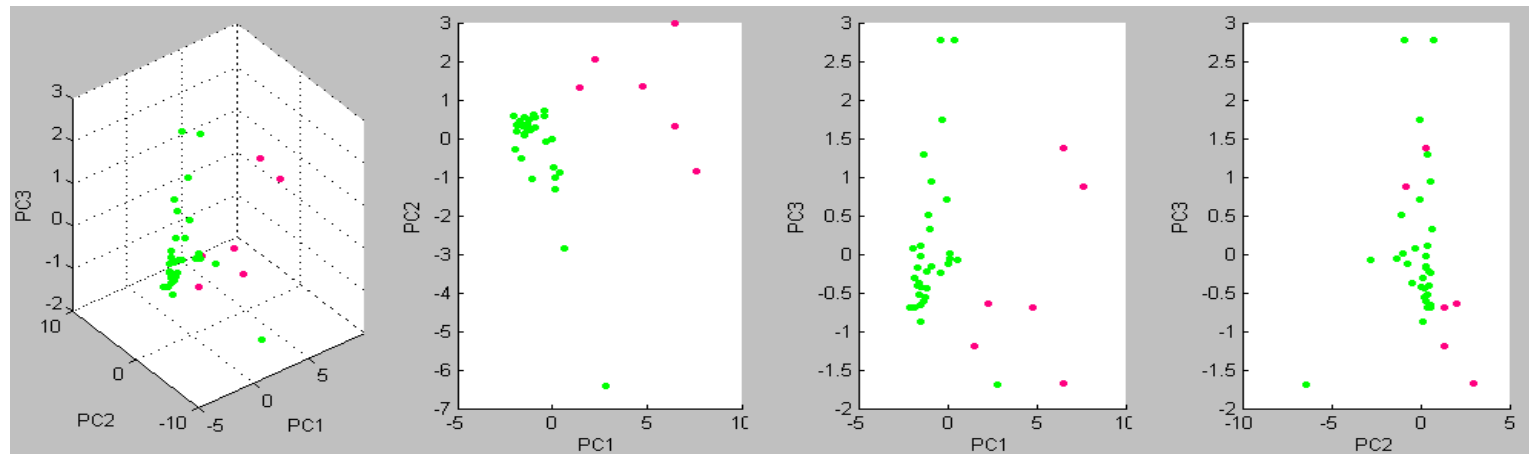
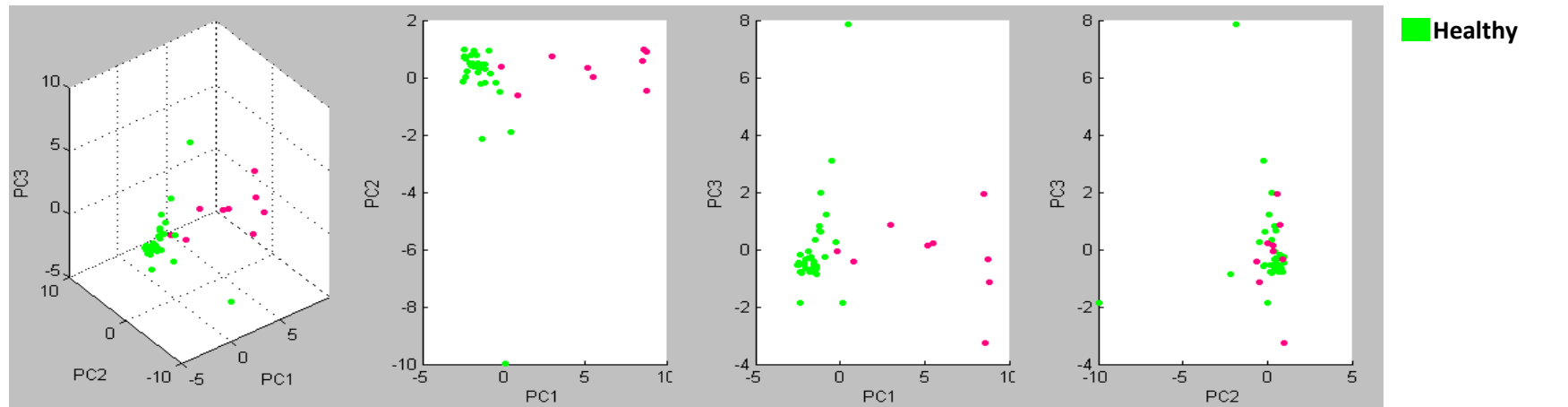


Figure 3.9 B: PCA plots of the wound bed versus healthy tissue proteome on Days 8 and 10. 3D and grouped equivalent 2D PCA plots were visualised for the wound bed ROI (pink) versus healthy tissue ROI (green).

C) Day 12



Day 16

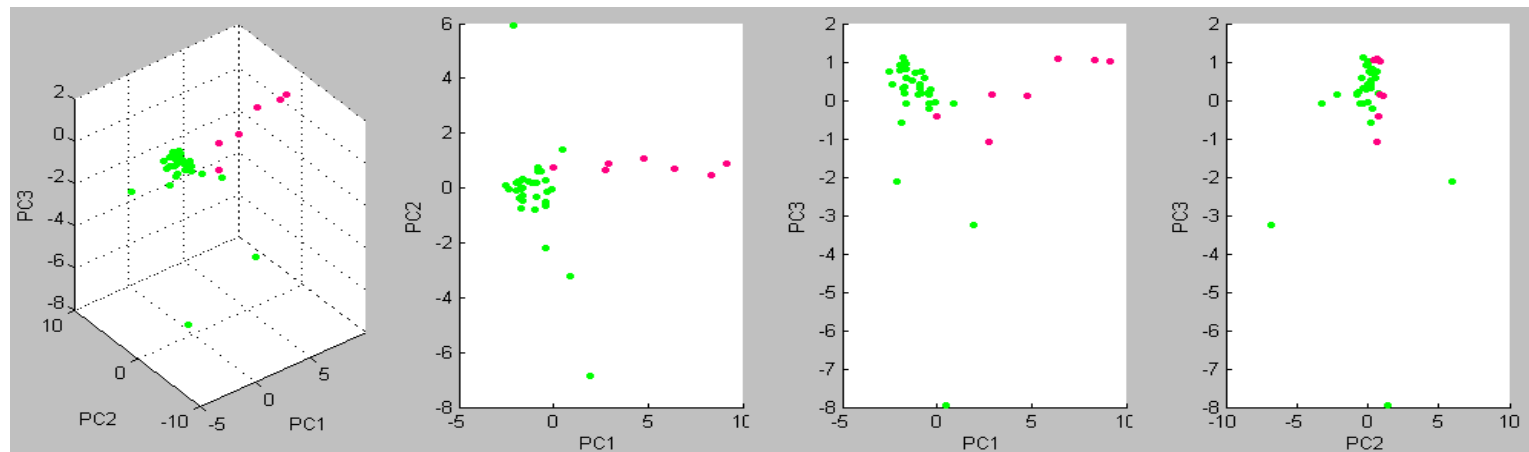
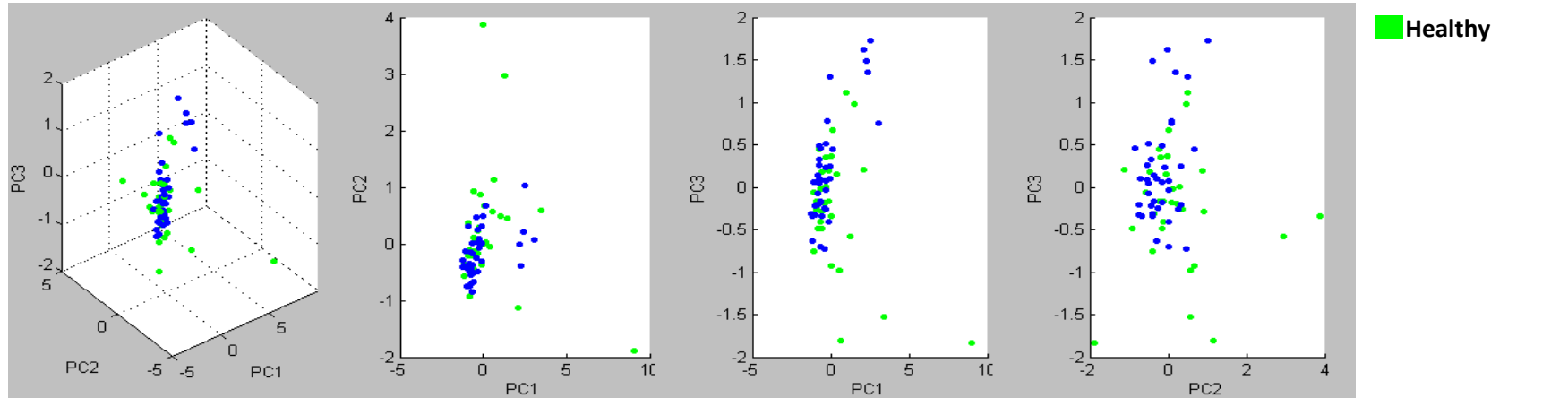


Figure 3.9 C: PCA plots of the wound bed versus healthy tissue proteome on Days 12 and 16. 3D and grouped equivalent 2D PCA plots were visualised for the wound bed ROI (pink) versus healthy tissue ROI (green).

A) Day 3



Day 5

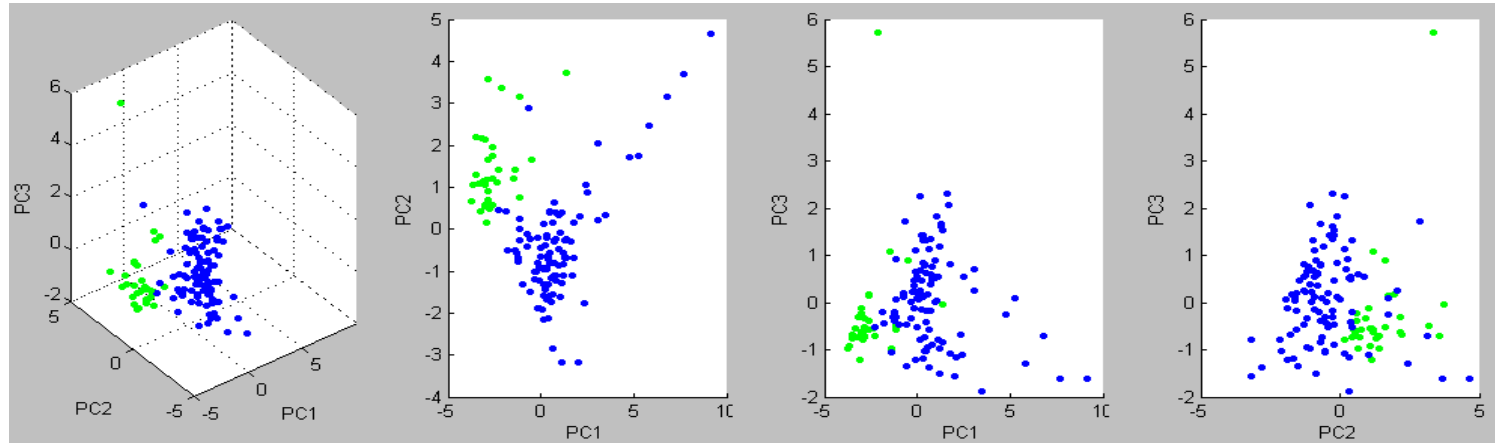
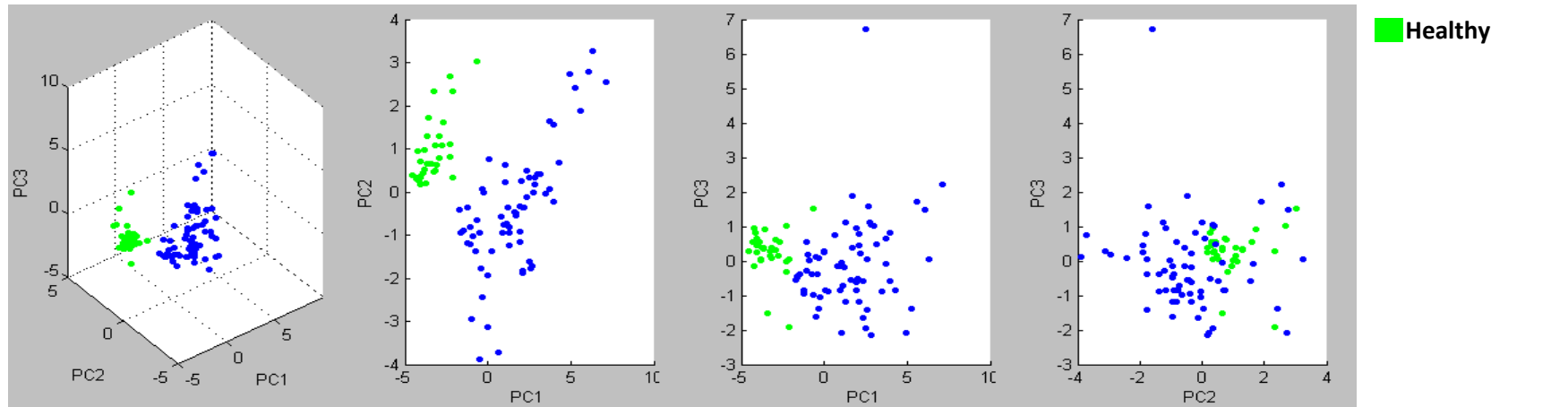


Figure 3.10 A: PCA plots of the wound edges versus healthy tissue proteome on Days 3 and 5. 3D and grouped equivalent 2D PCA plots were visualised for the wound edges ROI (blue) versus healthy tissue ROI (green).

B) Day 8



Day 10

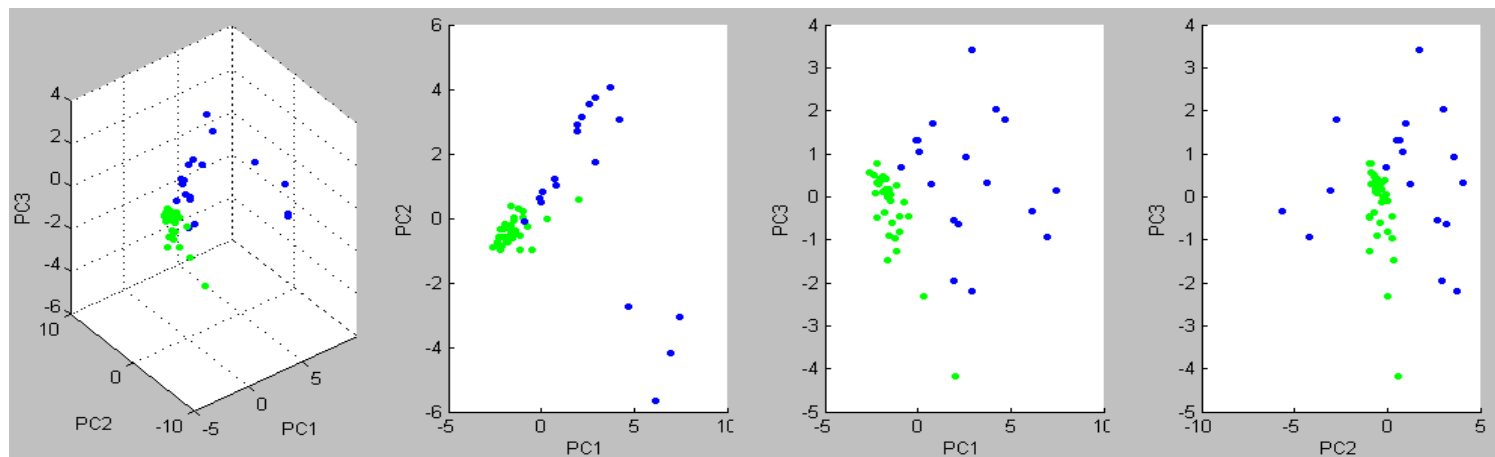
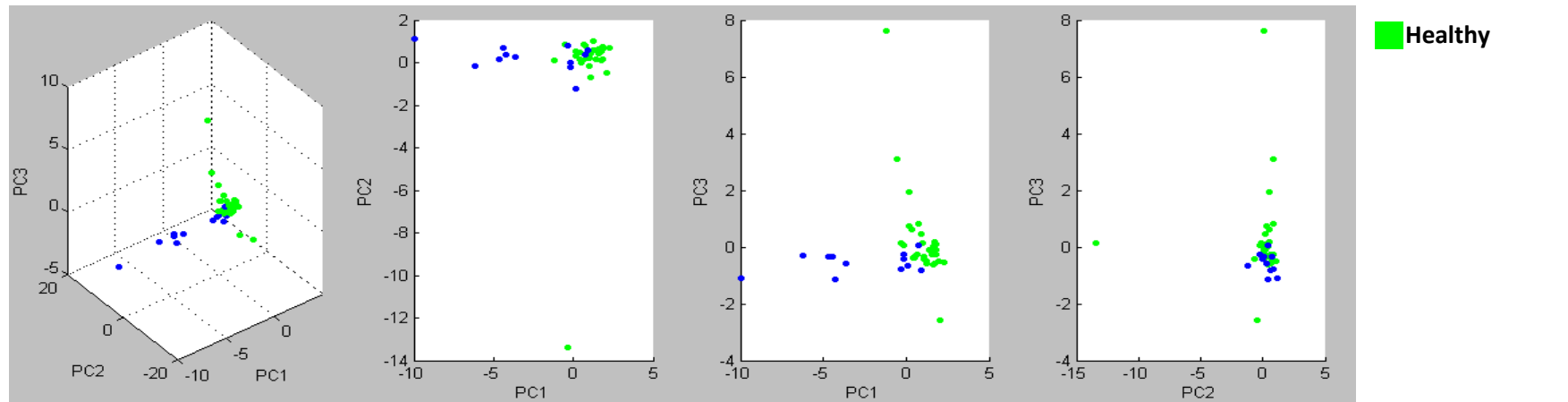


Figure 3.10 B: PCA plots of the wound edges versus healthy tissue proteome on Days 8 and 10. 3D and grouped equivalent 2D PCA plots were visualised for the wound edges ROI (blue) versus healthy tissue ROI (green).

C) Day 12



Day 16

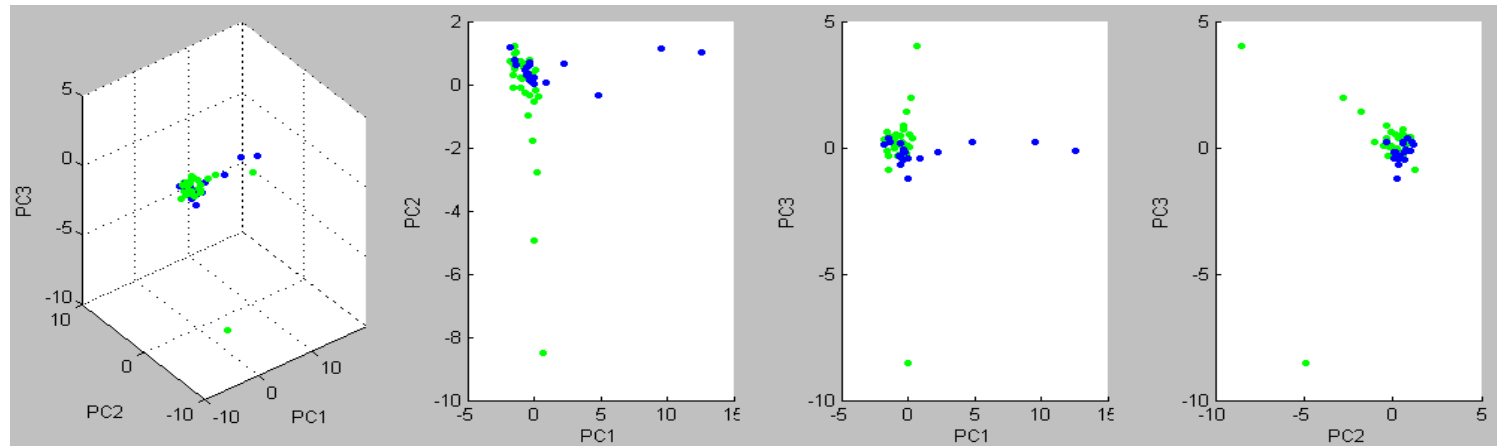


Figure 3.10 C: PCA plots of the wound edges versus healthy tissue proteome on Days 12 and 16. 3D and grouped equivalent 2D PCA plots were visualised for the wound edges ROI (blue) versus healthy tissue ROI (green).

PCA was successfully used to assess and track the continuously changing acute wound proteome using non-wounded, healthy tissue as a reference. At each of the wound excision time points, clear changes in the variance of the proteome features of both the wound bed and the wound edges could be observed (Figures 3.9 A, B, C and 3.10 A, B, C).

On Day 3 the PCA plots showed strong similarity from both the wound bed and edges with the healthy tissue (Figures 3.9 A and 3.10 A). This indicated that although not comparable to healthy tissue (which would not be expected in a Day 3 wound due to haemostasis and initiation of the inflammatory phase), there was still a clear similarity between wound ROIs and the non-wounded tissue ROI. Two days later at Day 5, the PCA plots showed significant changes.

Day 5 showed no overlap between the wound bed and healthy tissue with a clear separation of the two datasets in both the 3D and 2D (PC 1 versus (vs) PC 2, and PC 1 vs PC 3) plots. This implied that at this time point the wound bed tissue could be easily distinguished from surrounding healthy tissue with statistical significance despite not having the identity of the proteins or peptides involved (Figure 3.9 A). The same observation was made for the wound edges which showed clear differences from the healthy tissue on the PC 1 vs PC 2 plot of Day 5, and a small overlap in the remaining plots (Figure 3.10 A). A significant increase in the variance of both the wound bed and wound edges could be seen compared to Day 3. In addition, at this time point, features in the wound edges showed more variance than the healthy tissue. This increase in variance could be due to increased wound-associated biological activity or progression of wound healing phases. The reason for the significant change over just two days was probably due to the full onset of the inflammatory phase of acute wound healing, which would be expected at this time point (immediately upon injury to at least six days following wounding) (see Section 1.1.3.1) (Broughton *et al.*, 2006, Rittié, 2016). This is supported by the histological analysis of Day 5 wound tissue where a dense inflammatory cell infiltrate was visualised along the wound in sections stained with H&E (Figure 2.8 E-F). During the inflammatory phase the acute wound environment would not represent healthy tissue due to the presence of increased numbers of inflammatory-associated cells such as neutrophils, lymphocytes, monocytes, macrophages and inflammatory molecules

including the various enzymes, hydrolysis products and cytokines. The PCA plots not only supported but also visualised this physiological response.

On Day 8 the wound bed showed a marked increase in variance compared to Day 5 with no overlap of the healthy tissue in the PC 1 vs PC 2 and PC 1 vs PC 3 plots but with some overlap seen in the 3D and remaining 2D plot (Figure 3.9 B). The wound edges also showed an increase in the overall data variance at this time, with no correlation to the healthy tissue observed, except in the PC 2 vs PC 3 plot (Figure 3.10 B). The switch from the inflammatory to proliferation phase of acute wound healing was probably responsible for the increased variance in the wound tissue features at this time. Proliferation is reported to overlap with and follow a subsiding inflammatory phase starting as early as Day 4 through to Day 14 post-wounding. As inflammation subsides, proliferation is enhanced with the main aim being that of wound closure. The increased variance in the wound features seen between Day 5 and Day 8 was most likely due to initiation of proliferative phase processes such as deposition of granulation tissue, angiogenesis and cell migration over the provisional matrix supporting re-epithelialisation (Broughton *et al.*, 2006, Guo and DiPietro, 2010, Velnar *et al.*, 2009). This progression from the inflammatory phase on Day 5 to the proliferation phase on Day 8 was supported visually by the gross anatomy as seen in the wound photographs taken on these days just prior to wound excision (see Section 2.3.1, Figure 2.4). In the images it could clearly be seen that the wound on Day 5 was an open, uncovered gaping space, appearing significantly different on Day 8 where the wound was closed with a well-established scab, indicative of proliferation having occurred. These observations were also confirmed histologically in Chapter 2 (see Section 2.3.4, Figure 2.8 E-L) where distinct differences and progression of proliferation could be seen from Day 5 to Day 8.

On Day 10 a large decrease in feature variance was seen in both the wound bed and wound edges compared to Day 8, with an increased similarity to the healthy tissue ROI in both wound ROIs (Figures 3.9 B and 3.10 B). It appeared that the molecular processes that occurred within the wound decreased in activity with more of a baseline effect being observed. This could indicate further reduction in inflammatory mediators, a reduction in proliferative processes and potentially the start of the remodelling phase. Remodelling occurs approximately from Day 8 (see Section 1.1.3.3) and the initiation of this process may have been the reason for the slight

overlap of wound tissue features with healthy tissue due to the fact that sparse type III and IV collagen was beginning to be replaced with thicker type I collagen, which is found as the dominant collagen in non-wounded, healthy skin tissue (Broughton *et al.*, 2006).

Day 12 showed the wound bed ROI features correlating more with the healthy tissue than on Day 10 (Figure 3.9 C), where the wound edges also showed an increase in such correlation with a simultaneous significant decrease in variance (Figure 3.10 C). The increased similarity of the wound tissue to the non-wounded, healthy tissue was most likely due to wound remodelling, where sparse type collagen was increasingly replaced with type I collagen. This was confirmed in the histological analysis where the replacement of type III collagen, with thicker, more organised fibrous type I collagen was evident (Section 2.3.4, Figure 2.8 P).

On the last day of the study, Day 16, the wound edges showed the greatest similarity to the healthy tissue, which was to be expected as by this stage the wound opening had been filled with new tissue, and the wound edges were diffuse, being more difficult to discern from healthy tissue. A significant decrease in wound edge variance was also seen at this time, where the similar wound edge and healthy tissue ROIs almost formed a small, contained region in the PCA plot (Figure 3.10 C). The wound bed features on the other hand, being the deepest part of the original wound would be expected to take longer to correlate completely with non-wounded tissue. This was visualised on the PCA plots where a decrease in wound bed molecular variance was seen on Day 16 compared to Day 12, as well as some overlap with the healthy tissue (Figure 3.9 C).

In summary the PCA plots of both the wound bed and wound edge ROIs showed a large overlap of the wound tissues with the non-wounded healthy tissue on Day 3. On Day 5 a clear separation existed between the wound tissue and the healthy tissue, with a significant increase in wound tissue variance. On Day 8 the variance in the wound bed increased significantly while the wound edge data appeared similar to that of Day 5 but with a slightly decreased correlation to the healthy tissue. Two days later Day 10 showed a significant decrease in variance for both the wound bed and edges, with a further decrease in variance of wound tissue on Day 12 accompanied by a slightly closer correlation to the healthy tissue. By Day 16 wound tissue feature

variance was at its lowest point over the 16 days and the wound edges showed almost complete overlap with the non-wounded tissue, representing an almost completely healed wound.

Based on data variation and lack of similarity to the non-wounded healthy tissue, Day 8 was considered as the peak of proteomic wound healing activity where the wound tissue at this time least resembled the healthy tissue proteome and was the most complete representation of the active acute wound healing environment. This was supported by the mass spectra results of the wound regions (Figure 3.6) as well as by the average wound mass profiles generated (Figure 3.7), where feature detection and relative variance were shown to be at their peak on Day 8. Interestingly, previous research has stated that the transitions from the inflammatory phase (Day 5) to the proliferation phase (Day 8) in acute wound healing is of key importance for the successful completion of the wound healing process and is a critical phase transition in achieving healed wounds. Accumulating evidence is suggesting that a compromised transition between these two phases is the cause of non-healing wounds and healing disorders (see Section 1.1.4) (Eming *et al.*, 2007, Landén *et al.*, 2016). In this kinetic-based PCA, Day 8, closely followed by Day 5, represented the peak of variance in the wound proteome relative to healthy tissue over the 16-day healing process. This suggests that targeting this time point, or even both time points for wound healing phase transition analysis, may aid in accelerating future therapeutic developments for wound healing.

3.3.2.3 Loading plots

When using ClinProTools, PCA results are not only shown in PCA plots but are also available as the associated loading plots, which share the same principal components. Within the loading plots each point represents one feature and each plot contains as many points as features detected in the dataset based on statistical and peak statistics settings (Sections 3.2.3.2 and 3.2.3.6 respectively). Loading plots allow identification of the masses of the individual features contributing most to variance in the dataset. That is, each data point on the loading plot represents a single feature in the average mass spectra of the samples and displays the loading of each feature on the first three principal components. These plots show how PCs are related to the original features, where features that are far away from the central

data cloud contribute most to the variance within that dataset. The axes of the loading plots record the loadings between -1 and 1, where load 1 shows the loadings for PC 1, load 2 the loadings for PC 2 etc. During the PCA calculations features are attributed different loadings dependent on the amount of variance of a PC that is explained by individual features. Loading plots reveal the masses of potential features of interest for consideration and possible further analysis.

The PCA plots of the kinetic changes in features during acute wound healing visualised the variance occurring in a continuously changing wound proteome, however the masses of the peptides and/or proteins responsible for such variance were not identified. Loading plots coupled to each PCA plot (in Section 3.3.2.2) were therefore generated in order to reveal the masses of greatest significance in the acute wound healing process by highlighting the masses of those features showing the greatest variance relative to non-wounded healthy tissue and thus most representative of wound healing. Taken a step further, combination plots were created where the data from the six excision time points as well as the constant region of healthy tissue were plotted on a single set of axes for the wound bed and the wound edges. This allowed the most variant wound healing features over the 16-day study to be visualised using PCA and their masses revealed in the associated loading plots (Figures 3.11-3.13).

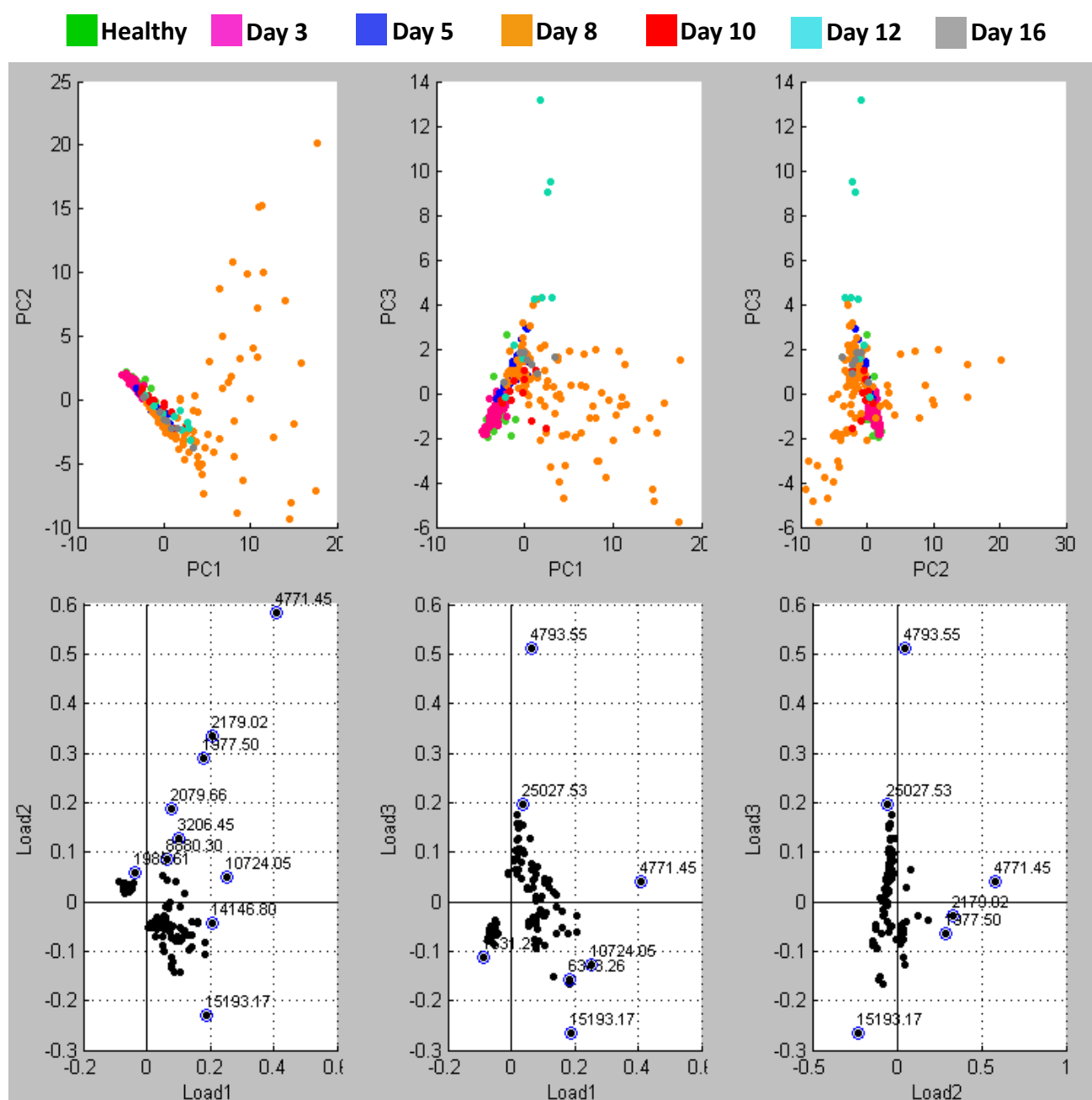


Figure 3.11: 2D combination PCA plots and associated loading plots of the wound bed vs healthy tissue over 16 days. 2D PCA plots (top row) are coupled to 2D loading plots (bottom row) where the mass identification of features (m/z) responsible for the variance in the combined datasets can be shown. Datasets compare mass spectra from non-wounded healthy tissue to the wound bed of Days 3, 5, 8, 10, 12 and 16. The healthy tissue and six wound time points are coloured according to the key at the top of the image.

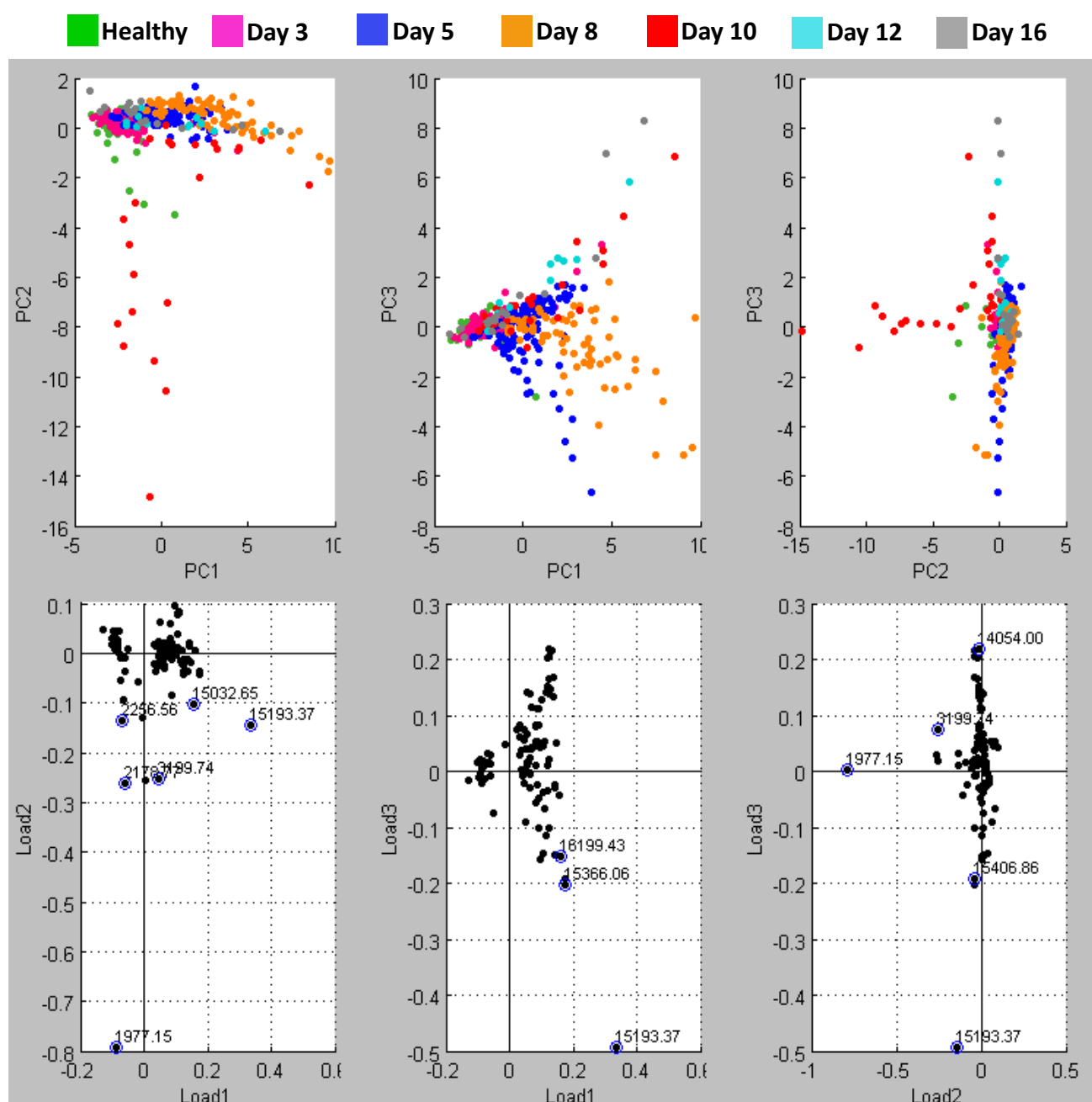


Figure 3.12: 2D combination PCA plots and associated loading plots of the wound edges vs healthy tissue over 16 days. 2D PCA plots (top row) are coupled to 2D loading plots (bottom row) where the mass identification of features (m/z) responsible for the variance in the combined datasets can be shown. Datasets compare mass spectra from non-wounded healthy tissue to the wound edges of Days 3, 5, 8, 10, 12 and 16. The healthy tissue and six wound time points are coloured according to the key at the top of the image.

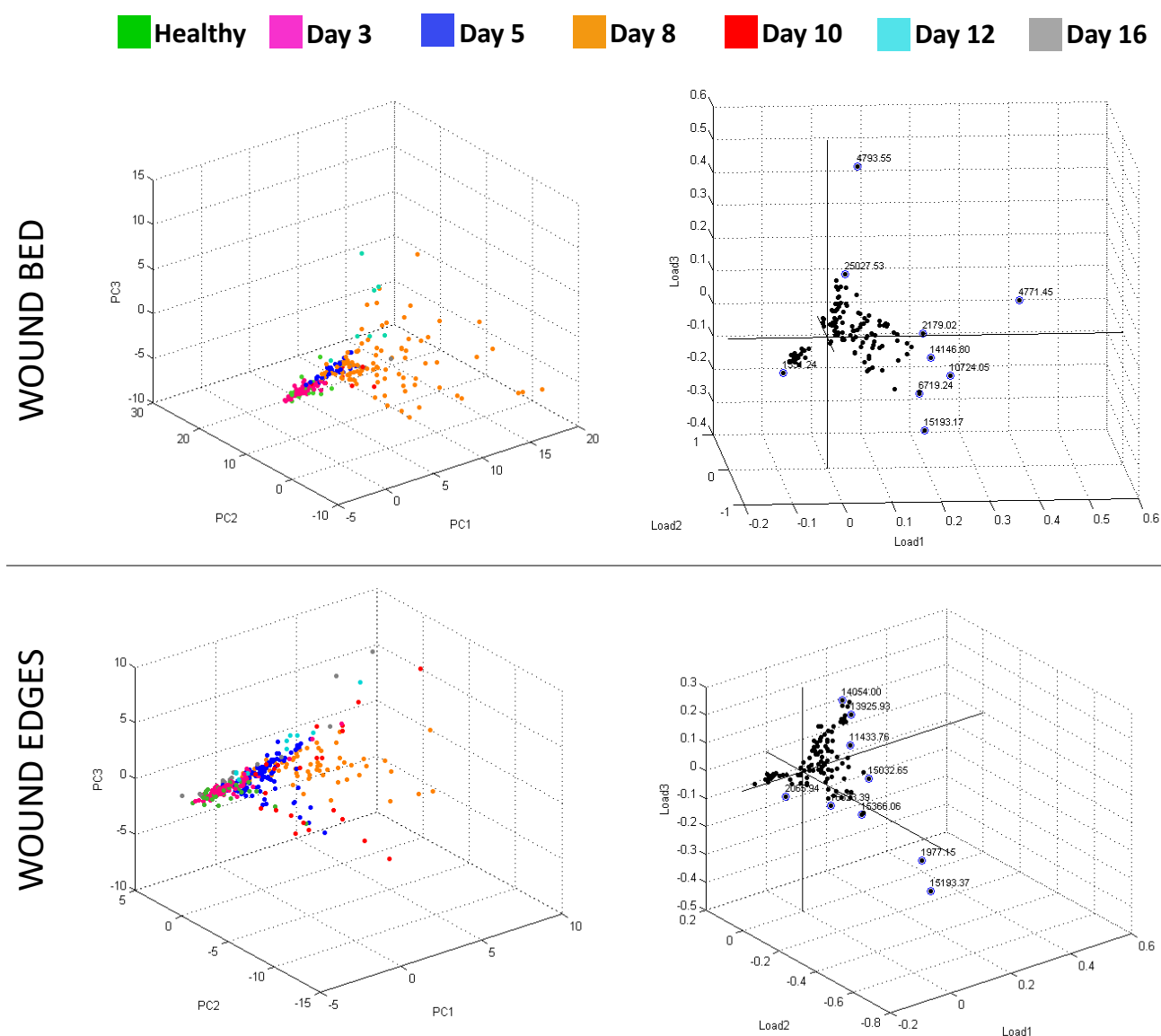


Figure 3.13: 3D combination PCA plots and associated loading plots of the wound bed and edges vs healthy tissue over 16 days. Combined 3D PCA plots with the respective coupled 3D loading plots of the wound bed (top row) and wound edges (bottom row) revealing the masses of features showing the greatest variance across the first three principal components for non-wounded healthy tissue and for the wound beds and edges on Days 3, 5, 8, 10, 12 and 16. The healthy tissue and six wound time points are coloured according to the key at the top of the image.

Combination PCA plots coupled with loading plots of mass spectra from the wound bed and edge ROIs from Days 3, 5, 8, 10, 12 and 16, and a non-wounded healthy tissue ROI were created. This could visualise the most variant features in the positive ions detected in the acute wound healing process relative to healthy tissue.

In theory the more variant a molecule is compared to non-wounded tissue, the greater its association may be to the healing process and the greater the potential for that feature being used as a unique feature for normal, acute wound healing.

When analysing the combination PCA plots, the same overall patterns were seen as in the previous individual time point analyses. As with the kinetic-based PCA plots of the wound bed vs healthy tissue (Figure 3.9) these 2D and 3D combination plots clearly showed Day 8 as having the least coincidence with the healthy tissue and a substantially higher variance compared to the wound beds from any of the other days of biopsy (Figures 3.11 and 3.13). Day 8 (shown in orange) had the largest distribution in all the plots, indicative of high variance and activity at this time point in the healing process, while the remainder of the days and the healthy tissue showed substantial similarity and minor variance in comparison. The 2D combination plots of the wound edge ROIs (Figure 3.12) supported what was observed in the kinetic-based PCA plots of wound edge ROIs vs healthy tissue ROI (Figure 3.10) where Day 8, closely followed by Day 5, showed great variance over the 16-day healing process. Of interest in the 2D combination plots, is that Day 10 (shown in red) also seemed to show significant variance for PC 2 and therefore in the PC 1 vs PC 2 and PC 2 vs PC 3 plots (Figure 3.12). In the 3D PCA plots however this variance seemed relatively small and the Day 8 (orange) data point variance outweighed that of Day 10 (red), with Day 5 (dark blue) also showing a higher variance and less correlation to the healthy tissue, matching what was previously seen (Figure 3.13).

The 3D combination loading plots (Figure 3.13) were used to generate a filtered, differential feature set that revealed mean masses of m/z 4771.45, 4793.55 (a possible sodium salt of 4771.45), 6719.24, 10724.05 and 15193.17 as being most variant from the central data cloud for the wound bed, and features with mean masses of m/z 1977.15, 14054.00, 15032.65, 15193.37 and 15366.06 for the wound edges. Due to the fact that these were combination plots, the resultant variance was relative and therefore it could not be concluded that the aforementioned masses were seen as variant features relative to healthy tissue for all the time points.

To determine the specific time points at which these isolated masses showed greatest variance and thus may have been associated with the healing process, singular day post-wounding loading plots were analysed. The loading plots coupled

to the individual PCA plots (Figures 3.9 and 3.10) were analysed for these targeted masses across both the wound bed and edge ROIs. The search was done using a mass error of $\pm 0.25\%$. Masses m/z 6719 and 10724 were variant on Day 8, while m/z 4772 and 15033 showed variance on Day 8 and Day 10. Mass m/z 4794 did not appear as an outlier from the central data cloud in any of the individual time point PCA plots, while being a prominent variant in the combination 3D plot (Figure 3.13). This means that only relative to the healthy tissue and combined time-point wound samples did m/z 4794 appear different and therefore may be representative of a very specific healing process occurring. These masses (m/z 6719, 10724, 4772, 15033 and 4794) being prominent on Days 8 and 10 are most likely associated with the proliferative phase and potentially the later part of the inflammatory phase in acute wound healing. Mass m/z 14054 showed variance on Days 10, 12 and 16 indicative of a role in later wound healing processes such as advancing proliferation and remodelling. Lastly, mass m/z 15366 was found as a variant at all time points except for Day 3, but masses m/z 1977 and 15193 showed variance across all wound healing time points being Days 3, 5, 8, 10, 12 and 16. These three masses may be useful as being representative of wound tissue in general, showing variance in healing tissue compared to non-wounded tissue throughout the 16-day study. However due to being present throughout all four healing phases these features lose the potential for acting as phase-specific markers to monitor changes during wound healing. Although masses m/z 15193 and 15366 were thought to possibly be porcine haemoglobin monomers it was found that this was not the case as porcine haemoglobin subunits α and β have masses of 15039 and 16166 Da respectively (TheUniProtConsortium., 2014).

3.3.2.4 Influence plots

The result of a PCA provides multidimensional information that can be presented in numerous different ways such as through 2D and 3D PCA plots, loading plots and influence plots. Influence plots show the influence of each feature (a data point in the PCA plot representing a mass spectrum) on the data distribution within that PCA model. The influence plots in this study included the number of principal components needed to explain 95% of the variance in the spectra set (therefore although linked to the previous PCA plots shown, these influence plots take into account more than just the first three PCs). Influence plots are used as a tool to identify potential outliers

in the PCA model. The vertical axis represents the residual, that measures how far away a feature is from the PCA model data cluster (*i.e.* distance to the model) and the horizontal axis measures how far away a feature is from the centre of the PCA model, measuring the leverage of a feature in the model. Outliers of extreme deviation will have a high leverage on the model as they will have a strong influence on the PCA dataset and may dominate an entire PC.

As the different PCA analyses of the wound bed ROIs and the wound edge ROIs rendered similar time point related changes, *i.e.* highlighting Day 8 as the peak of activity in the wound proteome (Figures 3.11 – 3.13), these separate ROI analyses were combined to provide a single whole wound dataset for influence plot analyses. The combined ROI influence plot generated from the whole wound PCA datasets for each of the six time points using the non-wounded healthy tissue as reference, is shown in Figure 3.14. The plot utilised multiple PCs required so as to explain 95% of the data variance.

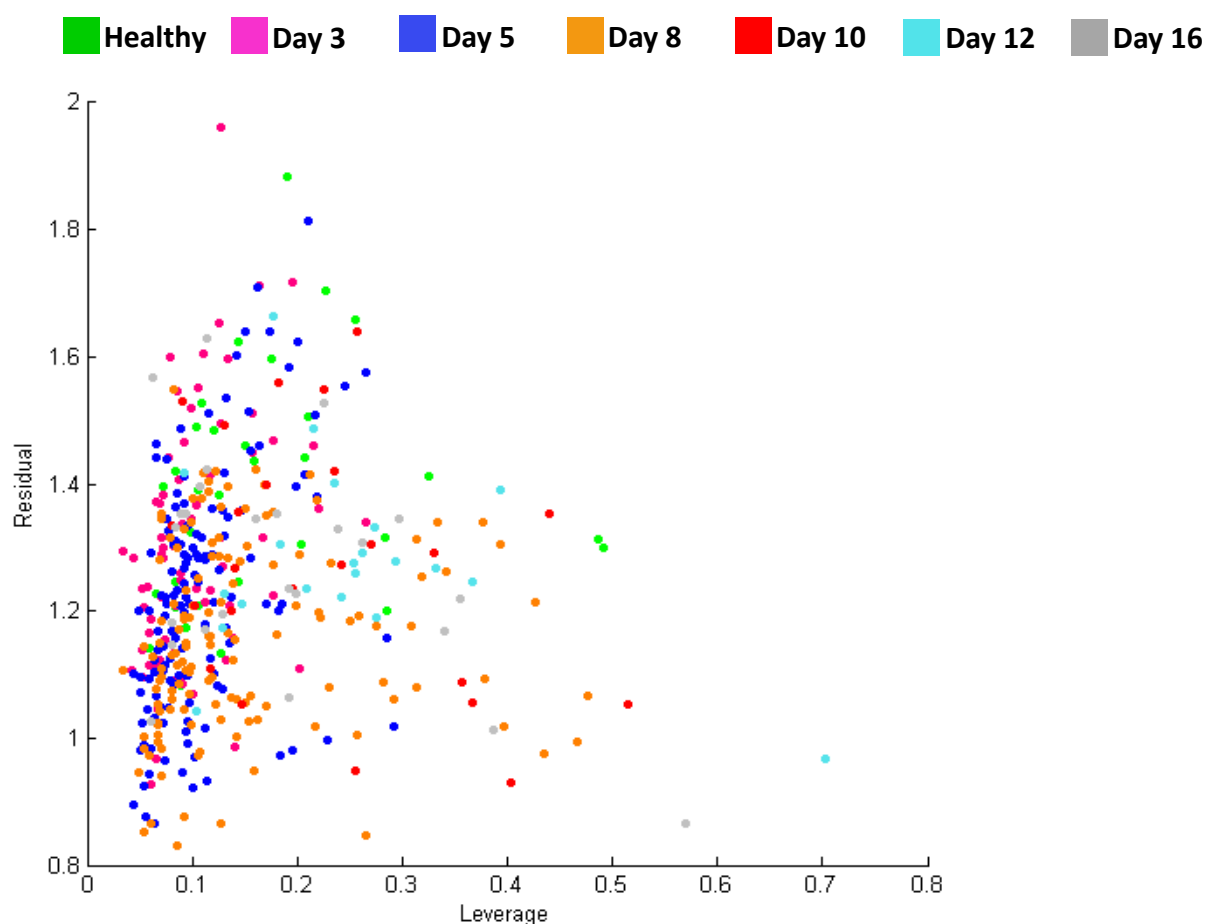


Figure 3.14: Influence plot of healthy tissue versus wound tissue from Days 3, 5, 8, 10, 12 and 16. The influence plot visualises the features associated with acute wounds and healthy tissue, with the majority of features showing an influence with a residual (y-axis) from 0.8 – 1.4 and a leverage (x-axis) of 0 – 0.15. Residual and leverage outliers can be seen with Day 5 (dark blue) dominating the residual and Day 8 (orange) dominating the leverage based on outlier feature number. The healthy tissue and six wound time points are shown in colours according to the key at the top of the image.

Influence plots identify outliers in a dataset by measuring the distance of a data point from a PCA model data cluster created utilising the number of principal components required to explain 95% of variance in the dataset. Outliers are those features which do not correspond to the model into which the majority of the features fit. The greater the residual and/or leverage of a data point, the greater the influence of that point on the model. An influence plot comparing the mass spectra of features from the healthy non-wounded tissue to whole wounds (wound bed and edges combined) on Days 3, 5, 8, 10, 12 and 16 was generated. The resultant influence plot clearly

showed healing time-based differences in the influence of a limited number of features on the acute wound healing PCA model over the 16 days. The majority of features spanned a residual value from 0.8 – 1.4 and a leverage value from 0.05 – 0.15 with the outlier features showing greater influence and lying outside of these ranges on both axes (Figure 3.14). A number of features from all sampling days with a residual higher than 1.4, some almost reaching a residual value of 2, could be observed. Dominating this influence range were features from Day 5 (dark blue), followed by Day 3 and healthy tissue (green). The Day 5 features with high residual values supported this time point as one of high relative variance which is most likely linked to the dominance of inflammatory processes as previously discussed (Section 3.4.2.2). The fact that healthy tissue was shown to have outliers suggested that the residual seen in wound tissue may not necessarily be completely attributed to healing processes and may have been partially linked to natural tissue variance. Healthy tissue should also correlate with a portion of the wound features as all samples originate from the same porcine skin tissue and wounds are surrounded by healthy tissue, including the dominating extracellular matrix. This was observed in the overlap of healthy and wound features in the plot.

Features from Days 8, 10, 12 and 16 (seen in orange, red, light blue and grey respectively) with outliers spanning a leverage range of 0.2 – 0.7 were seen on the x-axis of the influence plot. The Day 8 (orange) features dominated the leverage group of outliers in terms of number. Even though a single feature from Day 12 showed the greatest leverage on the healing model, the multiple features, and greatest relative number of features showing leverage on the model were from Day 8, suggestive of a greater overall influence on the variance of the acute wound PCA model. The high incidence of features from Day 8 appearing as outliers supported this time point as representing the peak in wound proteome variance. This implied that the proteome of Day 8 during healing activity was most different from that of healthy tissue. These results again support that Day 8 showed variance across multiple features and that the changes seen in the kinetic healing model were not due to chance or a single strong outlier that could be a contaminant or foreign protein. The results from the influence plot analysis supports the overall conclusion from the PCA plots (Figures 3.9 – 3.13) identifying Day 8 as the peak of proteomic wound healing activity.

3.3.3 Pseudo-gels

PCA allowed for the unsupervised visualisation of the proteome feature variance occurring at different time points during acute wound healing over a 16-day period. While the relative extent of the variance as well as some of the features responsible for the variance could be tracked, the actual changes measured could not be identified. Pseudo-gels visualise molecular feature changes in multiple sample classes through relative quantitative variance including molecule absence and/or presence (Figure 3.15).

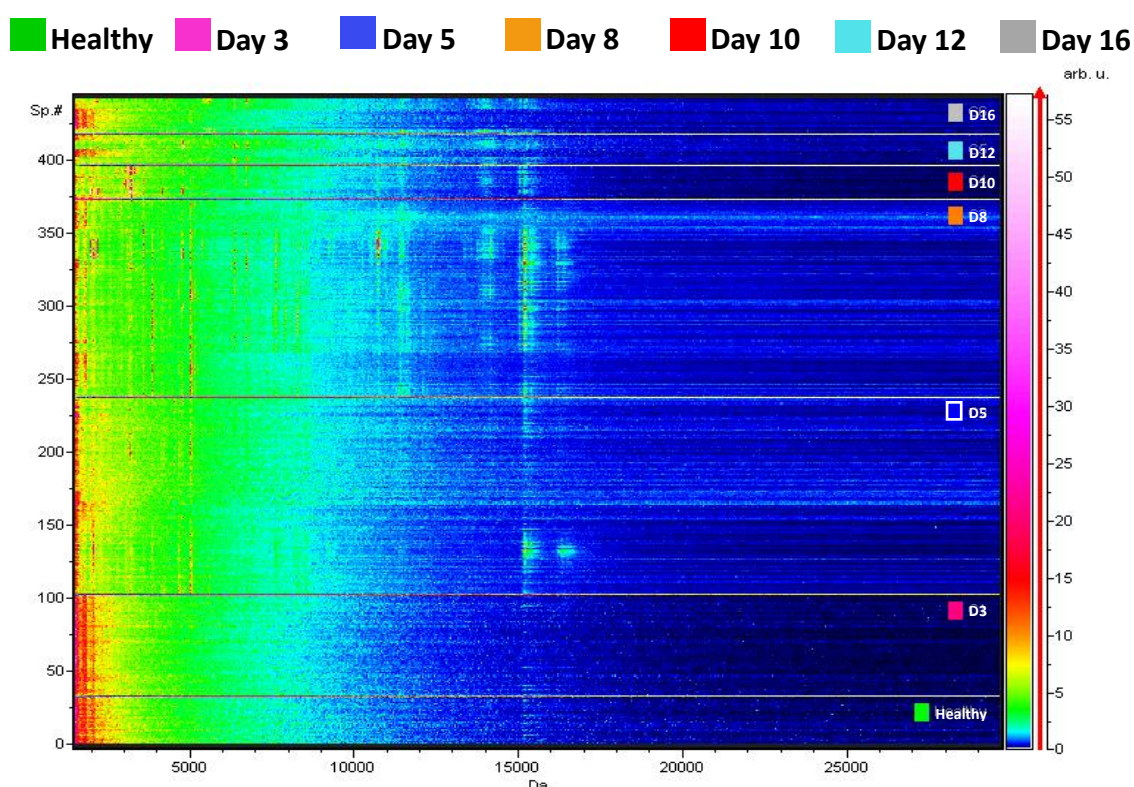


Figure 3.15: Pseudo-gel of healthy tissue and whole wound tissue from Days 3, 5, 8, 10, 12 and 16. This diagram presents the comparative data from the different samples in terms of the mass to charge ratio (1500 – 30000 Da) and the normalised intensity of each feature. Quantitative differences are clearly visible when tracking the changes of the acute wound proteome over select days during wound healing in comparison to the non-wounded, healthy tissue. The x-axis shows the molecular weight in Da (equivalent to m/z); the left y-axis shows the spectrum number originating from subsequent spectra loading (Sp.#); the right y-axis indicates normalised feature intensity through a coloured intensity scale in arbitrary units (arb. u.). The healthy tissue and six wound time points are separated by white lines on the gel and sets of spectra are identified by different colour square blocks according to the key shown at the top of the image.

The proteomic changes occurring over the 16 days of acute wound healing were clearly visualised on the pseudo-gel through quantitative differences in the normalised spectral intensities as well as absence and presence of specific features. Day 3 very closely quantitatively resembled the non-wounded healthy tissue especially with respect to the presence of small peptides in the low molecular weight region (1500 – 2500 Da) showing a high relative intensity. The intensity of the small peptides decreased from Day 5 and only started to increase again from Day 12 through to Day 16. On Day 5 quantitative differences were obvious compared to the healthy tissue, the most notable being the significant increase of features around the 15000 Da mass. Although present throughout the wound on Day 5, a significant increase in these proteins was seen from spectra number 125, which was most likely originating from the wound edges based on the mass spectra results shown in Figure 3.6, where an increase in this mass was seen in the right wound edge, as well as the increased wound edge variance observed in the PCA plots on Day 5 (Figure 3.10). There was also an increase at around 5200 Da on Day 5 compared to Day 3, where this small protein continued to increase and become more prominent on Day 8. This data was supported by the increase of this mass seen on Days 5 and 8 in Figure 3.6. Small proteins spanning from about 7500 – 9000 Da increased on Day 5 and remained at elevated levels through to Day 16.

A sudden increase in the feature of mass 3900 Da was observed on Day 8 but decreases again by Day 10. A prominent increase of the feature at 15000 Da on Day 8 compared to Day 5 could be seen in most Day 8 spectra. Other quantitative increases in proteins on Day 8 included those at 10700 and 11500 Da, which could clearly be seen in the wound mass spectra on Day 8 in Figure 3.6, as well as at 14000 Da. Proteins around 16500 Da increased on Day 8 as compared to Day 5, appearing in more sample spectra. The masses at 10700, 11500 and 14000 Da were found at a comparable intensity two days later on Day 10, while the proteins in the 15000 Da range decreased in intensity with those at 16500 Da being substantially decreased. A significant increase of the feature at 3500 Da was also seen on Day 10. On Days 12 and 16, the lower molecular weight peptides and proteins began to increase in intensity to more closely resemble the profile seen on Day 3 and in the healthy tissue. The masses around 10700, 11500, 14000 and 15000 Da remained present but decreased in intensity in the wound.

The pseudo-gel offered a visual graphic of the mass distribution and intensities which gives another dimension of insight into the kinetics of wound healing through quantitative molecular tracking over the six healing time points measured. Large quantitative changes occurring in different peptides and proteins were noted although minor quantitative changes in the mass range of 1500 – 6000 Da could not be discerned due to the fact that most of the detected features fell within this mass range and had relatively similar intensity signals. Quantitative changes that could be discerned were not necessarily significant changes or major contributors to the variance in the datasets previously seen.

To confirm which features were contributing to significant variance, masses from the pseudo-gel were compared to the variant masses identified in the loading plots where some correlation was seen. Masses of m/z 10724, 14054, 15033, 15193 and 15366 were all identified as outliers in the loading plots and showed quantitative changes in the pseudo-gel. In addition, the mass m/z 1977 which exhibited high variance in the loading plots showed large changes in relative intensity on the pseudo-gel compared to other features on the gel. While the pseudo-gel and loading plots visualised large amounts of data based on kinetic changes in molecular feature makeup of the acute wound healing process, they did not provide the statistical information required to determine the statistical significance of such changes.

3.3.4 Peak Statistics

3.3.4.1 PCA data analysis

While PCA (with all its associated plots) and pseudo-gels act as useful and sometimes vital screening tools in analyses of large comparable datasets of multiple features such as those resulting from an extended kinetic study of acute wound healing, they do not necessarily reveal all potential features or information of interest. While these techniques highlight and categorise important features associated with wound healing, they do not provide any significance for the identified outlier. In order to determine the importance of the features highlighted in the PCA and pseudo-gel analysis, as well as to reveal other features as potential acute wound healing markers, peak statistics were performed on a region of healthy tissue compared to wound tissue from Day 3, 5, 8, 10, 12 and 16.

Three peak statistic reports were generated for the wound bed ROIs, the wound edge ROIs and on the combined wound region (combined bed and edge ROIs) respectively, across the spectra of the healthy tissue and the six selected wound time points. A total of 126, 109 and 112 peak picked features for the wound bed, wound edges and whole wounds respectively, were detected according to the feature identification criteria set (Section 3.2.3.2) with the averaged intensity for each feature in the healthy tissue and each wound time point reported (Appendices III - V). These data were then reduced to a manageable differential feature set using PCA and associated loading plots to reveal the mean masses contributing most to the variance. ClinProTools software was used to create a minimised kinetic profile of acute wound healing over 16 days, where features appearing to be specifically differentiating were highlighted through PCA and associated loading plots (only the 5 most differentiating features per analysis were selected) (Section 3.3.2.3). In order to determine the statistical significance of these features, the Anderson-Darling (AD) test was used to assess whether the average intensities across the healthy tissue and six time points per feature of the full dataset displayed a normal distribution. This was followed by assessment of the p-values provided by either an ANOVA or Kruskal-Wallis test depending on the data distribution result. The coefficient of variation (CV) was calculated to indicate data variability (Table 3.2).

Table 3.2: Kinetic peak statistics on differential loading plot values as potential acute wound healing markers

	Mass (m/z)	D Ave	PKW	PAD	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
WOUND BED	4771,45	29,8	< 0.000001	0	3,3 ± 40,3	3,37 ± 25,94	4,67 ± 39,22	33,04 ± 181,4	4,48 ± 49,58	4,65 ± 35,24	3,24 ± 17,21
	4793,55	24,3	0,000173	0	3,21 ± 22,2	3,23 ± 24,87	3,71 ± 20,02	4,1 ± 56,9	3,75 ± 25,4	27,52 ± 106,7	3,34 ± 15,37
	6719,24	5,46	< 0.000001	< 0.000001	2,07 ± 34,9	1,95 ± 27,68	2,86 ± 23,02	7,41 ± 107,3	3,67 ± 54,51	2,91 ± 33,9	4,64 ± 47,49
	10724,05	5,51	< 0.000001	< 0.000001	1,15 ± 28,7	1,06 ± 29,33	1,42 ± 29,32	6,57 ± 105,4	2,02 ± 59,65	1,58 ± 32,3	1,84 ± 31,41
	15193,17	4,39	< 0.000001	< 0.000001	1,23 ± 126	0,75 ± 73,38	1,49 ± 50,55	5,13 ± 98,45	2,17 ± 80,86	1,06 ± 32,65	2,03 ± 57,02
WOUND EDGES	1977,15	31,7	< 0.000001	0	13,55 ± 68,9	9,33 ± 18,43	6,27 ± 20,81	4,98 ± 23,02	36,7 ± 102,8	7,17 ± 25,92	6,45 ± 31,73
	14054	0,5	< 0.000001	0	0,62 ± 54,5	0,52 ± 39,77	0,69 ± 33,36	0,82 ± 28,31	1,02 ± 63,01	0,92 ± 49,21	1,02 ± 110,9
	15032,65	0,67	< 0.000001	< 0.000001	0,57 ± 53,9	0,58 ± 46,03	0,68 ± 34,49	1,14 ± 58,28	1,25 ± 52,56	0,68 ± 34,75	0,68 ± 41,96
	15193,37	3,18	< 0.000001	< 0.000001	1,2 ± 130	0,65 ± 42,36	1,66 ± 96,18	3,83 ± 93,68	2,04 ± 65,97	0,9 ± 26,79	0,78 ± 46,49
	15366,06	0,69	< 0.000001	< 0.000001	0,54 ± 64,2	0,48 ± 50,32	0,95 ± 65,81	1,17 ± 48,19	0,85 ± 46,09	0,65 ± 49,74	0,56 ± 57,19

(**D Ave**: Difference between the maximal and minimal average feature intensity for healthy tissue and all six time points (arb. u.); **PKW**: P-value calculated by the Kruskal-Wallis test; **PAD**: P-value of Anderson-Darling test; **Ave N ± % CV**: Feature intensity average of class *N* where 'H'= healthy tissue and 'D'= Day post-wounding; ± coefficient of variation in % of class *N*)

Table 3.2 displays the five feature masses that contributed most to the data variation in the wound bed and wound edge ROIs independently on selected days during the 16 days of repair as revealed from the loading plots. None of the feature masses of interest displayed a normal distribution according to the AD test and therefore the Kruskal-Wallis test was used to determine statistical significance. All features in the table showed $p < 0.000001$ except for m/z 4793.55 where $p < 0.000173$, for the average intensity values. This means that the difference in average intensity of each of these features between the healthy tissue sample and the wound ROI samples from Days 3, 5, 8, 10, 12 and 16 were different with a statistical significance of >99.999% confidence, providing further proof of a continuously changing wound proteome.

In the wound bed, features with masses m/z 4771.45 and 4793.55 showed the greatest relative difference between the maximal and minimal average feature intensity (D Ave) across all time points with m/z 4771.45 peaking in intensity on Day 8 and m/z 4793.55 on Day 12 (confirming the time point of maximal variation of m/z 4793.55 in Section 3.3.2.3). Due to this significantly higher D Ave compared to the other three features, m/z 4771.45 and 4793.55 presented as more reliable and selective proteomic features of acute wound healing, especially due to the inclusion of non-wounded healthy tissue as the comparator. The other features of interest associated with the wound bed, m/z 6719.24, 10724.05 and 15193.17 all showed a peak in intensity on Day 8 and showed significantly lower D Ave values across the remaining healing time points and the healthy tissue, implying relatively minor quantitative changes occurred in these features during the healing process. Interestingly, four out of the five wound bed masses being m/z 4771.45, 6719.24, 10724.05 and 15193.17 showed their highest CV values on Day 8, with m/z 4793.55 peaking on Day 12. This again highlighted wounds from Day 8 as showing the greatest difference from healthy tissue during wound healing compared to the other time points and suggested the occurrence of multiple healing-associated processes occurring at this time point. This is most probably because the biological processes associated with inflammation, proliferation and remodelling were all occurring simultaneously on Day 8, making this time point the most likely to identify progression of healing kinetics within an acute wound model if a single time point were to be chosen.

With respect to the wound edges, only two out of the five selected features seemed to show an intensity difference in D Ave to show potential as proteomic features of acute wound healing. Mass m/z 1977.15 showed the highest D Ave with a maximum average intensity and CV on Day 10. Interestingly, this feature showed a significantly higher intensity in healthy tissue compared to all wound time points except for Day 10, where an almost three-fold increase was seen. This suggests that this feature is associated with proliferation and/or remodelling activities and would therefore be an interesting molecule to track in non-healing chronic wounds. The second feature showing a large D Ave value was m/z 15193.37, being the same feature seen in the wound bed. Just as in the wound bed, this feature showed a maximum intensity on Day 8. The remaining three features in the wound edge, being m/z 14054.00, 15032.65 and 15366.06, all had small D Ave values indicating small changes in average intensity compared to all the other healing time points, and especially relative to the non-wounded healthy tissue. This rendered these three features as potentially weak for classifying the phases of acute wound healing.

Peak statistical analyses revealed the following six features as the strongest contenders as potential features of the acute wound healing phases: m/z 1977, 4772, 4794, 6719, 10724 and 15193. Furthermore, Day 8 was again shown to represent the peak differences in acute wound healing molecular activity and variability, being the time point to focus on if a single time point were to be selected as a representative model to indicate progression of acute wound healing for further analyses.

3.3.4.2 Kruskal-Wallis and difference average analyses

PCA data coupled to loading plots revealed features from the acute wound healing process for further statistical analyses, ultimately highlighting six features as potential wound healing markers. This approach however, is only one of the possible methods of identifying such features. An alternative statistical approach is to analyse the peak statistics of the healthy tissue and six wound time points, independent of PCA analysis and to use the Kruskal-Wallis test combined with difference average (D Ave) values to rank and discover potential markers of acute wound healing. This approach was applied to the peak statistic reports of the wound bed ROIs, the wound edge ROIs and the whole wound data (wound bed and wound edge

combined ROIs) (Appendices III - V). Only the top 2 ranking masses according to either the most significant Kruskal-Wallis p-value (PKW) or the largest D Ave difference were focussed upon as identifiers of potential features of interest (Table 3.3).

Table 3.3: Kinetic peak statistics ranked by PKW and D Ave analyses for the different ROIs as potential acute wound healing features

	PKW-ranked mass (<i>m/z</i>)		D Ave	PKW*	D Ave-ranked mass (<i>m/z</i>)		D Ave	PKW*
W.B	1.	1531.24	19.73	< 0.000001	1.	4771.45	29.8	< 0.000001
	2.	1756.52	7.89	< 0.000001	2.	4793.55	24.3	0.000173
W.E	1.	1758.56	7.13	< 0.000001	1.	1977.15	31.72	< 0.000001
	2.	1977.15	31.72	< 0.000001	2.	1531.65	16.15	< 0.000001
W.W	1.	1815.45	9.45	< 0.000001	1.	1531.49	18.56	< 0.000001
	2.	1723.05	6.77	< 0.000001	2.	1977.43	18.23	< 0.000001

*Data do not follow a normal distribution based on PAD thus PKW was used for evaluation.

(W.B: Wound bed; W.E: Wound edges; W.W: Whole wound; D Ave: Difference average; PKW: P-value Kruskal-Wallis.)

The Kruskal-Wallis test and the D Ave values from the peak statistic reports were used to identify the most prominent and likely features of acute wound healing. To achieve this the probability value obtained from the Kruskal-Wallis test (PKW) was used to reveal the two features with the greatest difference between the healthy tissue and the wound bed ROIs, wound edge ROIs and whole wound regions from Days 3, 5, 8, 10, 12 and 16 post-wounding. Where PKW values were comparable, masses were ranked based on the best distinguishing features. D Ave on the other hand ranked features based on the largest significant difference between the maximal and minimal average feature intensity of the healthy tissue and six wound time points.

The top-two feature average masses revealed by the PKW sort-order were *m/z* 1531 and 1756; 1758 and 1977; and 1815 and 1723 in the wound bed ROIs, wound edge ROIs and whole wounds ROIs respectively (Table 3.3). All these features had a p-value <0.000001 meaning that these features showed a significant difference in intensity between the healthy tissues and all six time points during wound healing.

Mass m/z 1531 was not only found to be the most distinguishing feature between the healthy tissue and six wound time points in the wound bed but ranked second for the wound edges and showed the highest average difference in whole wound tissue with a D Ave value of 18,56; highlighting this feature as a feature of interest. When applying a mass error of $\pm 0.25\%$ (as in Section 3.3.2.3) to the average mass values, masses m/z 1756 and 1758 seen in the wound bed and edges respectively, could show a double bond difference or may be considered as the same peptide. This was supported by very similar D Ave values as well as the fact that both masses did not occur simultaneously in a selected ROI. Peptide m/z 1977 was already revealed as a potential feature of interest through PCA and loading plot analysis and not only showed a highly significant difference in intensity but was also responsible for the high D Ave in the wound edge and whole wound analyses. The fact that m/z 1977 had been shown as a feature of interest in two different and independent analyses (PCA and peak statistics) highlighted its potential value as a marker of acute wound healing. Lastly, in the PKW rankings, the features of m/z 1815 and 1723 presented as the most significantly different peptides between the healthy tissue and six wound time points in the whole wound samples, making them features of interest. Interestingly, m/z 1815 may be the same feature as m/z 1977 with the loss of a conjugated sugar.

When analysing the D Ave results in the table, small protein features m/z 4772 and 4794 showed the largest average differences in the wound bed being 29,8 and 24,3 respectively. As with m/z 1977, both these features had already been identified as potential wound healing markers through the PCA and loading plot analysis and therefore showed promise as potential markers. Interestingly, m/z 4772 and 4794 may be a potential sodium adduct pair.

In summary, using peak statistics and ranking by most significant PKW and D Ave values revealed m/z 1531, 1723, 1756/8, 1815 as new potential unique wound healing features; and confirmed m/z 1977, 4772 and 4794 as potential wound healing features. Compared to the PCA and loading plot analysis, this statistical approach of identifying potential healing markers appears to have revealed features in the small peptide range. It is interesting at this point to take note of the mass regions where large quantitative changes were visualised on the pseudo-gel (see Section 3.3.3). Such changes were especially seen from 1500 – 2000 Da, which

covers the range of the newly identified features of interest. The peak statistics thus confirmed that the changes seen visually on the pseudo-gel were in fact statistically significant ($p < 0.000001$) and represented some of the largest intensity changes when comparing healthy tissue to that of wounded tissue over the 16 days of healing.

By comparison, the rankings of the D Ave (from largest to smallest) for the remaining features of interest initially identified in the PCA analysis were as follows: m/z 6719 and 10724 both from the wound bed (see Table 3.2) ranked 24th and 23rd out of 126 features respectively and m/z 15193 revealed in both the wound bed and wound edges (Table 3.2) ranked 33/126 features in the wound bed and 28/109 features in the wound edges. With respect to PKW rankings m/z 6719, 10724 and 15193 ranked 61st, 22nd and 25th respectively out of 126 features in the wound bed, and m/z 15193 ranked 20/109 in the wound edges.

Statistical analyses were successfully used to reveal potential markers of acute wound healing. This was accomplished through two independent techniques being PCA combined with loading plots, and peak statistics analysis. Each technique revealed features showing high contribution to variance and significant difference in intensity between the healthy tissue and the six different time points over the 16 days of healing, with the same three features being highlighted by the different statistical approaches (m/z 1977, 4772 and 4794). Following analysis, 10 features of interest were identified as having the highest potential to act as markers of the progression of acute wound healing and are as follows (ranked by mass from smallest to largest): m/z 1531, 1723, 1756/8, 1815, 1977, 4772, 4794, 6719, 10724 and 15193.

3.3.5 2D Peak distributions

Up until this point, analyses of various features have involved the average intensity of these features per region of interest and have not visualised individual features. 2D peak distribution analysis shows the features from all individual spectra collected and not just the averaged quantitative values. This allows visualisation of the spatial distribution of the data points from the healthy tissue and from wound Days 3, 5, 8, 10, 12 and 16 based on two selected features of interest falling on the x and y axes.

The 2D distribution plot provides a basis for the assessment of features best differentiating the healthy tissue and six wound time points from each other.

To compare the abilities of the different statistical tests, being the Kruskal-Wallis test and PCA, to distinguish the features from the healthy tissue and six time points during wound healing, the first two features of each sort-order (ranked by most significant PKW and most variant PCA feature respectively) from whole wound analysis were selected as the feature combination for the 2D distribution plots. The greatest significant D Ave values were also assessed for sample differentiation and classification ability.

The Kruskal-Wallis ranked probability values and the D Ave assessment values reported in Table 3.3 were used with m/z 1815 and 1723 for Kruskal-Wallis probability, and m/z 1531 and 1977 for D Ave respectively. Assessments were performed on whole wound data where a 3D combination PCA plot coupled to a loading plot of whole wound data was created to reveal the most variable features (Figure 3.16). In doing so, six features contributing most to variance were used in further calculations. In order to determine the two single features contributing most to the variance during the healing process by PCA statistics for the 2D peak distribution plots, feature contribution calculations were performed on the six most variable features identified from the loading plot. Each feature was assigned three loading values directly corresponding to the three PCs (PC1, PC2 and PC3). According to the variance plot of this data, PC1, PC2 and PC3 explained 31%, 12% and 8% of the variance respectively. Thus, the single contribution (SC) of a feature per PC to the overall dataset was equal to the product of the feature's loading value and the PC contribution. The total contribution (TC) of each feature was the sum of the feature's three SC values. Thus, the two features with the highest absolute TC value were deemed as the most significant and were revealed as m/z 10724 and 15193 (Table 3.4).

In addition to testing the ability of the different statistical tests in distinguishing the healthy tissue and six wound time points, it was of interest to compare and investigate features of interest highlighted through quantitative changes seen on pseudo-gels (Figure 3.15). To test this, a mass region of interest was selected (m/z 15100 – 15500) where significant quantitative changes could be seen between the

spectra of healthy tissue and the six time points during wound healing. Features showing the greatest significant D Ave within this mass range according to the peak statistic report were then selected for 2D peak distribution analysis. These two features were m/z 15193 vs 15400. The 2D peak distribution plots testing the abilities of the Kruskal-Wallis test, PCA, largest D Ave and pseudo-gels to differentiate kinetic acute wound healing are illustrated in Figure 3.17.

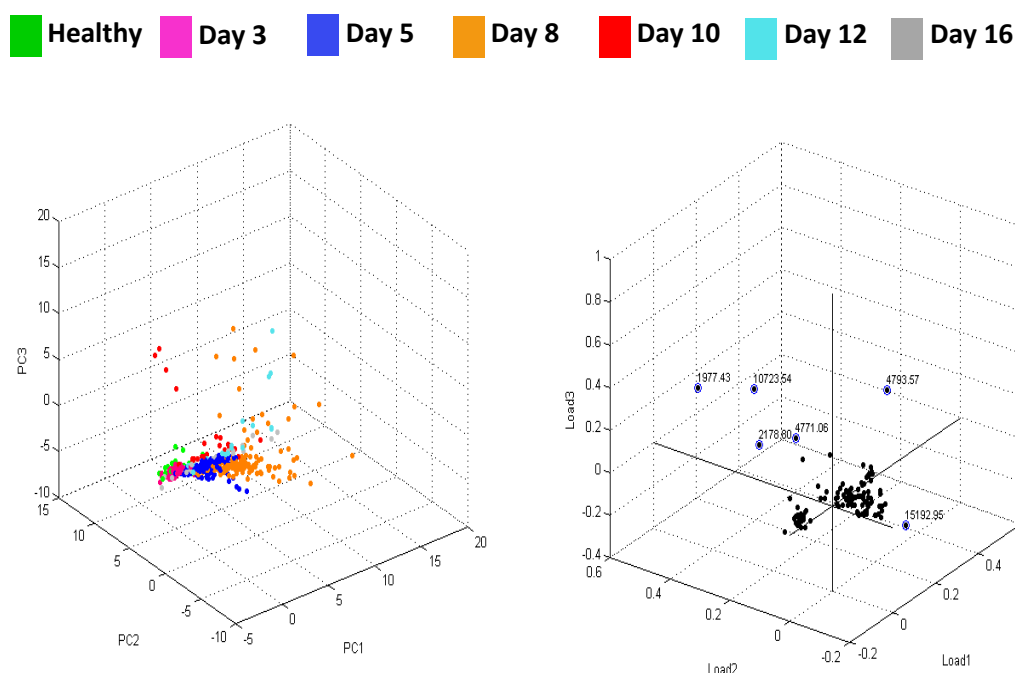


Figure 3.16: 3D PCA combination plot and associated loading plot of whole wounds vs healthy tissue over 16 days. Combined 3D PCA plots with the respective associated 3D loading plots of whole wound regions revealing masses of features showing the greatest variance across the first three principal components for healthy tissue and from wounds of Days 3, 5, 8, 10, 12 and 16. The healthy tissue and six wound time points are coloured according to the key at the top of the image.

Table 3.4: Variance contribution of differential PCA features of interest

Mass (m/z)	1977.43	SC	2178.80	SC	4771.06	SC	4793.57	SC	10723.54	SC	15192.95	SC
Loading 1	0.066	2.046	0.061	1.891	0.082	2.542	0.034	1.060	0.410	12.710	0.335	10.385
Loading 2	0.497	5.964	0.283	3.396	0.181	2.172	-0.158	-1.896	0.556	6.672	-0.005	-0.600
Loading 3	0.495	3.960	0.104	0.832	0.174	1.392	0.602	4.816	-0.0125	-0.100	-0.322	-2.576
TC		11.970		6.119		6.106		7.772		19.482		13.021

Negative values only indicate the direction of variance scattering on the loading plots. SC: Single contribution; TC: Total contribution. SC= loading value x PC variance explained (%). TC= sum of SC values. PC1= 31%; PC2=12%; PC3=8%.

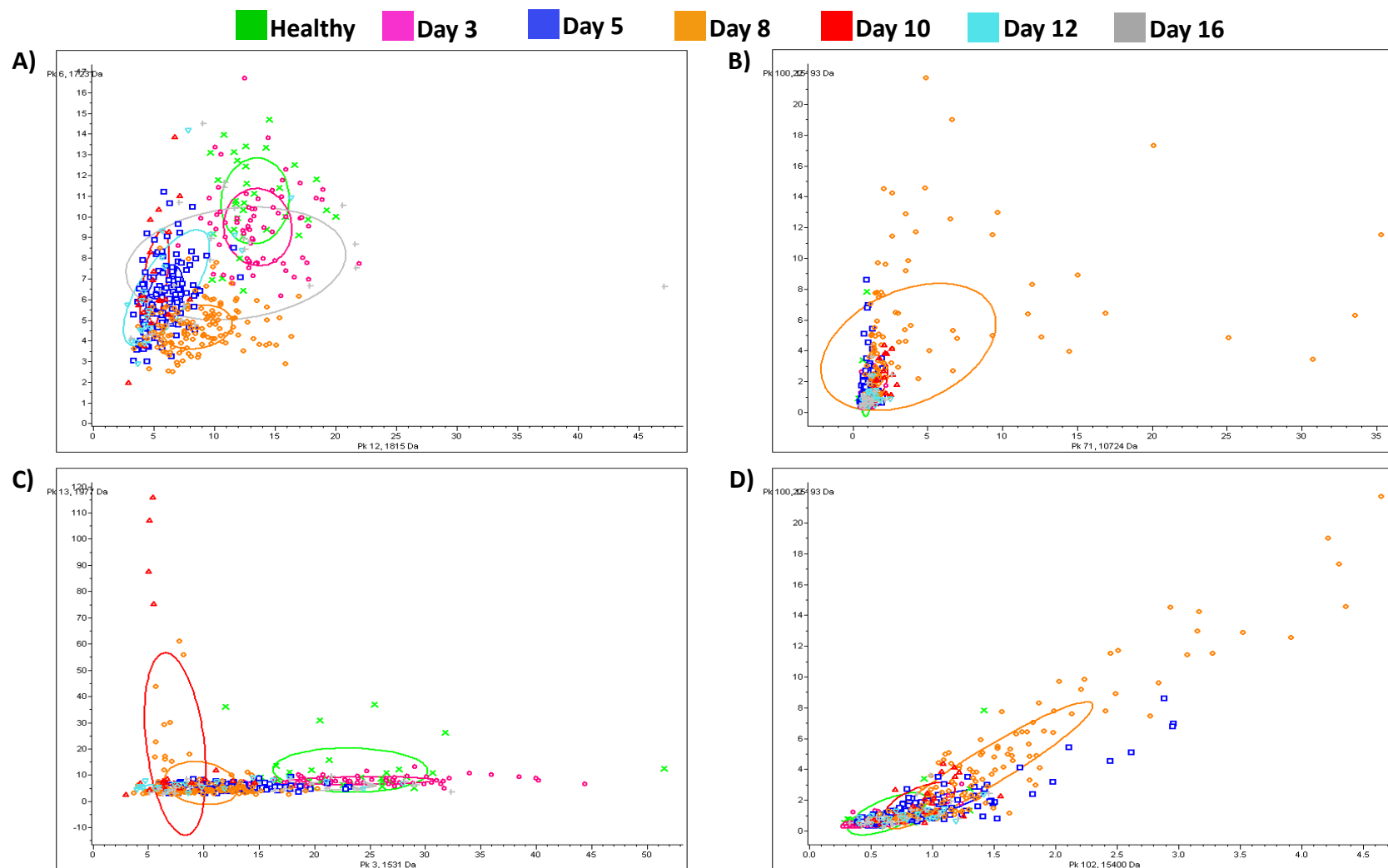


Figure 3.17: 2D peak distribution plots of features of interest. 2D peak distributions of the two top-ranked features in whole wounds by (A) PKW value: m/z 1815 vs 1723; (B) PCA value: m/z 10724 vs 15193; (C) D Ave value: m/z 1531 vs 1977; and (D) pseudo-gel quantitative change: m/z 15400 vs 15193. The healthy tissue and six wound time points are shown according to the colour key. Ellipses represent the standard deviation of the time point average of the feature intensities. Axes measurements are in arbitrary units chosen automatically to fit the plot optimally in the plane.

2D peak distribution was used to assess which features best distinguished healthy tissue from that of wounded tissue and the different time points of healing using feature intensity changes. Feature candidates were selected based on three statistical rankings (PKW, PCA and D Ave) as well as relative quantitative changes displayed in pseudo-gels. The first statistical ranking was that by PKW, where the two most significantly different and best distinguishing features between the healthy tissue and six wound time points were selected, being m/z 1815 and 1723. First and second ranked features are shown on the x and y axes respectively for all peak distribution plots (Figure 3.17 A). PCA, the second statistical approach, ranked features based on their contribution to variance within the dataset and found m/z 10824 and 15193 to be the greatest contributing features (Figure 3.17 B). The third statistical ranking was based on the largest, significant D Ave across the sample time points being features m/z 1531 and 1977 (Figure 3.17 C). Lastly, features that showed a relatively large quantitative change between sample time points in the pseudo-gel (Section 3.3.3) were used to create mass ranges of interest, in this case being from 15100 – 15500 Da. Peak statistics were then applied to identify the two features showing the greatest D Ave in this mass range and revealed m/z 15400 and 15193 (Figure 3.17 D).

Figure 3.17 A based on PKW showed time-based differences in the selected feature intensities. Only Day 3 was similar to the non-wounded healthy tissue, which supported what was seen in the PCA of the wound bed and edges vs healthy tissue (Figures 3.9 and 3.10). The remaining time points overlapped with one another to varying degrees. Days 10, 12 and 16 completely overlapped with one another and for the most part also with Days 5 and 8. Day 8 however, showed the greatest spread across the datasets (in feature number) closely followed by Day 5, with peptides m/z 1815 and 1723 being the greatest contributors to the variance on Day 8 and Day 5 respectively. A few features from Day 10 also displayed significant variance in peptide m/z 1723, while some features from Day 16 presented as relatively extreme outliers, resulting in a high standard deviation at this time point.

The PCA-based distribution plot (Figure 3.17 B) did not differentiate between the healthy tissue or the different time points of healing where significant overlap was seen between the data points, except for Day 8. This plot highlighted and confirmed

the large number of differentiating features that occurred on Day 8, with large quantitative changes in both m/z 10724 and 15193.

The peak distribution plot ranked by largest D Ave (Figure 3.17 C) showed a sequential yet overlapping distribution in feature m/z 1531, decreasing in intensity as healing progressed. This feature's distribution did not differentiate wound time points and healthy tissue. Peptide m/z 1977 (y-axis), differentiated some Day 8 and to an even greater extent Day 10, features from remaining time points and healthy tissue. Interestingly, the peak statistics analysis revealed m/z 1977 as showing the greatest relative average intensity on Day 10 (Table 3.2) but this peak distribution analysis indicated that this high average was due to only four individual feature outliers, also explaining the large standard deviation shown by the red ellipse on the plot (Figure 3.17 C). This standard deviation range included most of the features with high variance on Day 8 and thus if this feature were to be used as a marker, distinguishing Day 8 from Day 10 would be improbable. The wound healing process during the switch to proliferation and remodelling could be distinguished from non-wounded tissue and potentially from non-healing, chronic wound tissue that would not enter the remodelling phase.

The last 2D peak distribution plot (Figure 3.17 D), the pseudo-gel analysis, revealed relative quantitative change over the 16-day wound healing study but did not provide an indication of the extent of the variance or spread of the features. The 2D distribution plot of m/z 15400 vs 15193 showed Days 5 and 8 as the only time points displaying significant variance with relative increases in intensity on both days. The intensity increases observed in m/z 15193 were substantially larger than those in m/z 15400, making m/z 15193 the feature with more potential as a possible healing (Day 5 – 8) marker.

While the PKW, PCA and D Ave plots (Figures 3.17 A – C) showed some overlap between the healthy tissue and wound time points, the PKW-ranked plot showed the least similarity between all samples and could better differentiate the wound and non-wounded healthy tissue. The PCA-ranked plot showed high correlation between the samples, except for the high variance on Day 8. Despite far greater variance seen on Days 8 and 10 on the D Ave ranked plot compared to the PKW and PCA-ranked plots, healthy tissue could not be differentiated from that of wounded tissue

and overall greater overlap was seen between different time points. Additionally, the relative spread of Day 8 data points was more significant in the PCA-ranked plot, allowing for easier differentiation in a model setting.

Considering these outcomes, features ranked according to the highest contribution to variance based on PCA presented the most favourable distinguishing features of acute wound healing. Even though features with the most significant PKW differentiated the healing phases better for acute wound healing and from non-wounded tissue, this differentiation could not confidently classify unknown status wound samples to specific phases nor characterise tissue as an acute wound. Although only Day 8 showed a significant difference in the PCA-based 2D peak distribution plot compared to non-wounded tissue, it was this time point that was found to be the height of acute wound healing activity and thus arguably the most representative of the advancing healing process.

The 2D peak distribution plots highlighted Day 8 as being the healing time point with the most variance overall, as well as the most differentiated from non-wounded, healthy tissue. All plots showed overlap to some degree between the healthy tissue and the six wound time points, but all samples are endogenous porcine tissue encompassing overlapping phases of wound healing. In addition, the excised wounds were unavoidably surrounded by varying amounts of healthy tissue, and some correlation between wounded and non-wounded tissue would always be present.

3.3.6 Spectral analysis

All MALDI-IMS analysis reported in this chapter was based on the ROIs selected, being the wound bed, wound edges and a region of non-wounded healthy tissue (Section 3.3.1). This approach defined regions of spectra on which data analysis was done. As these ROIs were selected manually, based on H&E histology, it could be argued that human error or bias was possibly influencing the definition of a wound region to some extent and thus may have affected the resultant features of interest. In this section the analytical approach was thus reversed, where instead of analysing pre-selected spatial data, whole tissue mass spectral data was analysed for spatial wound association. The overall average mass spectrum of each whole tissue

section, *i.e.* the section cut from the wound biopsies from Days 3, 5, 8, 10, 12 and 16, were manually scanned, feature by feature to determine whether there was any spatial association occurring in the wound area. Spatial association was defined as relative quantitative molecular changes associated with the wound. This was done to reveal any wound-associated features that may have been missed in previous statistical analyses, which could act as potential acute wound healing markers (Figure 3.18).

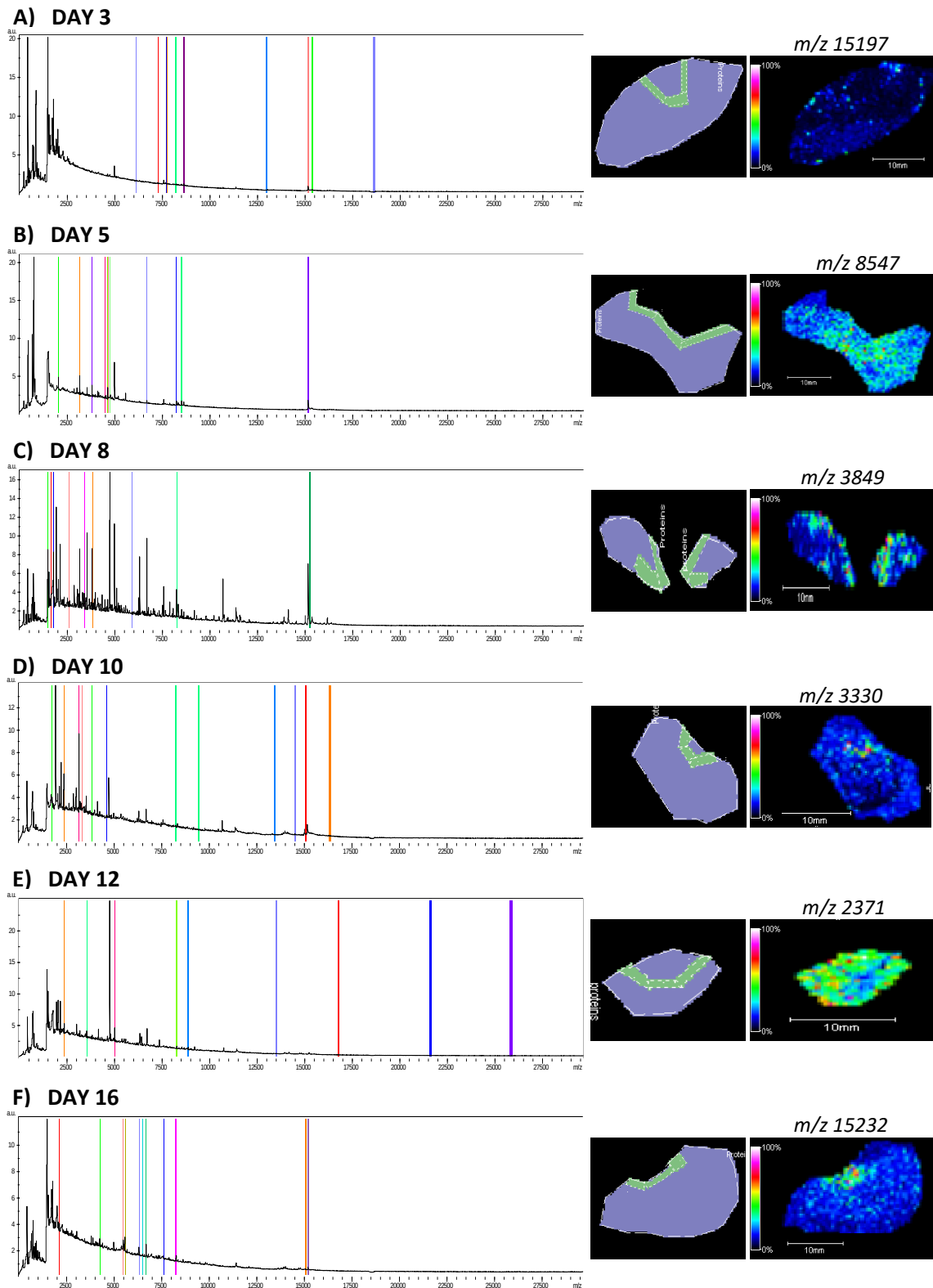


Figure 3.18: Selected wound-associated features through spatial quantitation. Proteins showing relative quantitative change in the wound region were found on Days 3, 5, 8, 10, 12, and 16 (A-F). Image sequence in A-F: Average mass spectrum of sample with lines indicating the masses showing large quantitative changes within the wound site; IMS scan template indicating the wound region in green; and IMS image showing the quantitative distribution of the protein with the highest association of the wound region. Colour intensity scale measures quantitation in arb. u.

Following the analysis of the average mass spectra of whole wound samples from Days 3, 5, 8, 10, 12 and 16, between four and nine features were highlighted per time point as having quantitative differences within the wound region compared to the surrounding, non-wounded tissue. None of the time points displayed features completely unique to wound tissue, while at some time points significant changes could not be visualised. For this reason, only the features that showed the greatest relative quantitative change in the wound area were illustrated in Figure 3.18.

Day 3 did not display any notable differences in protein quantitation between the wound and healthy tissue, which correlated with what was generally seen in the IMS statistical analyses already described in this chapter. The greatest visual change was for a protein with m/z 15197 and interestingly when applying a mass error of $\pm 0.25\%$, m/z 15193, which has already been identified as a feature of interest in this chapter, was included. This feature appeared to be absent from the wound on Day 3 but was found at very low levels in the surrounding tissue (Figure 3.18 A). On Day 5 this feature still showed only slight wound association and was thus marked in the mass spectra, but the greatest visual changes occurred in a small protein with m/z 8547. This small protein was dominant in only one wound edge and in the deeper, underlying tissues of the wound bed (Figure 3.18 B). The number of features observed on Day 8 with quantitative change associated with the wound decreased, however the relative intensity difference compared to surrounding tissue as well as specificity to the wound area, was increased compared to Day 5. The most representative Day 8 wound feature, m/z 3849, showed a large relative increase in abundance within the wound (Figure 3.18 C). Interestingly, at around m/z 3900 on the pseudo-gel (Section 3.3.3, Figure 3.15) a significant increase in intensity was seen on Day 8.

The small protein that showed the largest association with wound tissue on Day 10, m/z 3330, also appeared in the range where a significant increase was seen on the pseudo-gel on Day 10 (Figure 3.15). The increase in m/z 3330 seemed to be exclusively associated with the wound (Figure 3.18 D) being in the middle of the wound bed. This suggested that this molecule could be associated with proliferation, as the deepest part of the wound was being filled with new tissue to support the formation of the new epidermis as seen on Day 12 (Figure 2.8 M-P). Inspection of the mass spectrum on Day 12 did not reveal any features showing significant

quantitative changes associated with wound healing. This was in line with the results of the statistical analyses (PCA and peak statistics, Sections 3.3.2 and 3.3.5 respectively) where the features of the Day 12 wound showed significant overlap and correlation with that of the surrounding healthy tissue. The most quantitatively differentiating peptide between the wound region and surrounding tissue on Day 12 was found at m/z 2371, where this molecule was not present in what appeared to be new granulation tissue as seen from the associated histological staining (Figure 2.8 O). This quantitative difference may be used to show proliferation, however the area affected was small and therefore this could prove to be a challenge (Figure 3.18 E).

Lastly, Day 16 showed a decrease in the number of features showing quantitative change within the wound yet had high spatial specificity. Protein m/z 15232 was highly associated with the wound at this time and when applying the mass error of $\pm 0.25\%$, the mass range included m/z 15193. This not only matched an already highlighted protein of interest in this chapter, but there is a possibility that this feature is the same feature as that seen on Day 3. This small protein may thus be a marker of non-wounded tissue or more specifically newly formed healthy tissue and may be linked to the remodelling process as previous statistical analyses (Sections 3.3.2 and 3.3.5) showed considerable association of the wound with non-wounded healthy tissue at Days 3 and 16.

This reversed approach of scanning mass spectra for spatial association with the wound, revealed additional masses of interest as potential wound markers as well as confirmed previous results found through statistical tools. On Days 8 and 10 m/z 3849 and 3330 respectively, not only showed spatial association with the wound but matched the quantitative changes seen in the pseudo-gel (Figure 3.15). These two small proteins should therefore be added to the list of potential acute wound healing features. In addition, the mass spectra confirmed m/z 15193 as being a potential healing marker where clear spatial association was seen on Days 3 and 16. It is also interesting that the overall mass spectra at all healing time points, except on Day 12, showed a feature associated with the wound ROI in the m/z 15000 range (Figure 3.18 A-F).

3.4 Summary

MALDI-IMS made it possible to study the kinetic (time-based) and spatial small-proteome changes during acute wound healing by identifying ROIs in the wound and from surrounding healthy tissue for analysis. Selected ROIs allowed for the tracking of features in the wound bed and wound edges independently and relative to non-wounded, healthy tissue on selected days over a period of 16 days during which the wounds appeared to follow the normal healing process. Unidentified kinetic changes at the molecular level could be monitored for the acute wound healing process and were visualised within a single combined image for analysis.

PCA successfully reduced the large volume of complex, proteomic data of tissue samples collected at six time points so that the continuously changing wound proteome could be visualised and tracked in a manageable manner. The resultant PCA plots formed an optimised reduced profile of acute wound healing and its healing phases without requiring feature identification for potential future clinical use. Using non-wounded healthy tissue as a reference point, Day 8 (closely followed by Day 5) was revealed as being the peak of acute wound healing proteomic activity, showing the greatest variation and differentiation compared to wound tissue from Days 3, 5, 10, 12 and 16. This fact was supported by pseudo-gel quantitative analysis, peak statistics and 2D peak distribution analysis.

The 2D peak distribution analysis supported PCA as being the tool of choice when identifying potential markers of acute wound healing.

Day 8 being representative of the most differentiating features during acute wound healing and the greatest variance, would be the time point to focus on in the kinetic process of healing if a single sampling time had to be selected for further, more in-depth proteomic comparative study, such as in chronic wound analysis. Interestingly, based on literature and confirmed by histological analysis in this study (Section 2.3.4), Day 8 is representative of a subsiding inflammatory phase and the beginning of proliferation, a transition that is thought to be hindered in impaired healing processes. Previous studies have highlighted the importance of the transition between the inflammatory and proliferative phases, stating this to be a key step for successful healing. Targeting features unique to these phases and during phase transition may lead to future therapeutic intervention studies (Landén *et al.*, 2016).

This further supports the use of Day 8 wound tissue in future analysis of different wound healing regimens.

Based on PCA calculations, associated loading plots, peak statistics and spatial wound association, 12 averaged masses were revealed that could potentially be used as unique features defining acute wound healing phases in the porcine model: m/z 1531, 1723, 1756/8, 1815, 1977, 3330, 3849, 4772, 4794, 6719, 10724 and 15193.

Experiments would need to be repeated to confirm these features as robust and reliable markers of healthy acute wound healing. In addition, because these masses are averaged, repeat analyses would help to provide an accurate mass range for identification. In future studies, identification of these potential markers may also aid in defining their appropriateness as markers. Revealing markers of acute wound healing offers a potential diagnostic tool for monitoring impaired healing wounds and may be used to identify new key targets for drug development therapy.

Chapter 4: Metabolomic and Lipidomic MALDI Imaging Mass Spectrometry

4.1 Introduction

Matrix-assisted laser desorption ionisation imaging mass spectrometry (MALDI-IMS) is a powerful analytical technique coupling the sensitivity of mass spectrometry with the unique ability to assess spatial molecular distribution in an array of sample tissue types. The 'omics' data typically generated through this type of analysis consists of thousands of mass spectra, requiring focussed data reduction and processing for more precise interpretation and possible clinical applicability. In this chapter the capabilities of MALDI-IMS coupled with histology and multivariate statistical analysis to reveal acute wound healing features of interest over six selected time points during 16 days of healing is demonstrated across metabolomic and lipidomic bases (see Section 3.1 for detail).

4.2 Materials and Methods

4.2.1 Wound tissue preparation

The wound tissue used for the MALDI-based metabolite and lipid assays was taken from sequential histological sections from the snap frozen, biopsied wounds collected on the different selected days post-wounding as described in detail in Section 3.2.1 of Chapter 3.

4.2.2 MALDI-IMS analysis

Tissue sections were removed from the -80°C freezer and desiccated under vacuum for 30 minutes to allow the samples to dehydrate and equilibrate to room temperature. The slides were then marked with dots of liquid white-out to provide a spatial reference of the sections and were optically scanned using a HP Deskjet F4583 scanner.

Ionisable matrix was applied to tissue sections using the ImagePrep station (Bruker Daltonics, Bremen, Germany) where independent, pre-installed standard protocols for metabolite and lipid analysis were used as shown in Tables 4.1 and 4.2, respectively. The matrix solution used for metabolite analysis was prepared with 2,5-

dihydroxybenzoic acid (DHB) at 30 g/l in 50% methanol with 0.2% trifluoroacetic acid (TFA); while the matrix solution used for lipid analysis was prepared with α -cyano-4-hydroxycinnamic acid (CHCA) at 7g/l in 50% acetonitrile with 0.2% TFA (all reagents purchased from Sigma-Aldrich, St Louis, USA). Following matrix deposition, the prepared slides were loaded into an MTP slide adapter II (Bruker Daltonics, Bremen, Germany). MALDI-IMS analysis was performed using an UltrafleXtreme MALDI TOF/TOF system (Bruker Daltonics, Bremen, Germany) accumulating 200-400 laser shots per pixel at 1 kHz laser frequency with instrument control and data acquisition performed through FlexControl 3.4 software (Bruker Daltonics, Bremen, Germany). To yield optimal results, data for MALDI-IMS metabolite and lipid analyses were acquired in reflectron positive mode with parameter settings as per Table 4.3. FlexImaging 3.0 software (Bruker Daltonics, Bremen, Germany) was used for the visualisation, interpretation and analysis of the resultant MALDI image data, which was acquired at a spatial resolution of 200 μ m.

Table 4.1: ImagePrep protocol used for DHB application (Metabolome analysis)

DHB	Matrix thickness (fixed number of cycles OR sensor control (V) + number of cycles	Nebulise Spray power, modulation and spray time (fixed time (sec.) or sensor control (V))	Incubation time (sec.)	Complete Dry time
Phase 1: initialisation	0.2 V within 10 and 15 cycles	25%, 30% and 1.5 s	15 s	50 s
Phase 2: 3x2 cycles	6 cycles	25%, 30% and 0.05 V	30 s	Sensor control (every 2 nd cycle)
Phase 3: 3x4 cycles	12 cycles	25%, 30% and 0.05 V	30 s	Sensor control (every 4 th cycle)
Phase 4: 3x6 cycles	18 cycles	25%, 30% and 0.1 V	30 s	Sensor control (every 6 th cycle)
Phase 5: 5x10 cycles	50 cycles	25%, 30% and 0.15 V	30 s	Sensor control (every 10 th cycle)

Table 4.2: ImagePrep protocol used for CHCA application (Lipidome analysis)

CHCA	Matrix thickness (fixed number of cycles OR sensor control (V) + number of cycles	Nebulise Spray power, modulation and spray time (fixed time (sec.) or sensor control (V))	Incubation time (sec.)	Complete Dry time
Phase 1: initialisation	0.2 V within 10 and 15 cycles	15%, 40% and 2.5 s	15 s	65 s
Phase 2: 1 cycle	0.07 V within 3 and 10 cycles	25%, 40% and 0.05 V	30 s	Sensor control (every 1 st cycle)
Phase 3: 2 cycles	0.07 V within 6 and 12 cycles	25%, 45% and 0.1 V	30 s	Sensor control (every 2 th cycle)
Phase 4: 3 cycles	0.2 V within 9 and 30 cycles	25%, 45% and 0.2 V	30 s	Sensor control (every 3 rd cycle)
Phase 5: 4 cycles	0.6 V within 8 and 64 cycles	25%, 45% and 0.3 V	30 s	Sensor control (every 4 th cycle)

Table 4.3: Parameter settings for MALDI-IMS metabolite and lipid analyses

Parameters	Metabolites	Lipids
Laser power:	57%	54%
Laser repetition rate:	1 KHz	1 KHz
Detector gain:	17.5 kV	17.5 kV
<i>m/z</i> measurement range:	500 – 2000	500 – 1300
Matrix suppression:	500 Da	500 Da
Acquisition rate:	1 GS/s	1 GS/s

4.2.3 Data analysis

Metabolomic and lipidomic data analysis was performed in the same way as the proteomic analysis (described in detail in Section 3.2.3) with adjustments in the statistical settings for optimal metabolomic and lipidomic analyses. One limitation was that only the mass spectra in the range from 500 – 2000 Da for metabolites and 500 – 1300 Da for lipids were assessed with the lower limit of 500 Da due to the high background from the matrix ions below this mass.

4.3 Results and Discussion

4.3.1 Regions of interest

Similar to the proteomic analysis described in Chapter 3, regions of interest (ROIs) were defined for the wound bed and wound edges for images produced by MALDI-IMS analyses of the metabolome and lipidome. These regions were accurately identified utilising the sequential wound sections stained with H&E for histological reference and co-registration on the MALDI sample images (Figure 3.3). ROIs consisted of the wound bed, the left and right wound edges, and a region from the surrounding non-wounded healthy tissue.

Selected ROIs provided the advantage of tracking the kinetic spatial composition of metabolites and lipids in the wound bed and each of the edges relative to non-wounded tissue over the 16 days of healing. Mass spectra of each ROI on Days 3, 5, 8, 10, 12 and 16 were displayed on a single set of axes per time point to allow for easy visual tracking of a continuously changing acute wound metabolome (Figure 4.1) and lipidome (Figure 4.2).

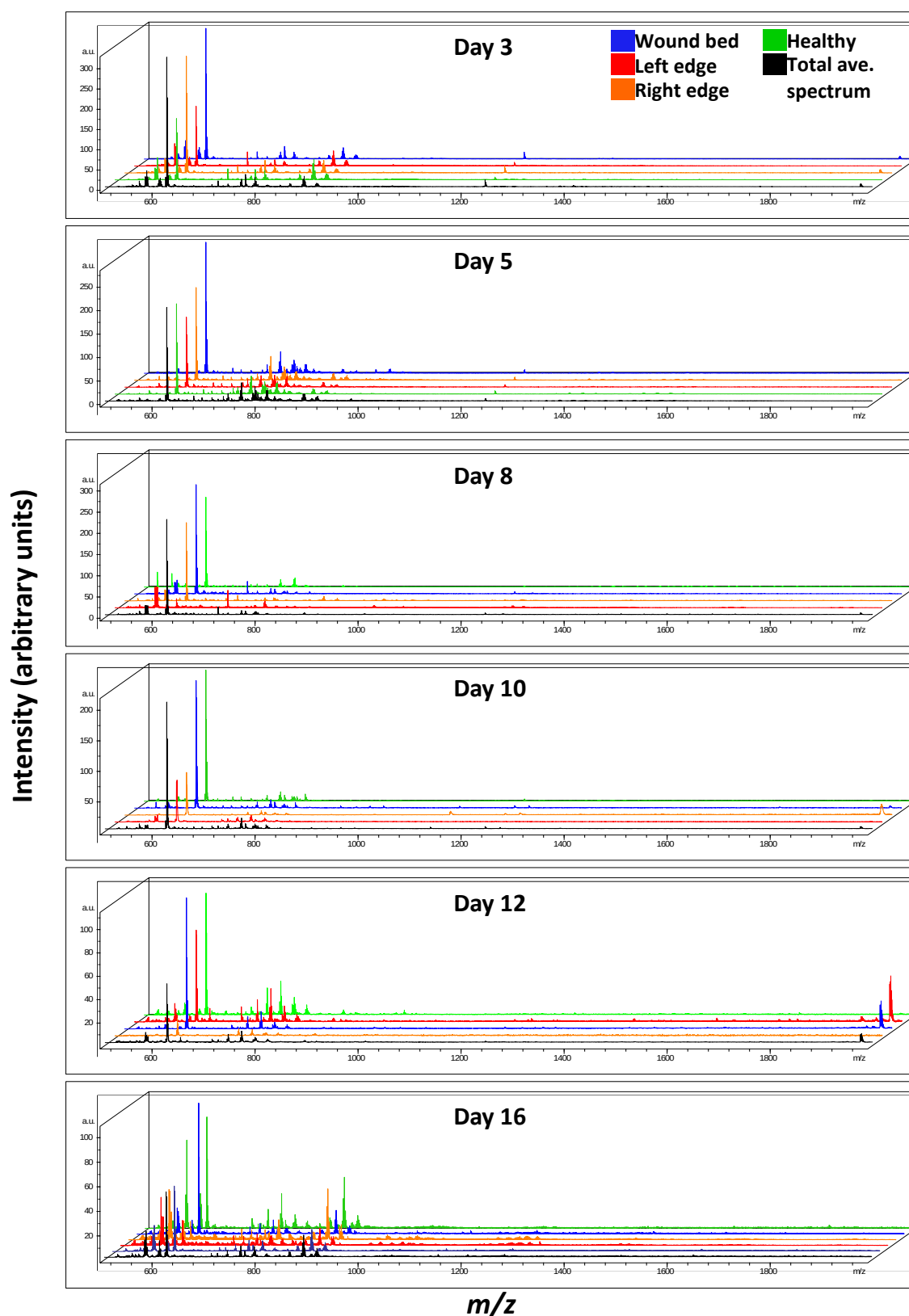


Figure 4.1: Summary of the wound metabolome intensities over 16 days. Averaged metabolite mass spectra of the wound bed, left and right wound edges, healthy tissue ROI's and a total average spectrum at 3, 5, 8, 10, 12 and 16 days post-wounding. Quantitative feature differences are evident across the six time points including total absence of specific features. The y-axis scale is automatically normalised to optimally fit each dataset.

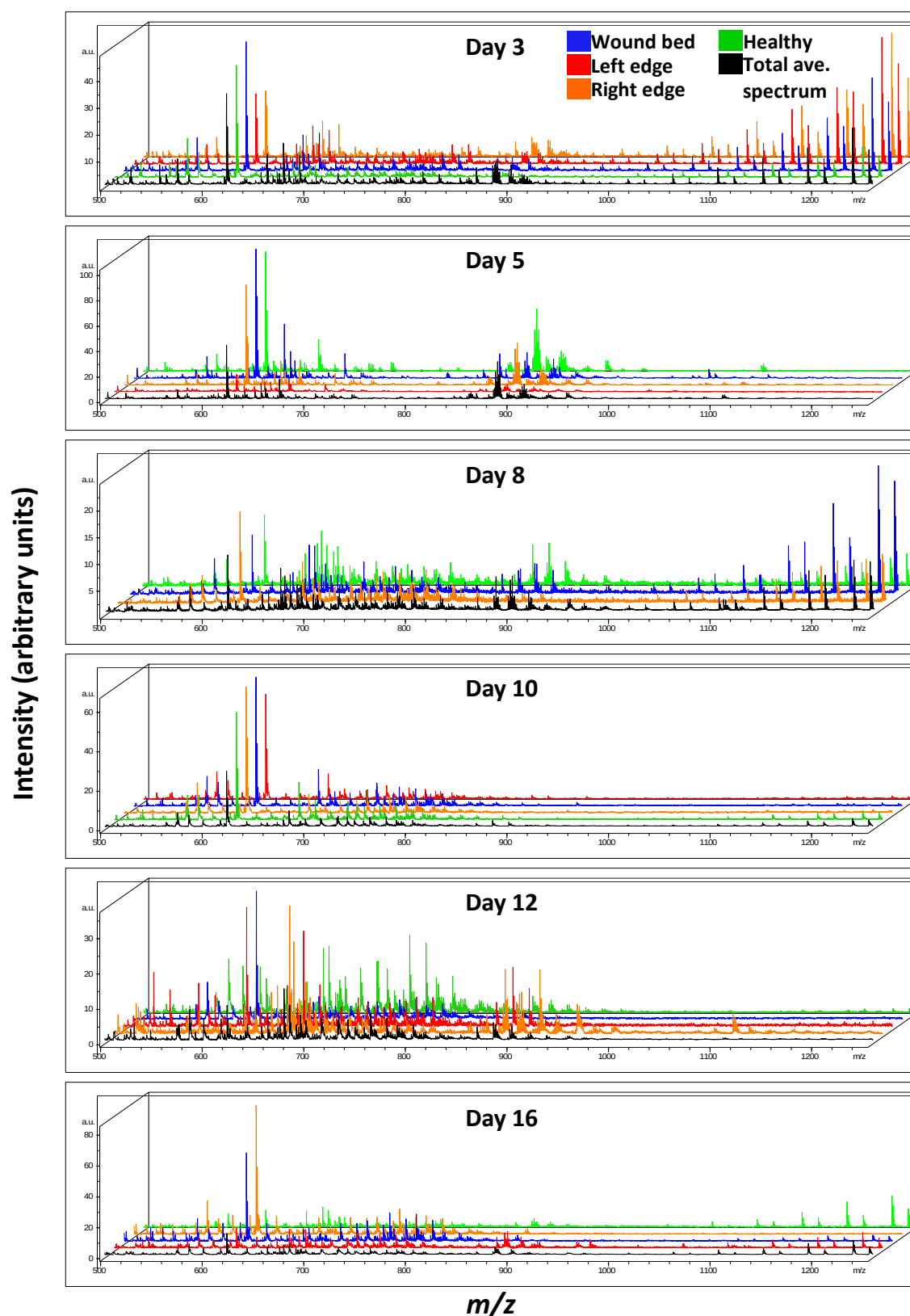


Figure 4.2: Summary of the wound lipidome intensities over 16 days. Average lipid mass spectra of the wound bed, left and right wound edges, healthy tissue and total average spectrum at 3, 5, 8, 10, 12 and 16 days post-wounding. Quantitative feature differences are evident across the six time points including total absence of specific features. The y-axis scale is automatically normalised to optimally fit each dataset.

Wound metabolome and lipidome profiles were generated over a period of 16 days at six different healing time points (Figures 4.1 and 4.2 respectively). The spatial advantage provided by MALDI-IMS allowed for the relative comparison of the wound bed, edges and healthy tissue within and between time points. Quantitative differences as well as the absence or presence of molecules were evident across the different ROIs on Days 3, 5, 8, 10, 12 and 16. The 'total average spectrum' (shown in black) represents the averaged mass spectrum of the whole tissue section analysed by MALDI-IMS *i.e.* not just the selected ROIs.

Imaging mass spectra revealed the majority of detected metabolites to be within a mass range of m/z 580 – 920 (Figure 4.1). Day 3 showed some intensity difference between the different ROIs, especially when comparing the wound tissue to the non-wounded, healthy tissue. The largest intensity changes were seen around m/z 630 and 1235, showing a lower intensity in healthy tissue relative to the wound bed and both edges. At approximately m/z 1970, a metabolite appeared to be present in the wound bed and right edge that was not seen in the non-wounded tissue and thus may be exclusively involved in the wound healing process.

By Day 5, the increased wound-associated intensity of m/z 630 was only seen in the wound bed, while wound edges were similar to the surrounding healthy tissue. A relative increase in a feature at m/z 780 was also seen in the wound tissue, with the additional appearance of features in the m/z 640 – 750 range, and at m/z 820. The features found on Day 3 at m/z 890 and 1235 had decreased significantly in intensity across all regions, while the m/z 1970 metabolites were no longer observed.

On Days 8 and 10, many of the features observed on Days 3 and 5 had substantially decreased in intensity, with low intensity spectra visible from m/z 800 and higher. The spectra around m/z 630 were still prominent on Days 8 and 10 but decreased in intensity in the wound tissue as time progressed, a trend that remained consistent in Day 12 and 16 spectra. After Day 8, this metabolite was found at comparable levels in the wound bed and healthy tissues through to Day 16. The increase in the metabolites found in the range m/z 640 – 820 on Day 5, all substantially decreased in intensity by Day 8 and remained at a relatively lower level throughout the rest of the 16-day study. The metabolites (m/z 630 – 820) showing a maximal relative intensity at Day 5 in the wound tissue compared to the healthy tissue, implied a role

in the inflammatory phase of wound healing, based on histological analysis of comparable tissue (Figure 2.8 E) and the sequence of healing phases (Broughton *et al.*, 2006). From Day 10 onward minor relative change could be seen when comparing the healthy tissue to wounded tissue, indicating closer correlation between the two tissue conditions, suggestive of progressive healing.

The features seen around m/z 890 and 900 on Day 3 that decreased by Day 5 and were no longer seen on Days 8, 10 and 12, reappeared on Day 16 at levels comparative to those of Day 3. These metabolites may be associated with healed and remodelled tissue as they were found before and after the degradative and enzymatic processes of inflammation and proliferation. Another metabolite that may also be involved in the proliferative or remodelling phases of acute wound healing was that found at approximately m/z 1970, where minor features were seen exclusively in the wound tissue on Day 3, and on Day 12.

The mass spectra of the complete 16-day acute wound lipidome were found to be distributed across the entire m/z 500 – 1300 analysis range (Figure 4.2). Due to its small tissue size, the Day 8 tissue section left wound edge was compromised during sample preparation and therefore this ROI was excluded from the analysis for this time point. Multiple differences in intensity could be seen through absence or presence of peaks in the mass spectra from the different ROIs within a particular sample and between the samples from different healing time points.

Day 3 showed minor differences between the different ROIs, except in the mass range of m/z 1100 – 1300 where multiple evenly spaced lipids with increasing intensity were found at a higher intensity in the wound tissue (especially the wound edges) as compared to the non-wounded healthy tissue. The lipids in this high lipid mass range reappeared on Day 8 and minimally on Day 16.

Other prominent features that appeared during the healing process within the lipidome included those around m/z 625, being increased in the wound initially on Day 3, with an increasing difference relative to the healthy tissue from Day 10 – 16. A cluster of lipids with m/z between 650 – 700 showed a wound-associated increase in intensity relative to healthy tissue from Day 3, peaking on Day 5 and reappearing, although decreased, on Day 12. The comparable intensities of these lipids within the healthy tissue on Day 16, suggested an association with healing. Another prominent

set of features were found between m/z 870 – 910, however despite showing a significant relative increase in the wound edges compared to the healthy tissue on Day 12, these lipids showed minor wound-associated differences during the remaining healing time points.

In addition to the mass spectra collected from each individual ROI being plotted, an average mass profile was also generated for selected ROIs for each wound time point during the study. Average mass profiles show the average spectra for the whole wound region *i.e.* the combination of the wound bed ROI and both wound edge ROIs. These average profiles offer a more focussed assessment for tracking the kinetics of the metabolome and lipidome during acute wound healing over 16 days using an averaged change for each of the six wound excision time points (Figures 4.3 and 4.4).

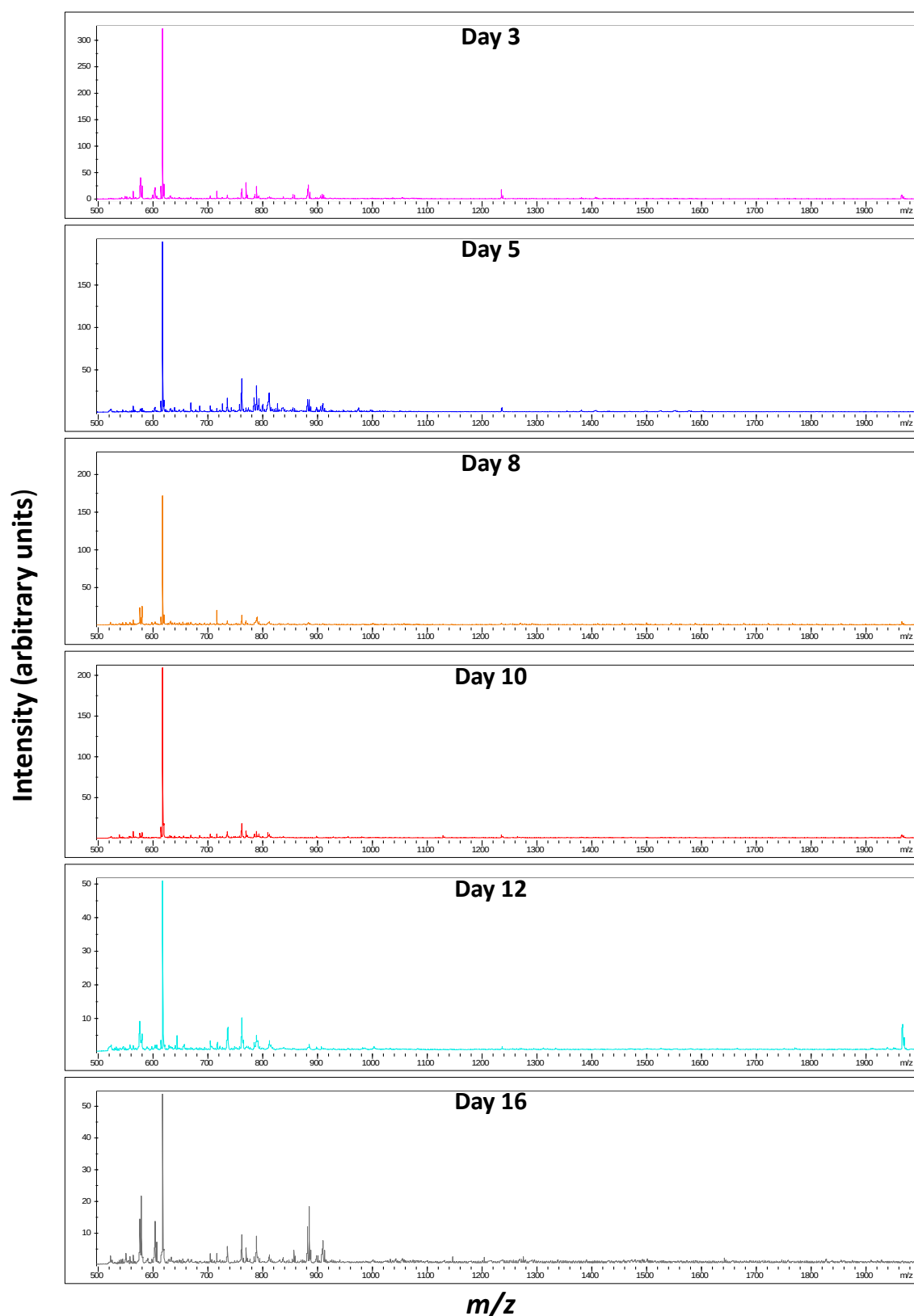


Figure 4.3: Average metabolomic mass profiles of the kinetic acute wound. Average mass profiles are shown tracking the metabolomic kinetics of the whole wound region on Days 3, 5, 8, 10, 12 and 16. Continuous changes in the wound metabolome are evident across the six time points. Masses below 500 Da are disregarded due to matrix effects. The scale of the y-axis is automatically normalised to optimally fit each individual dataset.

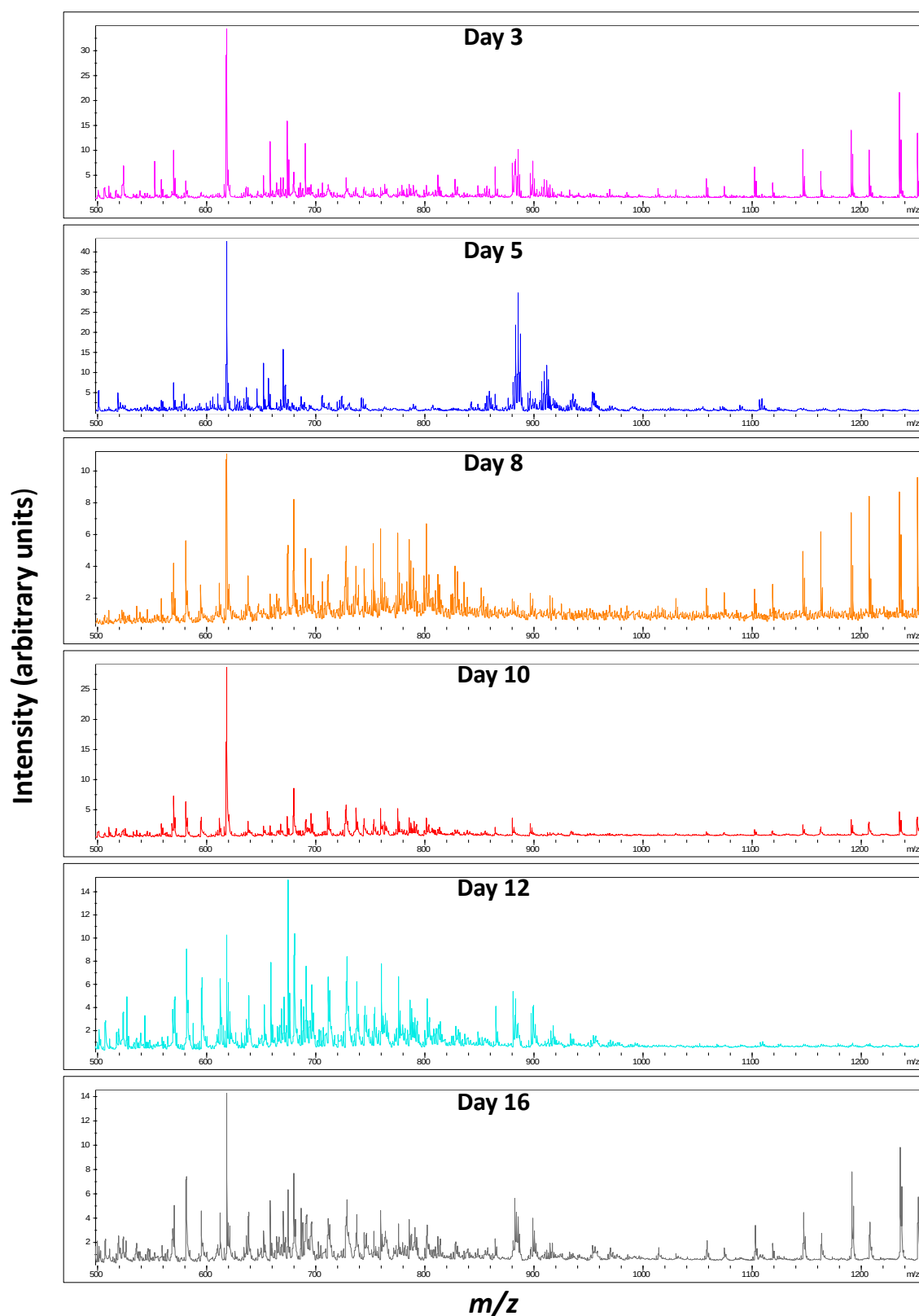


Figure 4.4: Average lipidomic mass profiles of the kinetic acute wound. Average mass profiles are shown tracking the lipidomic kinetics of the whole wound region on Days 3, 5, 8, 10, 12 and 16. Continuous changes in the wound lipidome are evident across the six time points. Masses below 500 Da are disregarded due to matrix effects. The scale of the y-axis is automatically normalised to optimally fit each individual dataset.

Metabolomic and lipidomic average mass profiles of whole wound regions (wound bed and both wound edges combined) across 16 days were created (Figures 4.3 and 4.4 respectively), providing a visualisation of a continuously changing overall healing environment. Both average mass profiles showed quantitative differences between each of the six wound excision time points across both 'omic' bases.

The metabolomic average mass profile showed mass spectra appearing similar in distribution over the 16 days. The majority of metabolomic kinetic changes occurring in the acute wound were intensity related, rather than an absence/presence of features between time points. From Day 3 to Day 5, there was a decreased intensity of major features, continuing into Day 8. Day 10 showed an increase in one prominent and continuously present metabolite, while remaining features showed little or no change. From Day 12 and continuing to Day 16, a substantial decrease in intensity in most of the detected metabolites was seen, implying an overall reduction in metabolomic activity. Although appearing similar to Day 3 mass spectra, Day 5 represented the height of wound healing metabolic activity, where the highest number of metabolite features were seen (Figure 4.3). This observation was supported by the individual wound ROIs and the averaged measurements compared to the non-wounded, healthy tissue in Figure 4.1.

In the lipidomic average mass profile, an increased intensity in the common features of Day 3 and Day 5 could be seen. On Day 8 however, feature intensity decreased substantially even though the number of detected features increased to more closely represent Day 3. On Day 10 many of the larger mass lipids dropped in intensity, with remaining dominant features either greatly increasing in intensity or remaining mostly unchanged. Day 12 saw intensity changes occurring in many of the features already present on Day 10. On Day 16 minor increases and decreases in intensity were seen where features appeared similar to those of Day 12, with the exception of the prominent reoccurrence of larger lipids also appearing on Days 3 and 8. The average lipidomic mass profile suggested that the peak of lipidomic changes occurred on Day 5 where the maximal intensities of multiple lipids were reached (Figure 4.4). This effect was also found in the individual wound regions of Day 5 in Figure 4.2.

Visualising the mass spectra of the kinetic metabolome and lipidome of acute wound healing over 16 days, has made it possible to track molecular changes occurring within acute wounds over time and across multiple ‘omic’ bases (including proteomics, discussed in Chapter 3). Variations were tracked through relative change (wound tissue versus healthy tissue), through wound region comparisons (wound bed versus wound edges) and through wound profiling by relative quantitation, absence and/or presence of metabolites and lipids over time. The ability to visualise the ‘omic’ changes occurring in a wound at six different time points within composite images provides a unique and useful tool for the interpretation of large and most often overwhelming datasets. This approach may be used to reveal time points or molecules of interest for further analysis, as a characterisation tool or model for the kinetics of acute wound healing in the porcine model.

4.3.2 Principal component analysis

As discussed in the proteomic analysis of Chapter 3 (see Section 3.3.2), MALDI-TOF technology has contributed greatly and revolutionised the biomarker research field. The large, multidimensional datasets produced from this type of mass spectrometric analysis however, require a reduction of data to an informative yet manageable volume. Principal component analysis (PCA) has the ability to reduce data dimensionality while ensuring that no critical information is lost.

4.3.2.1 Kinetic changes in acute wounds

PCA was utilised in the proteomic analysis to track the molecular kinetics of acute wound healing at six pre-selected time points over the 16 days of healing. This successfully revealed Day 8 as the peak of wound activity as shown through proteomic changes and identified this time point as one of interest, potentially being the most representative proteomic environment for confirmed acute wound healing (Section 3.3.2.2). The same approach was applied to the metabolomic and lipidomic mass spectral data to determine whether the peak of wound-related activity correlated kinetically across the three different ‘omic’ bases.

Acute wound kinetics were visualised across metabolomic and lipidomic bases using PCA. The continuously changing molecular environment of the wound was tracked on Days 3, 5, 8, 10, 12 and 16 where the wound bed and edges (left and right combined) were compared independently to a region of non-wounded healthy tissue.

The region of healthy tissue was selected from the Day 3 wound biopsy tissue for both the metabolomic and lipidomic analyses and was used as a consistent control throughout these analyses. Inter-pig biological variability was deemed insignificant in the PCA of the different healthy tissue samples (see Section 3.3.2.1) where Day 3 tissue was proven to be an accurate representation of non-wounded, healthy porcine tissue. By comparing the different wound tissue, based on healing time, to a control region of non-wounded tissue, the relative progression of healing could be measured and the time points best representing the acute wound metabolome and lipidome could be visualised. Due to the fact that the 2D PCA plots in Chapter 3 did not provide any additional pertinent information that was not already revealed in the 3D plots (Figures 3.9 and 3.10), only the 3D PCA plots for the metabolomic and lipidomic kinetic analyses are displayed. This has the added advantage of a more concise and focussed visual analysis (Figures 4.5 and 4.6).

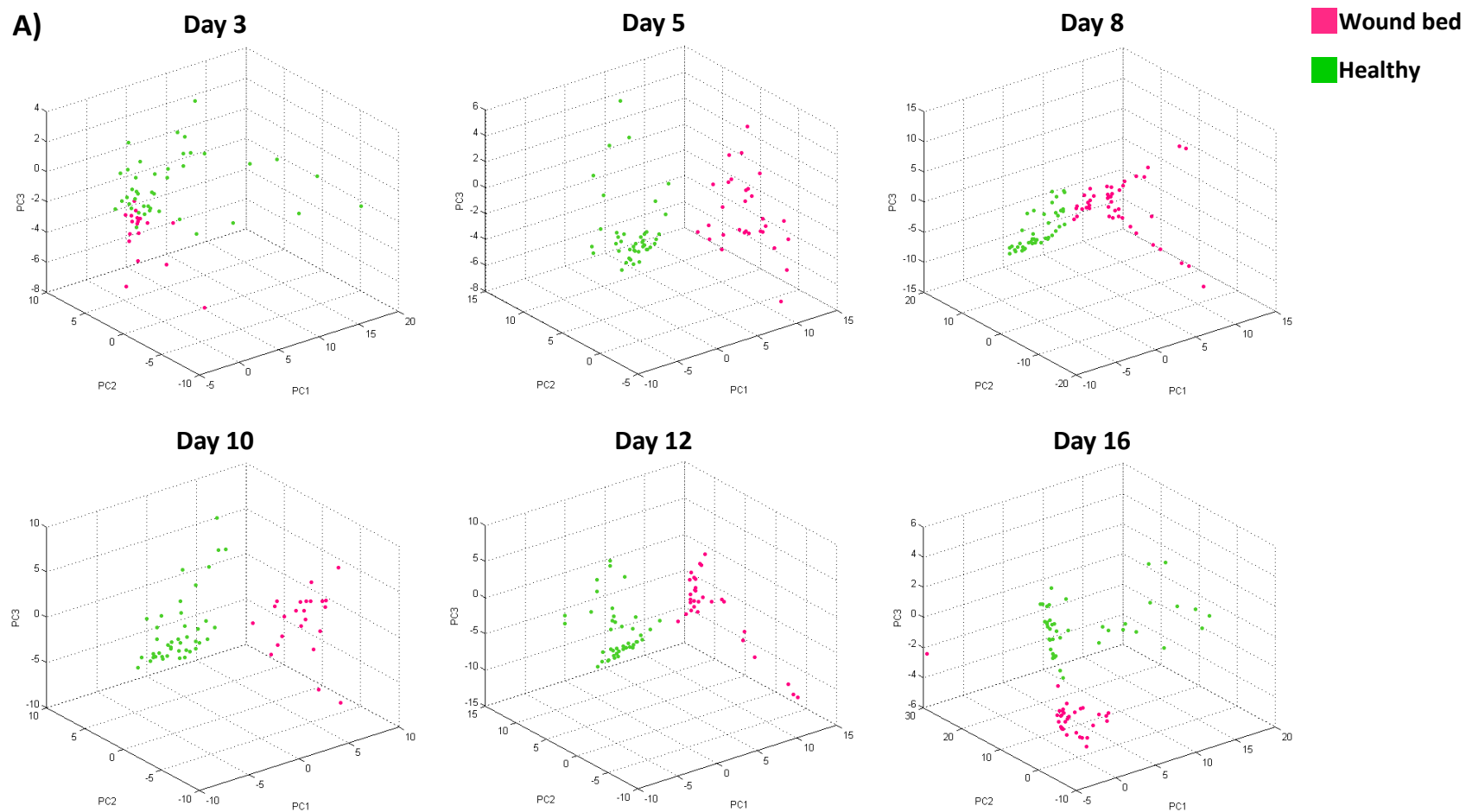


Figure 4.5 A: 3D PCA plots of the metabolomic wound bed ROIs versus healthy tissue over selected time points over 16 days. 3D PCA plots of the wound bed (pink) versus non-wounded, healthy tissue (green) on Days 3, 5, 8, 10, 12 and 16 are shown. Changes in the wound metabolome are seen at each of the six healing time points, with maximal variation occurring on Day 5.

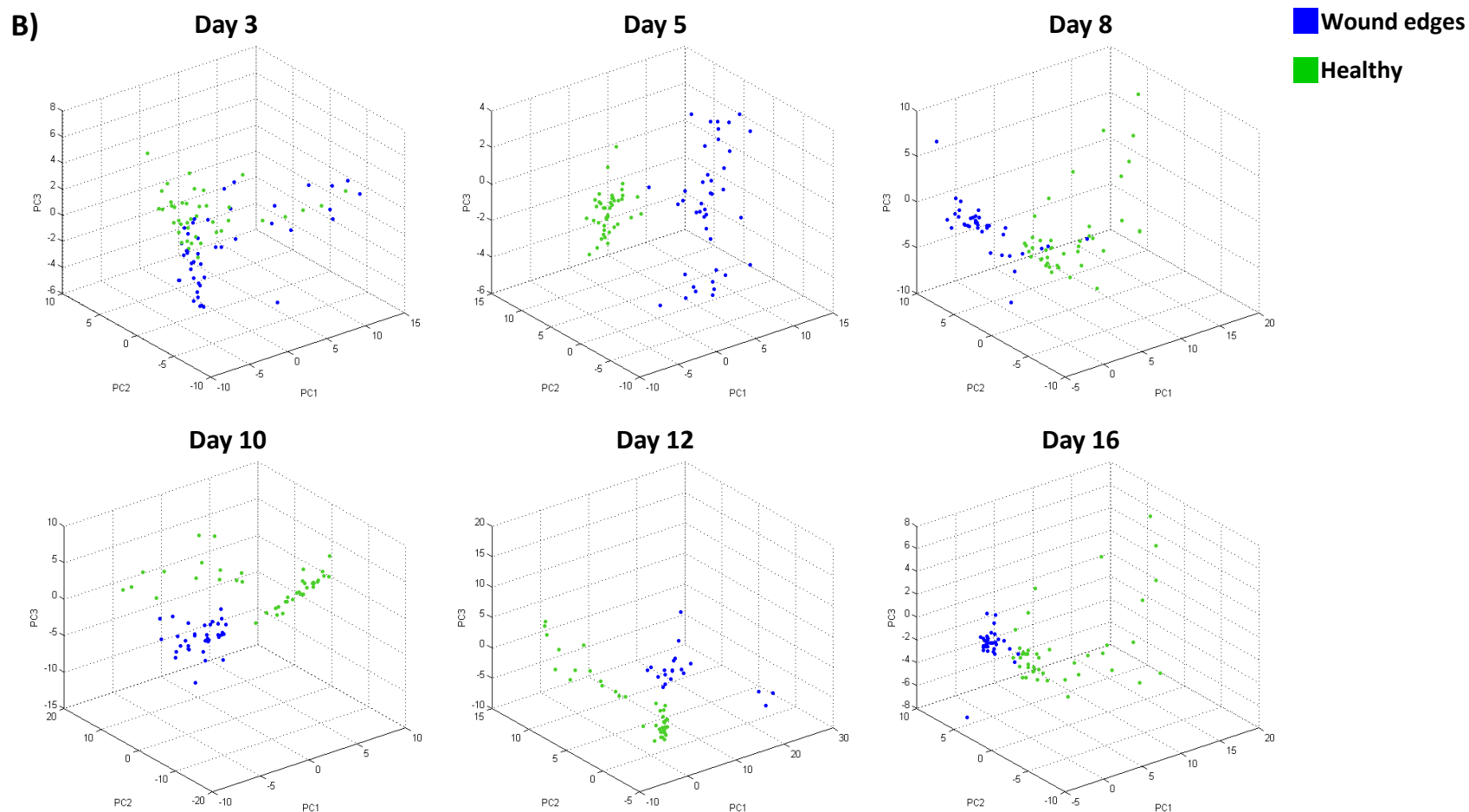


Figure 4.5 B: 3D PCA plots of the metabolomic wound edge ROIs versus healthy tissue over selected time points over 16 days. 3D PCA plots of the wound edges (blue) versus non-wounded, healthy tissue (green) on Days 3, 5, 8, 10, 12 and 16 are shown. Changes in the wound metabolome are seen at each of the six healing time points, with maximal variation occurring on Day 5.

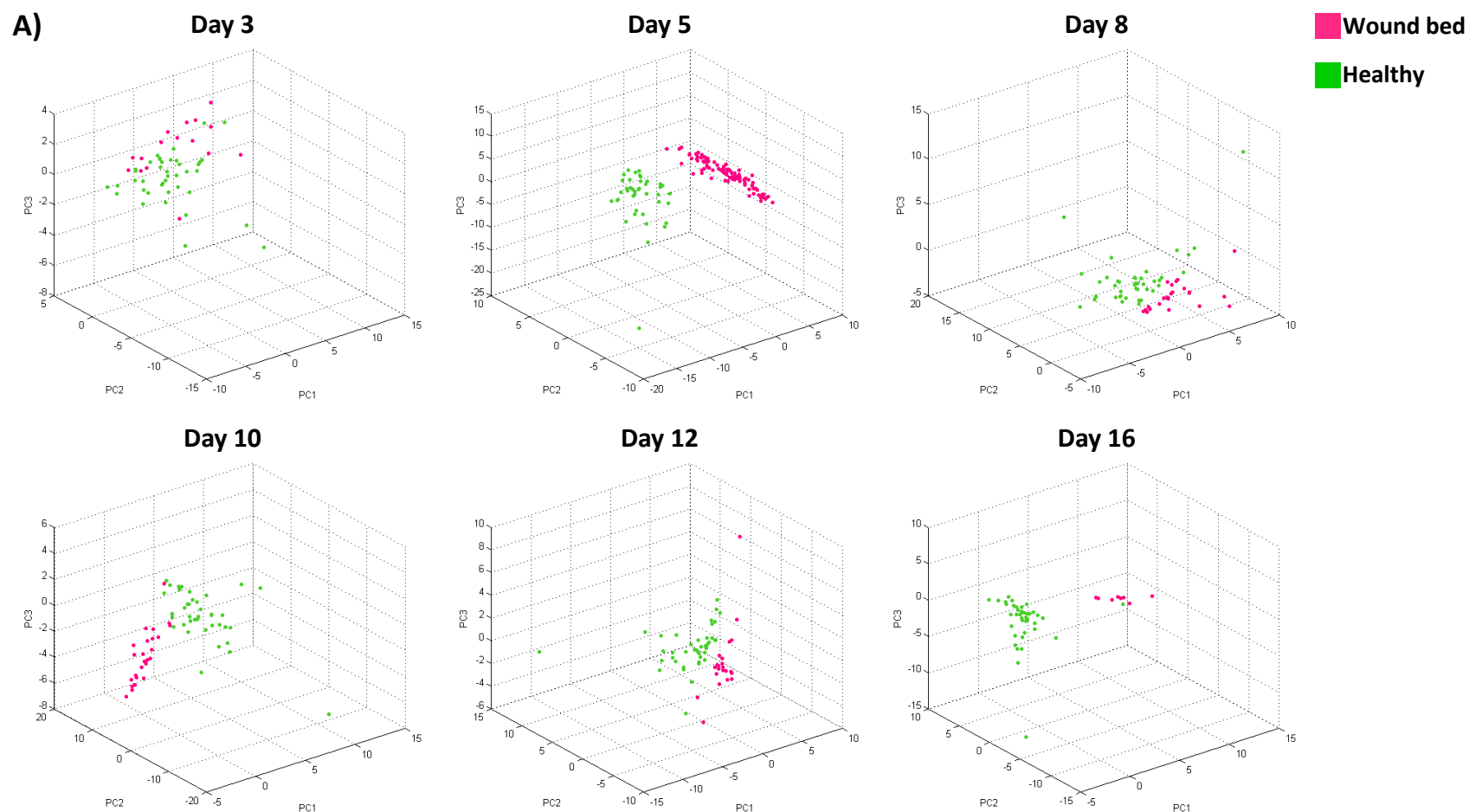


Figure 4.6 A: 3D PCA plots of the lipidomic wound bed ROIs versus healthy tissue over selected time points over 16 days. 3D PCA plots of the wound bed (pink) versus non-wounded, healthy tissue (green) on Days 3, 5, 8, 10, 12 and 16 are shown. Changes in the wound lipidome are seen at each of the six healing time points, with maximal variation occurring on Day 5.

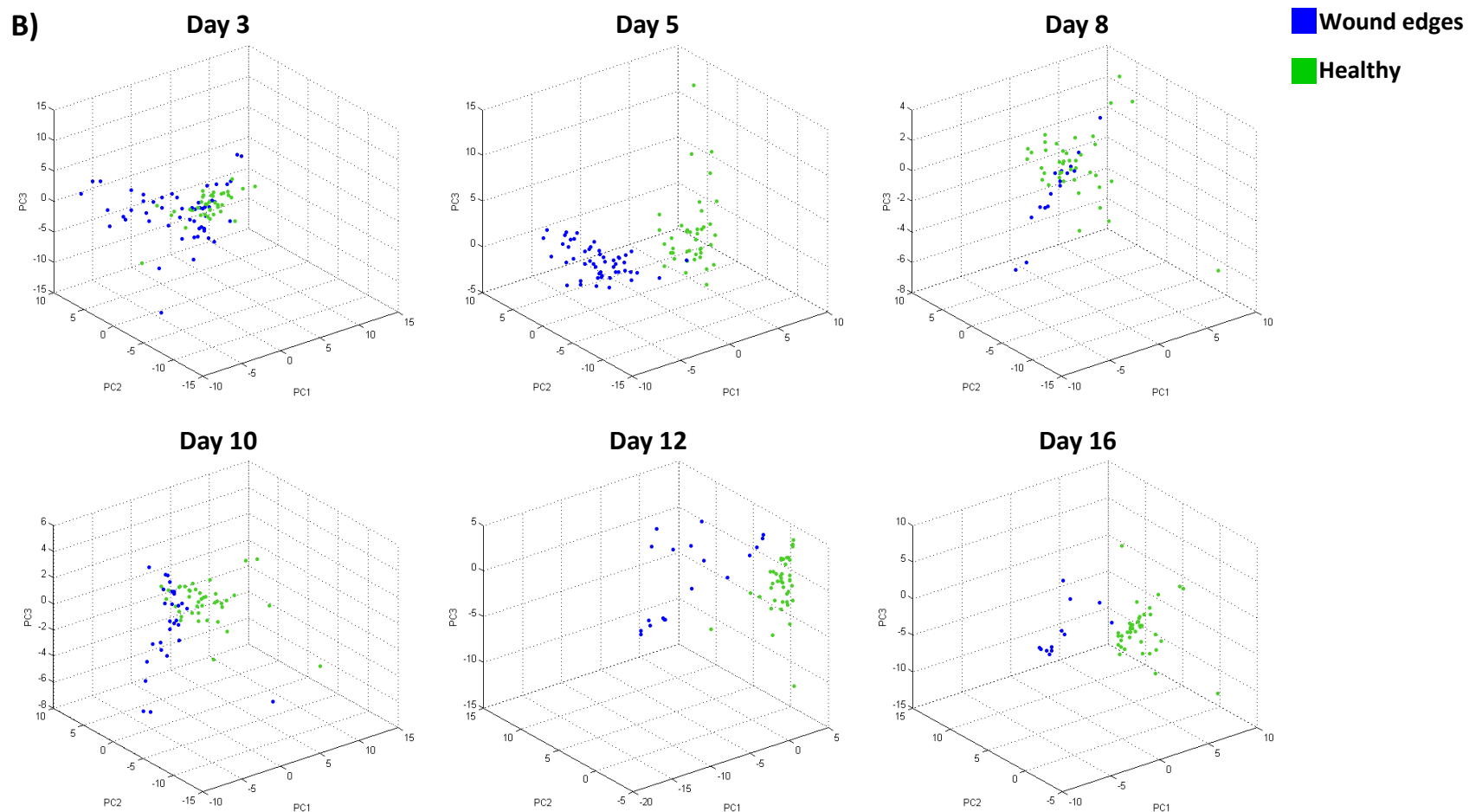


Figure 4.6 B: 3D PCA plots of the lipidomic wound edge ROIs versus healthy tissue over selected time points over 16 days. 3D PCA plots of the wound edges (blue) versus non-wounded, healthy tissue (green) on Days 3, 5, 8, 10, 12 and 16 are shown. Changes in the wound lipidome are seen at each of the six healing time points, with maximal variation occurring on Day 5.

Three-dimensional PCA plots successfully visualised the continuously changing metabolome and lipidome of the acute wound over selected time points over 16 days. Wound kinetics were tracked over six time points using non-wounded, healthy tissue as reference. Changes in the variance of the metabolome and lipidome in both the wound bed and edges was evident, as well as varying correlation of wound tissue to the healthy tissue (Figures 4.5 and 4.6 respectively).

The Day 3 wound metabolome showed a clear overlap between the non-wounded, healthy tissue and both the wound bed and edges. By Day 5 a complete separation between both wound tissue ROIs and the healthy tissue was seen, where the wound and healthy tissue could be statistically differentiated at this point. This Day 5 separation was also accompanied by a relative increase in wound tissue variance, especially in the wound bed. These wound data distribution patterns were suggestive of an inflammatory response being mounted on Day 3, which then progressed to reach maximal activity on Day 5. This is supported by the fact that the inflammatory phase of acute wound healing, according to literature, occurs any time from immediately after injury to approximately six days post-wound creation (sometimes even longer) (see Section 1.1.3.1) (Broughton *et al.*, 2006, Rittié, 2016). At the height of the inflammatory phase, significant inflammatory cell infiltration at the wound site would be expected, which would provide an explanation for the complete differentiation between wounded and non-wounded tissue. To support this, a dense inflammatory cell infiltrate associated with the wound was seen on histology on Day 5 (Figure 2.8 E).

On Day 8 the wound bed and especially edges became more closely representative of the healthy tissue, where minor overlap between features from the two tissue groups occurred. This increased tissue similarity was most likely due to the proliferative phase of wound healing, where processes such as new granulation tissue deposition and re-epithelialisation at the wound site occur while inflammatory processes shut down. Proliferation is reported to overlap with and follow a subsiding inflammatory phase starting 4 – 14 days post-wounding (as discussed in Chapter 3, Section 3.3.2.2). Wound tissue variation on Day 8 was found to decrease, especially in the wound edges.

Interestingly, Day 10 saw a complete separation between the healthy tissue and both wound tissue areas (bed and edges) on the PCA plots. This sudden differentiation could be due to an increase in proteolytic activity associated with the remodelling phase of wound healing (see Section 1.1.3.3). As time progressed and collagen type I was deposited into the wound in an organised manner, mimicking what is seen in non-wounded tissue, so too did the PCA plots of Days 12 and 16 show a closer correlation of the wound bed and edges to the healthy tissue. As wound remodelling progresses, the synthesis and degradation of different collagen types will slow, possibly explaining the significant but progressive decrease in wound bed and edge variation seen in the plots of Days 12 and 16. On Day 16 the healthy tissue and wounded tissue data points on the PCA plots did not overlap or correlate to a great extent, as the remodelling phase was still progressing and could last up to years (Broughton *et al.*, 2006).

The PCA plots of the acute wound lipidome revealed a similar data distribution to that of the metabolome. Day 3 showed similarity between the wound and healthy tissue, mostly in the wound edges, where Day 5 displayed a clear separation between both wound tissue regions and the healthy tissue. This is especially true in the wound bed where no obvious data overlap occurred, and a relatively large distance separated the two tissue types in the PCA plot. The greatest wound variation was seen on Day 5, with an increase in the number of data points visualised, mostly in the wound bed. As with the metabolic PCA assessment, the distinct differentiation and maximal data variation in the wound tissue at this time point was most likely due to the peak of inflammatory phase activity, based on theoretical acute healing phase time lines (Broughton *et al.*, 2006) and the histological observations of comparable wound tissue (Figure 2.8 E).

Day 8 showed an increased correlation between the wound and healthy tissues, especially in the wound edges. This increased wound tissue correlation was most likely due to proliferative healing phase activity, with new granulation tissue deposition in the wound space and re-epithelialisation providing an explanation for the closer representation of wound tissue to healthy tissue (Figure 2.8 J and L). Unlike the metabolites, a clear separation between wound and healthy tissue was not seen on Day 10. At this time both the wound bed and wound edge vs healthy tissue PCA plots appeared very similar to those of Day 8 with a slight increase in

wound variance. This suggested that the wound-associated activity seen in the metabolome on Day 10 (suspected to be linked to the remodelling phase and being proteolytic in nature) did not affect the wound lipidome to a comparable extent. The Day 12 and 16 lipidomic plots showed a progressive decrease in wound bed and edge variation indicative of decreasing healing-associated activity and progression of wound healing. By Day 16, the wound tissue was not yet similar to the non-wounded healthy tissue, suggestive of an ongoing remodelling phase in the wound lipidome at this time.

In summary, the kinetics of the metabolome and lipidome of the acute wound relative to healthy tissue over 16 days displayed similar data patterns on 3D PCA plots. Day 3 showed some similarity between the wound and healthy tissue, slightly more so in the wound edges. Day 5 showed the greatest relative wound variation and differentiation with no similarity between the tissue groups occurring. On Day 8, a decrease in wound differentiation and variation was seen, especially in the lipidome. As the acute wound healing process progressed a decrease in wound tissue variation was seen, while similarity to non-wounded tissue seemed to stabilise overall from Day 10 with minor differences being seen from then onwards. By Day 16 wound tissue variance (bed and edges) was at its lowest across both 'omic' bases, indicative of a progressed wound healing process.

Based on wound data variation and lack of similarity to the non-wounded healthy tissue, PCA revealed Day 5 as the peak of acute wound healing activity in both the wound metabolome and lipidome. Day 5 may be considered as the most complete representation of the active acute wound healing environment across these two 'omic' bases. These findings are supported by the mass spectra results of the individual ROIs (Figures 4.1 and 4.2) as well as by the average mass profile results (Figures 4.3 and 4.4) where distinct relative differences in feature intensities, compared to the remaining wound excision time points were seen. It is thought that impaired wound healing occurs due to being 'trapped' in the inflammatory phase of healing, which was confirmed by the histological analysis in this study as the phase occurring on Day 5 (Section 2.3.4 and Figure 2.8 E – F) (Eming *et al.*, 2007, Landén *et al.*, 2016). Performing further in-depth analysis on Day 5 wound tissue in the porcine model could potentially reveal useful therapeutic metabolomic or lipidomic drug targets for the development of new treatment options in chronic wounds.

PCA plots highlight relative variation and Day 5 showed the greatest variation and differentiation compared to non-wounded healthy tissue, but this does not necessarily mean that Day 5 showed the greatest variance relative to the other days during wound healing *i.e.* within the whole kinetic healing process. Combination 3D PCA plots were created containing the spectra from all six wound excision days and from the healthy tissue for both metabolomic and lipidomic bases. For comparative purposes, data from the proteomic analysis was also included (Figure 4.7).

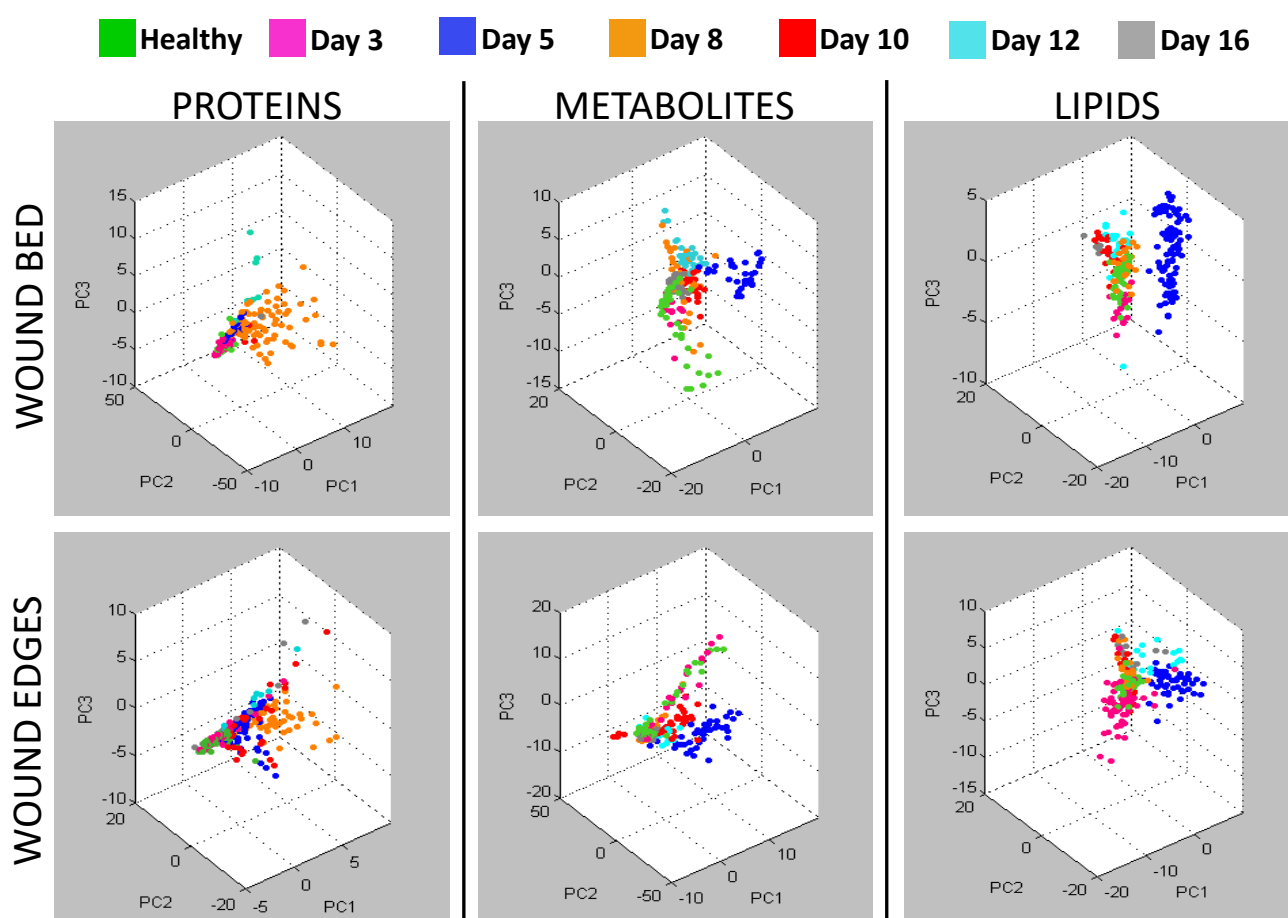


Figure 4.7: Proteomic, metabolomic and lipidomic based 3D combination PCA plots of the changes in the acute wound over 16 days. Combination PCA plots of the wound bed and edges (rows) relative to non-wounded, healthy tissue on Days 3, 5, 8, 10, 12 and 16 across proteomic, metabolomic and lipidomic bases (columns). Plots clearly reveal the maximal wound-related activity to occur on Day 8 (orange) for proteomic changes and on Day 5 (dark blue) for both metabolomic and lipidomic changes. The healthy tissue and six selected time points are identified according to the colour key at the top of the image.

Three-dimensional combination PCA plots of data across the 16 days of the acute wound proteome, metabolome and lipidome were created to visualise and isolate the time points of most variation during the acute wound healing process. Maximal wound-related activity was the healing time point showing the greatest amount of relative variation, and the greatest degree of differentiation compared to the healthy tissue. In the proteomic analysis of Chapter 3, Day 8 was already revealed as the tissue representing the peak of proteomic wound healing activity (Figure 3.13). The 3D combination PCA plots (Figure 4.7) clearly visualise Day 5 as showing the greatest relative variance and healthy tissue differentiation compared to Days 3, 8, 10, 12 and 16, and confirm the result seen in the individual PCA (Figures 4.5 and 4.6). Individual plots were not as informative as the combination plots in discerning the most variable and differentiated healing time points, especially between Days 5, 8 and 10 for the metabolomic analysis. In both the metabolome and lipidome, the wound bed and wound edge data of Day 5 showed little similarity to the healthy tissue. The Day 5 wound bed metabolome showed minimal similarity to Days 8 and 12, while the lipidome showed a distinct separation and no similarity to any of the remaining time points. The wound edges in both 'omic' bases of Day 5 showed relatively minimal similarity to the remaining wound tissue time points.

When comparing all three 'omic' bases, the wound proteome seemed to be lagging one time point behind the metabolome and lipidome in acute wound healing peak activity (being Day 8 and Day 5 respectively).

The kinetic PCA did not reveal any conclusive difference between the wound bed and edge regions across both 'omic' bases at any time point. Further metabolomic and lipidomic analyses were therefore done on whole wound datasets (*i.e.* wound bed and edges combined).

4.3.2.2 Loading plots

As described in Chapter 3 the results generated from PCA are not only interpretable in PCA plots but also in the linked loading plots, which share the same principal components and allow for the identification of data points by mass (m/z) (see Section 3.3.2.3).

Through loading plot analysis, features responsible for the greatest variance in the dataset can be identified by their mass and be considered as potential markers of

acute wound healing pending further, confirmatory analysis. In order to identify the variant features visualised in the acute wound PCA plots, loading plots were generated and coupled to each PCA plot (Section 4.3.2.1). In this way the metabolites and lipids of greatest contribution to the variance in the PCA model of the acute wound healing process were revealed. The loading plot analyses shown are of whole wound data with all six wound excision time points over 16 days plotted in combination on a single set of axes (Figure 4.8).

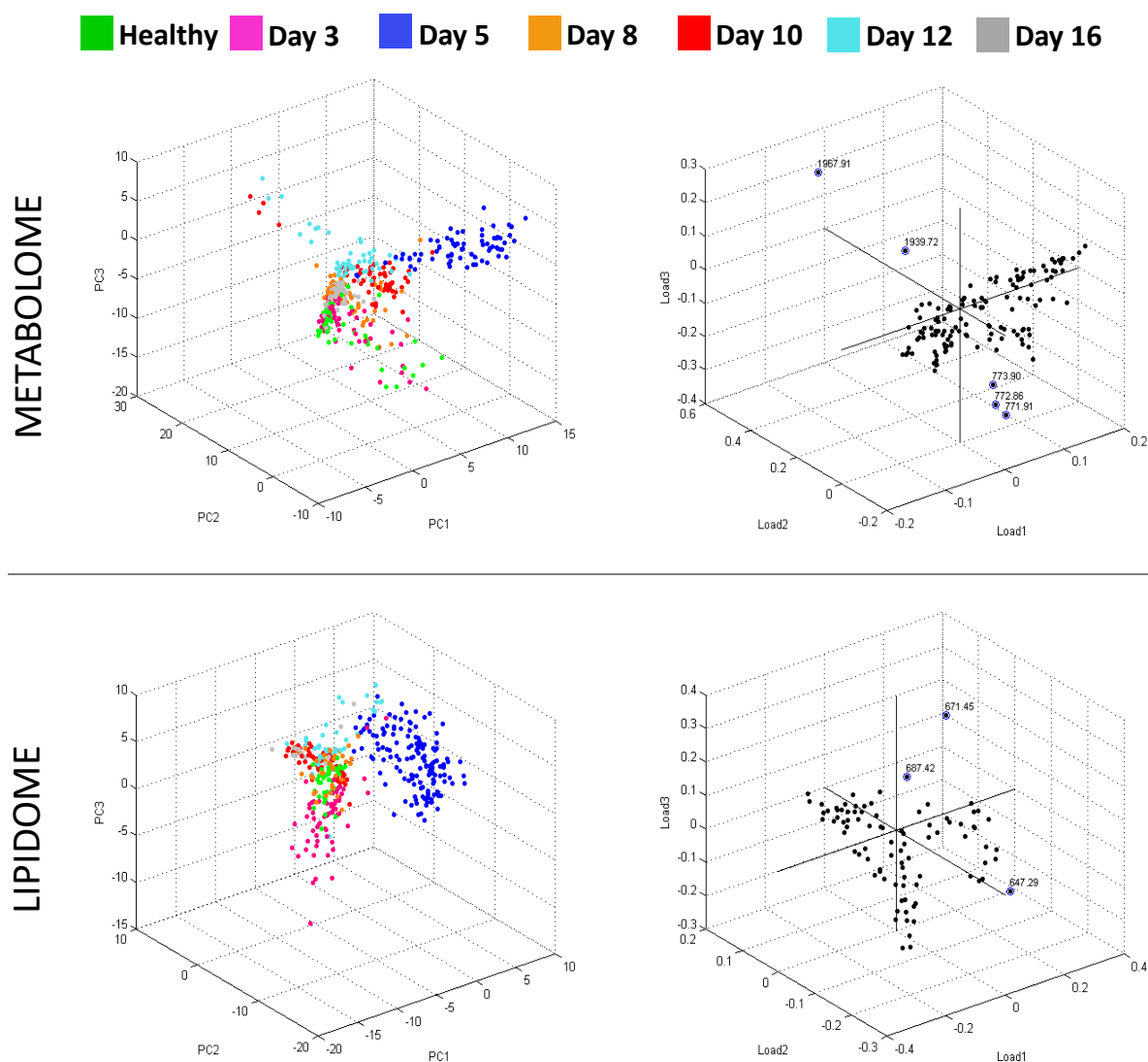


Figure 4.8: 3D combination PCA and the associated loading plots of whole wound tissue vs healthy tissue across the metabolome and lipidome over 16 days. Combined 3D PCA plots with the respective associated 3D loading plots of whole wounds across metabolomic (top row) and lipidomic (bottom row) bases revealing the masses of features showing the greatest variance across the first three principal components for non-wounded healthy tissue and for the wounds of Days 3, 5, 8, 10, 12 and 16. The healthy tissue and six selected time points are identified according to the colour key at the top of the image.

Three-dimensional combination PCA plots coupled with loading plots, of whole wound tissue from the six wound healing time points and non-wounded healthy tissue, were created across metabolomic and lipidomic bases. These plots visualised the most variant metabolite and lipid features detected in the acute wound healing process relative to healthy tissue (Figure 4.8). In theory the more variant a molecule is compared to non-wounded tissue, the greater its association may be to the healing process and the greater the potential for it being used as a feature associated with normal, acute wound healing.

The metabolomic PCA plot of whole wound tissue correlated to what was seen in the separate wound bed and edge analysis (Figure 4.7), where Day 5 showed the greatest amount of relative variance and no overlap with the non-wounded healthy tissue, highlighting the redundancy of individual wound ROI analysis in this case. While Days 10 and 12 showed distinct variability and no correlation with the healthy tissue in the PCA plot, Day 5 presented a larger number of variable data points and therefore greater variation. It is of interest that selected features from Days 10 and 12 show distinctly different patterns of variation in alternative principal components compared to Day 5 on the 3D PCA plot. This observation highlights two separate and clearly differential metabolomic acute-wound related activities occurring on Day 5 and again on Days 10 and 12.

The 3D combination metabolomic loading plot (Figure 4.8) was used to generate a filtered, differential feature set revealing the three most variant molecules with mean masses of m/z 1967.91, 1939.72 and 771.91. These three metabolites were found to be the greatest distance and the most isolated from the central data cloud. To determine the time points at which these isolated masses showed variance relative to healthy tissue and may have been associated with the healing process, loading plots coupled to the individual PCA plots (Figure 4.5) were analysed across both the wound bed and edges. The analysis was performed using a mass error of $\pm 0.25\%$.

Mass m/z 772 showed variance on wound Days 5, 8 10 and 12 in both the wound bed and edges, covering multiple time points and highlighting this metabolite's potential as a general marker of the healing process. The feature with m/z 1940 showed variance on Days 8 and 12 in the wound bed only. This metabolite was significantly more variable on Day 12 (as seen on the combination plot in Figure 4.8)

suggesting that it is related to the early phases of remodelling, which begins approximately 8 days post-wound creation. Supporting this is the fact that this metabolite only showed large variance in the wound bed, where remodelling-related activities, such as tissue deposition, dominate (Broughton *et al.*, 2006). Mass m/z 1968 was found to show variance in the individual PCA plots of Days 3, 8, 10 and 12, making this metabolite a potential overall marker of acute wound healing (comparable to m/z 772). The highest relative variation of m/z 1968 was seen on Day 12, suggesting progressive quantitative change as the healing process advanced. It is of interest to note that wound-associated features with large variance were also seen in the metabolome kinetics (Section 4.3.1 and Figure 4.1) in these mass ranges at the same time point.

The 3D PCA plots of the wound lipidome kinetics using either whole wound tissue (Figure 4.8) or the separate wound bed and wound edge ROIs (Figure 4.7), provided comparable results, highlighting Day 5 as the healing time point with most variability and representative of a changing acute wound lipidomic environment. In Figure 4.8 a clear differentiation can be seen between data points from Day 5, and those of the remaining healing time points and healthy tissue. While Day 12 showed some similarity to Day 5 features, no other time points or the healthy tissue showed similarity.

The combination 3D loading plot (Figure 4.8) of the changing acute wound lipidome identified a variant feature set with masses m/z 647.29, 671.45 and 687.42 being well separated from the central data cloud. The analysis of singular loading plots (using individual time point PCA plots, Figure 4.6) with a mass error of $\pm 0.25\%$ revealed minor variance of the feature with m/z 687 in the wound bed on Day 8, but otherwise Day 5 was the only time point where these three lipids were all expressed as variants. This suggests that these lipids are probably associated with the inflammatory phase of wound healing, which would peak around Day 5 and then subside by Day 8 (Broughton *et al.*, 2006). Interestingly, the Day 5 wound lipidome (Figure 4.2) showed higher intensities of many features in the m/z 645 – 700 range compared to non-wounded tissue (as discussed in Section 4.3.1) with lipids m/z 647, 671 and 687 all included in this mass range.

4.3.2.3 Influence plots

As explained in Chapter 3 the data generated from PCA can be interpreted through multiple means including the PCA and loading plots already shown as well as through influence plots, which show the influence of each mass spectrum on the current PCA model (see Section 3.3.2.4 for further detail).

Influence plots were generated on combined, whole wound PCA datasets across metabolomic and lipidomic bases for each of the six selected time points and the non-wounded, healthy tissue. Each plot utilised multiple PCs that accounted for at least 95% of the data variance (Figure 4.9).

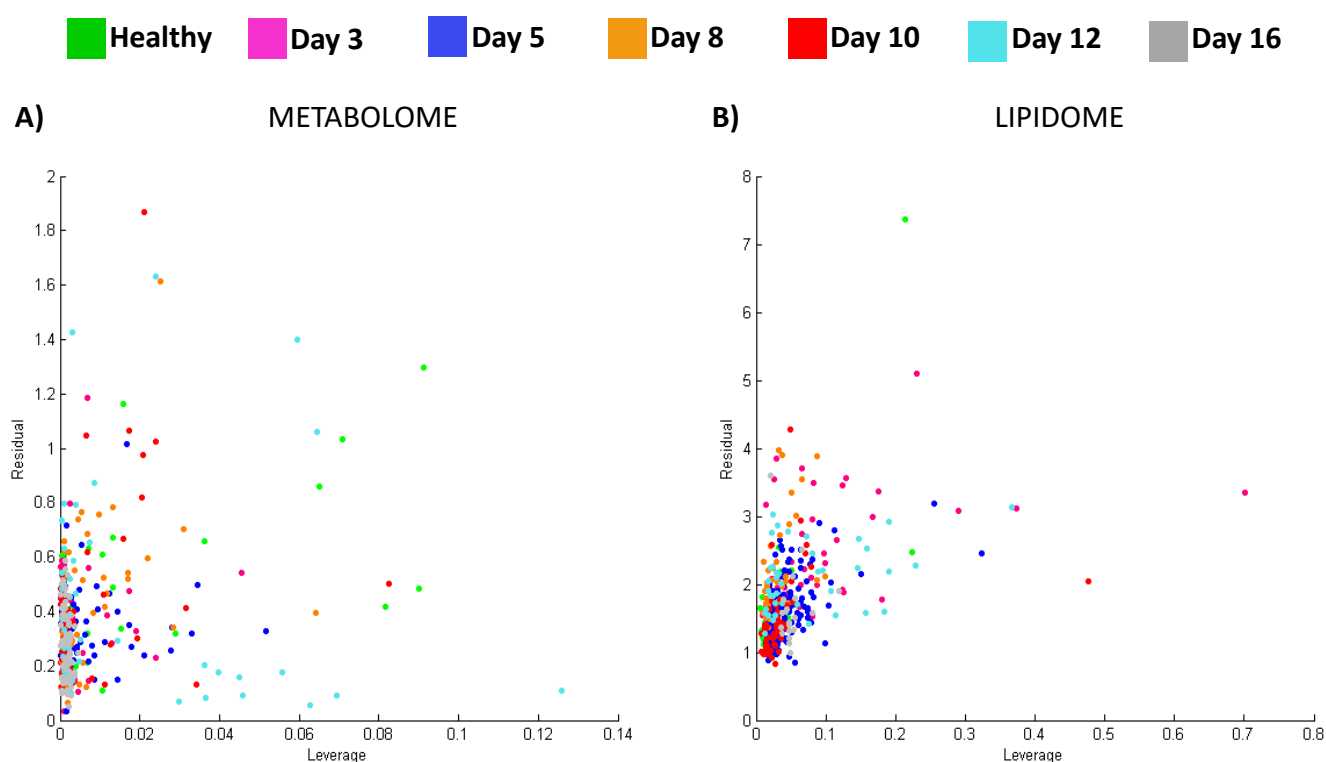


Figure 4.9: Influence plot of healthy tissue versus wound tissue from Days 3, 5, 8, 10, 12 and 16 across the metabolome and lipidome. The influence plots visualise the features associated with differences between acute wounds and healthy tissue for the (A) metabolome and (B) lipidome. The features of the acute wound metabolome show substantially less variation than those of the lipidome. The leverage and residual outliers are mostly Day 3 (pink), Day 5 (dark blue), Day 12 (light blue) and healthy tissue (green) metabolomic features; and Day 3 (pink), Day 5 (dark blue) and healthy tissue (green) for lipidomic features. The healthy tissue and six selected time points are shown in colours according to the colour key at the top of the image.

Influence plots identify outliers in a dataset by measuring the distance of a data point from the current PCA model cluster. Outliers are those points which do not correspond to the model fitted to the majority of the data. The greater the residual or leverage of a data point, the more influence that point has on the PCA model. Influence plots were created using the metabolomic or lipidomic mass spectra of healthy non-wounded tissue compared to whole wound tissue (wound bed and both edge ROIs combined) from Days 3, 5, 8, 10, 12 and 16.

The metabolomic influence plot showed that the majority of features over the 16 days comparing acute wound and healthy tissue cluster well with very small differences. A small residual scale was seen from approximately 0 – 0.8 and a very small leverage scale from approximately 0 – 0.02, with a limited number of outliers showing increased influence outside these ranges on both axes (Figure 4.9 A). The number of residual outliers was relatively small and included features from healthy tissue, implying a degree of natural tissue variance rather than variance induced by healing activity. Day 12 (light blue) features dominated the leveraging outliers but showed low residual therefore the overall influence on the model was not large. Other outliers were found to be mainly from Days 3 (pink) and to a lesser extent Day 5 (dark blue). The overall features of the acute wound healing metabolome kinetics showed little influence on the variation of the PCA model.

The lipidomic influence plot displayed far greater differences and a larger spread of the majority of features between each of the acute wound time points and the healthy tissue with a residual scale spanning 0.9 – 3 and a leverage scale spanning 0 – 0.1, with few outliers present on each axis (Figure 4.9 B). Residual outliers were mainly from Day 3 (pink), and 8 (orange) features, while leverage outliers were dominated by Day 12 (light blue) features, with limited Day 5 and especially Day 3 features showing strong influence on the variation of the PCA model.

The influence plots from all three 'omic' bases in this study (including the proteomic plot in Chapter 3) revealed the degree of variance that the different 'omic' features contributed following PCA. When comparing the influence plots of the proteome (Figure 3.14), metabolome and lipidome (Figure 4.9) to one another, it became evident that the metabolomic kinetics during acute wound healing showed substantially less variation compared to the proteomic and lipidomic datasets. This is

not only based on residual outliers but on the extent of the spread of the data in the metabolomic influence plot, where feature leverage is 5 to 10 times less than those of the lipidomic and proteomic influence plots respectively. The wound proteome kinetics showed the greatest variance between the healthy tissue and six selected time points, displaying leverage twice the extent of the lipidomic counterpart. This suggests that the acute wound proteome in the porcine model may offer greater potential in detecting specific healing phase markers as opposed to the metabolome and lipidome.

4.3.3 Pseudo-gels

As discussed in Chapter 3 (Section 3.3.3), while PCA visualised and revealed the mass identities of metabolite and lipid features responsible for the variance in the 16-day healing process, the relative changes in intensities could not be seen. Pseudo-gels can visualise these relative feature changes for all the samples in the continuously changing molecular wound environment by plotting relative quantitative variance in a single image (Figure 4.10).

The continuously changing metabolome and lipidome of the acute wound over 16 days could clearly be visualised through plotting the quantitative changes in the normalised spectral intensities as well as molecular absence and/or presence of features between the different time points relative to a region of healthy tissue in the pseudo-gels.

The pseudo-gel of the wound metabolome kinetics (Figure 4.10 A) indicated Day 3 wound tissue to most closely resemble the healthy tissue, confirming the PCA plot results where considerable similarity between healthy tissue and Day 3 wound features was seen (Figures 4.5, 4.7 and 4.8). One of the quantitative differences between the two samples on the gel was the appearance of a metabolite around 1970 Da in some of the spectra of Day 3, which was not present in the healthy tissue. Metabolite 1970 Da appeared again on Day 8, through to Day 10 peaking on Day 12 before disappearing on Day 16. The loading plot analysis (Section 4.3.2.2) revealed metabolite m/z 1968 to be variable in the healing process and a potential marker of acute wound healing.

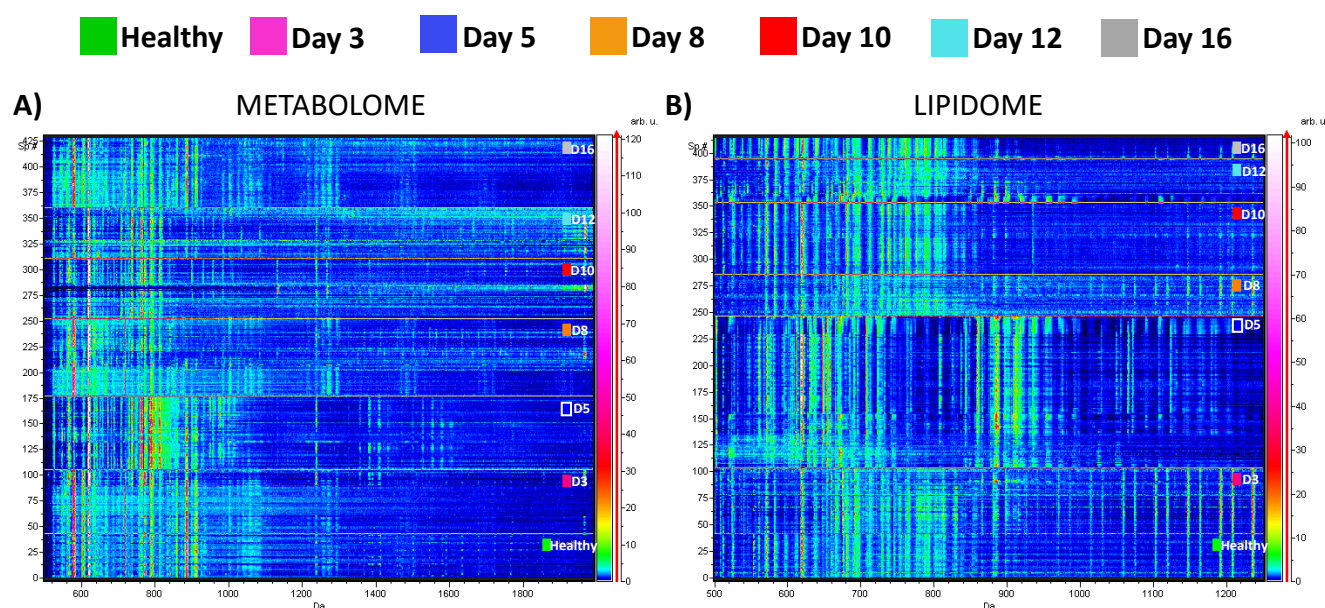


Figure 4.10: Pseudo-gels of healthy tissue and whole wound tissue from Days 3, 5, 8, 10, 12 and 16 across metabolomic and lipidomic bases. The pseudo-gels present comparative data from the different samples in terms of the mass to charge ratio (500 – 2000 Da for metabolites and 500 – 1300 Da for lipids) of the normalised intensity of each feature. Quantitative differences in different mass ranges are clearly seen when tracking the acute wound kinetics for both the (A) metabolome and (B) lipidome over the six selected healing time points and healthy tissue. The x-axis shows the molecular weight in Da (equivalent to m/z); the left y-axis shows the running spectrum number originating from loading spectra (Sp#); the right y-axis indicates normalised feature intensity through a normalised colour intensity scale in arbitrary units (arb. u.). The healthy tissue and each set of the six healing time points are separated by white lines on the gel with spectra sets identified by different colour square blocks according to the key shown at the top of the image.

On Day 5, a relative signal decrease was seen around 580 and 610 Da. Metabolite 610 Da continued decreasing through Days 8, 10 and 12 and represented levels seen in non-wounded tissue on Day 16. A notable increase was seen in the mass range 730 – 840 Da on Day 5 (also seen on Figure 4.1). Based on the pseudo-gel, these metabolites were responsible for most of the variance seen on Day 5 and theoretically (confirmed from histology results) are most likely features associated with inflammatory processes occurring during the early wound healing process (Broughton *et al.*, 2006). It is interesting to take note of the mass 772 Da revealed by the loading plot analysis, as a potential marker of healing (Section 4.3.2.2) that falls into this mass range. Other distinct changes seen in the metabolome from Day 5 onwards included a decrease in intensity around 890 and 900 Da compared to Day 3

and the healthy tissue, which then continued decreasing up to Day 10 and 12 and reappeared on Day 16 at levels representative of those in healthy tissue. This quantitative trend implies that these metabolites play a role in the process of acute wound healing, most likely being associated with newly formed tissue and remodelling. Additional exclusive changes seen on Day 5 were quantitative increases in metabolites between 940 – 1000 Da, 1390 – 1420 Da and 1540 – 1620 Da.

Distinct quantitative increases beginning on Day 8 and increasing into Days 10 and 12 were found at 1920 – 1960 Da. This mass range includes the metabolite of 1940 Da revealed in the loading plot analysis of Section 4.3.2.2.

The pseudo-gel of the wound lipidome kinetics (Figure 4.10 B) revealed a similar quantitative lipidomic profile between healthy tissue and Day 3 wound tissue, with minor differences seen between the two groups. This supports the results of the PCA plots where substantial overlap between healthy tissue and Day 3 data points was seen (Figures 4.6 – 4.8). Some of the more prominent differences on the gel included a decrease in lipids around 595 and 615 Da on Day 3, as well as a minor increase around 880 Da.

On Day 5, relatively large quantitative changes occurred showing the most variation compared to the remaining wound and healthy tissues. Multiple features showed differences in intensity on Day 5 including an increase in lipid 615 Da that remained elevated up to Day 16, as well as increases of lipids in the 620 – 680 Da range, which decreased again from Day 8 – 16. Lipids spanning 690 – 710 Da were found to decrease in intensity on Day 5 but then continuously increased from Day 8, 10 and 12, peaking on Day 16. Lipids between 775 – 810 Da were almost absent on Day 5, reappearing on Day 8 and remaining at consistent levels from Day 10 onwards. The highly variable lipids revealed by the loading plots analysis (Section 4.3.2.2), lipids *m/z* 647, 671 and 687, all fell within the mass ranges where relative quantitative change on the pseudo-gel could be seen. Some of the largest relative quantitative increases were seen between 880 – 960 Da on Day 5. All these lipids decreased significantly on Day 8 with the majority being absent on Day 10 and reappearing in limited spectra on Days 12 and 16.

Lipids seen between 1060 – 1235 Da were present in healthy tissue and on Days 3 and 8, with low intensity on Day 16, but were absent on Days 5, 10 and 12. The sudden appearance and disappearance of these unusually large lipids between samples from different time points was not an expected natural occurrence, and is thought to be due to the minor contamination with OCT compound used during the sample processing to attach the tissue samples to the cryotome chuck. Potential contamination of the cut surface of the samples during cryosectioning is a probable source of these features. The masses of these features follow mass differences and increasing intensity typical of a polymeric compound mass envelope. These masses coincide with and fall into the reported mass envelope of the OCT polymer from m/z 1000 - 2000 (Weston and Hummon, 2013). As a result, the features observed in this mass range were considered as being non-tissue related and disregarded in all the data analyses including the PCA and subsequent statistical testing.

The pseudo-gels offered an alternative normalised graphic of the mass distribution and intensities of the continuously changing metabolomic and lipidomic wound healing environment. Both metabolomic and lipidomic pseudo-gels showed Day 5 as the peak difference from the healthy tissue and from the other sampling time points. The three most variable metabolites and lipids during the healing process based on PCA calculations and loading plot analysis were visualised on the pseudo-gels as masses showing variation amongst others (Section 4.3.2.2 and Figure 4.8). Although pseudo-gels are a useful profiling tool and offer a unique molecular overview, statistical analysis is required to determine the validity and extent of such visual variance.

4.3.4 Peak Statistics

4.3.4.1 PCA data analysis

PCA (with all its associated plots) and pseudo-gels were used to visualise and assess the acute wound metabolome and lipidome kinetics over 16 days, across six selected wound excision time points and in doing so revealed various metabolites and lipids of interest associated with wound healing. In theory, the more variant a molecule and the less correlation to non-wounded tissue the more representative it may be of a wound healing associated feature and the more interest it evokes as a

potential healing marker and future target for drug development. To determine the importance of the molecules highlighted in the PCA and pseudo-gel analyses, and to potentially reveal other metabolites and lipids as promising acute wound healing features, peak statistics were performed between healthy tissue and wound tissue from Days 3, 5, 8, 10, 12 and 16.

Peak statistic reports were generated on whole wound tissue spectra (wound bed and edge ROIs combined) of all six selected time points and the healthy, non-wounded tissue region across both metabolomic and lipidomic bases. A total of 132 and 88 differentiated peaks made up the metabolomic and lipidomic reports respectively. Features were picked according to the peak calculations set (Section 3.2.3.2) where the average intensity for each feature in each sample group was recorded (Appendices VI - VII).

As shown earlier in the chapter, this data was filtered to a manageable differential peak set across both 'omic' bases using PCA analysis and the 3D views provided by loadings plots to reveal the mean masses of greatest variance. Ultimately, minimised kinetic profiles of the acute wound metabolome and lipidome over the 16 days of healing were created, where features appearing to contribute to differentiation were highlighted through PCA and resultant loading plots (only the three most differentiating features were focussed on) (Section 4.3.2.2). In order to determine the statistical significance of these variant features, the Anderson-Darling (AD) test was used to assess whether the spectral data followed a parametric distribution (*i.e.* normal distribution), followed by assessment of the p-values provided by the relevant statistical test, either being ANOVA or Kruskal-Wallis. The coefficient of variation (CV) was also calculated to measure data variability across the healthy tissue and six selected time points (Table 4.4).

Table 4.4: Kinetic peak statistics on differential loading plot values as potential acute wound healing markers across metabolomic and lipidomic bases

	Mass (m/z)	D Ave	PKW	PAD	Ave H $\pm$ % CV	Ave D3 $\pm$ % CV	Ave D5 $\pm$ % CV	Ave D8 $\pm$ % CV	Ave D10 $\pm$ % CV	Ave D12 $\pm$ % CV	Ave D16 $\pm$ % CV
METABOLITES	771.91	23.69	< 0.000001	< 0.000001	26.9 $\pm$ 134.15	18.66 $\pm$ 112.02	8.87 $\pm$ 74.41	8.46 $\pm$ 109.7	12.82 $\pm$ 84.53	3.21 $\pm$ 93.49	4.67 $\pm$ 83.15
	1939.72	3.54	< 0.000001	< 0.000001	1.07 $\pm$ 27.65	1.03 $\pm$ 34.24	1.12 $\pm$ 36.85	1.46 $\pm$ 56.64	2.37 $\pm$ 120.89	4.57 $\pm$ 86.45	1.36 $\pm$ 39.39
	1967.91	27.76	< 0.000001	< 0.000001	1.04 $\pm$ 24.52	4.42 $\pm$ 151.47	1.27 $\pm$ 31.97	4.64 $\pm$ 215.22	14.22 $\pm$ 247.07	28.8 $\pm$ 160.8	1.3 $\pm$ 40.61
LIPIDS	647.29	25.19	< 0.000001	< 0.000001	1.78 $\pm$ 22.1	2.47 $\pm$ 41.44	26.54 $\pm$ 62.46	1.84 $\pm$ 32.17	1.35 $\pm$ 17.63	1.46 $\pm$ 76.63	1.73 $\pm$ 44.16
	671.45	6.73	< 0.000001	< 0.000001	1.77 $\pm$ 29.32	3.02 $\pm$ 147.13	8.1 $\pm$ 108.05	2.82 $\pm$ 107.04	1.38 $\pm$ 25.7	8.02 $\pm$ 147.8	3.4 $\pm$ 189.94
	687.42	2.53	< 0.000001	< 0.000001	2.04 $\pm$ 31.38	2.74 $\pm$ 59.93	2.46 $\pm$ 56.11	3.17 $\pm$ 89.84	1.53 $\pm$ 26.37	4.06 $\pm$ 120.26	3.72 $\pm$ 100.43

(**D Ave**: Difference between the maximal and minimal average feature intensity of healthy tissue and all six time points (arb. u.); **PKW**: P-value calculated by the Kruskal-Wallis test; **PAD**: P-value of Anderson-Darling test; **Ave N $\pm$ % CV**: Feature intensity average of class *N* where 'H'= healthy tissue and 'D'= Day post-wounding; $\pm$ coefficient of variation in % of class *N*)

Table 4.4 displays the three feature masses contributing most to the data variation in whole wound tissue of the wound metabolome and lipidome as revealed by loading plots over the 16 days of healing. None of these metabolites or lipids showed a normal distribution according to the AD test and therefore the Kruskal-Wallis test was used to determine statistical significance. All features in the table showed $p < 0.000001$ for the average intensity values across the non-wounded, healthy tissue and wound tissue from Days 3, 5, 8, 10, 12 and 16. The difference in average intensity between the healthy tissue and six selected time points was statistically significant with >99.999% confidence, evidence of a continuously changing wound metabolome and lipidome.

In the metabolome m/z 1967.91 and 771.91 had relatively high D Ave values (difference between the maximal and minimal average feature intensity across all time points) compared to m/z 1939.72, with intensities of 27.76 and 23.69 compared to 3.54 respectively. This suggested that m/z 1967.91 and 771.91 may be more robust markers of the acute wound healing process. This is especially due to the relatively large quantitative differences between the healthy and wound tissue samples.

The large D Ave of m/z 1967.91 was due to a maximal intensity on Day 12 compared to a minimal intensity in the healthy tissue, where an almost 27-fold difference occurred and where an increase was first noted on Day 10. This intensity pattern implies that m/z 1967.91 is associated with the remodelling phase of acute wound healing and may have potential as an acute wound phase marker. Such a marker may be used in the analysis of chronic wounds that appear to not reach the remodelling phase of healing (Broughton *et al.*, 2006, Landén *et al.*, 2016, Velnar *et al.*, 2009). This quantitative trend was highlighted in the pseudo-gels (Section 4.3.3 and Figure 4.10 A) and discussed in the PCA section (Section 4.3.2.2) where m/z 1967.91 was found to be variant on Days 3, 8, 10 and 12 on the singular loading plots (one healing time point versus healthy tissue per plot; not shown). High CV values corresponded to these same time points, peaking on Days 8 and 10 suggestive of mounting biological activity that began to decrease on Day 12 and continued decreasing to Day 16.

Metabolite m/z 771.91 showed the largest intensity difference between Day 12 (minimal intensity) and the healthy tissue (maximal intensity). This suggested lower levels of m/z 771.91 were associated with acute wound healing, where 1.4 – 8.4 times less of the metabolite was found in healing tissue across the six selected time points. This metabolite may play a role in processes that occur in non-wounded tissue explaining the decreases seen on wound Days 3, 5 and 8 through the height of haemostasis and inflammation. On Day 10 during the peak of proliferation and when new tissue had been deposited in the wound space, m/z 771.91 levels increased by approximately 50%. Two days later on Day 12 during remodelling when thin collagen fibres were being enzymatically removed, the levels decreased 4-fold, slowly beginning to increase on Day 16 as new collagen type I synthesis overshadowed degradation (supported histologically in Figure 2.8). Metabolite m/z 771.91 was found to show variance relative to healthy tissue in the single time point loading plots discussed in Section 4.3.2.2, that was not immediately obvious in the pseudo-gels, highlighting the importance of peak statistics analysis. Interestingly, the highest CV value of m/z 771.91 was seen in the healthy tissue, supporting the theory that this metabolite is associated with processes occurring naturally in non-wounded tissue.

The lipidome peak statistics (Table 4.4), showed one out of the three variant feature masses to have a relatively large intensity difference when comparing wound to healthy tissue. Although several isomers of the lipids are possible and that MS/MS data was not collected where typical fragmentation could confirm the masses of the individual fatty acids, the empirical mass of some lipid features could be determined and the most likely structure could be implied from the empirical mass. This was done through Lipid Maps website (Wellcome Trust, 2019). Lipid m/z 647.29 (PA(14:0/18:1 1-tetradecanoyl-2-octadecenoyl)-sn-glycero-3-phosphate) showed a D Ave value of 25.19, substantially higher than the other D Ave values of 6.73 and 2.53 of lipids m/z 671.45 (PA(16:0/18:3)1-hexadecanoyl-2-(octadecatrienoyl)-glycero-3-phosphate) and 687.42 (PG(12:0/18:4) 1-dodecanoyl-2-(octadecatetraenoyl)-glycero-3-phospho-(1'-sn-glycerol) respectively. This suggests that m/z 647.29 may not only be a useful feature of acute wound healing but due to major intensity differences between the six selected time points, a potentially reliable wound phase marker. This lipid was found to show between an 11 – 20-fold intensity increase on

Day 5 compared to all remaining wound days, implying an association with the inflammatory phase of acute wound healing, making it an interesting feature to study in chronic wounds. Chronic wounds are said to be trapped in the inflammatory phase of healing (Section 1.1.4) where a potential inflammatory feature would be an interesting target to identify and study over time in a non-healing wound environment (Broughton *et al.*, 2006, Landén *et al.*, 2016).

Interestingly, although relatively high on Day 5, the CV of lipid m/z 647.29 was maximal on Day 12. Inflammation was still present in the wound on Day 12 in this study (Figure 2.8) and this may be a possible explanation for the presence of this lipid, with inflammatory processes decreasing and varying levels of the lipid being found. The CV values of all the wound tissue time points (except Day 10) were higher than in non-wounded tissue and this again suggests a role of lipid m/z 647.29 in the acute wound healing process.

Lipids m/z 671.45 and especially m/z 687.42 displayed substantially less intensity variance when comparing the healthy tissue and six selected time points, displaying less potential as robust and detectable features of wound tissue. Intensity of lipid m/z 671.45 was found to peak on Day 5 together with lipid m/z 647.29. Both lipids correspond to the changing 16-day trends revealed by PCA where the height of variation in the acute wound lipidome was seen on Day 5 (Section 4.3.2). The largest variation of both lipids on Day 5 was clearly visualised in the lipidomic loading plot (Figure 4.8) as well as on the pseudo-gel of the wound lipidome (Figure 4.10 B).

4.3.4.2 Kruskal-Wallis and difference average analyses

An alternative method to filter and highlight variant masses for statistical analysis as potential acute wound healing features, is to utilise peak statistics independently of PCA. The Kruskal-Wallis test and D Ave values across the healthy tissue and six selected wound healing time points were used to rank and reveal potential wound healing features. This approach was applied to the peak statistic reports of whole wound data (bed and edge ROIs combined) (Appendices VI - VII). Only the top-two highest ranked and significantly different (outside of the $\pm 0.25\%$ mass error) features, according to either the most significant PKW or the largest D Ave were considered as potential molecules of interest (Table 4.5).

Table 4.5: Kinetic peak statistics of metabolites and lipids ranked by PKW and D Ave analyses as potential acute wound healing features

	PKW-ranked mass (<i>m/z</i>)	D Ave	PKW	D Ave-ranked mass (<i>m/z</i>)	D Ave	PKW
Metabolites	1. 884.01	41.97	<0.000001	1. 619.23	208.23	<0.000001
	2. 910.11	13.93	<0.000001	2. 582.16	58.64	<0.000001
Lipids	1. 886.10	14.79	<0.000001	1. 619.31	56.48	<0.000001
	2. 611.14	14.65	<0.000001	2. 647.29	25.19	<0.000001

*Data do not follow a normal distribution based on PAD thus PKW was used for evaluation. (D Ave: Difference average; PKW: P-value Kruskal-Wallis.)

The Kruskal-Wallis test and the D Ave values from the peak statistic reports were used to identify the most prominent and likely metabolomic and lipidomic features of acute wound healing. PKW was used to reveal both the top-two metabolites and lipids that showed the most significant statistical difference between the healthy tissue and wound tissue from Days 3, 5, 8, 10, 12 and 16. Where PKW values were comparable, masses were ranked based on the best distinguishing features. D Ave was independently used to rank metabolites and lipids based on the largest significant difference between the maximal and minimal average feature intensity across the healthy tissue and six selected time points.

The top-two metabolomic feature average masses revealed by the PKW sort-order were *m/z* 884.01 and 910.11, where those found to have the highest D Ave values were *m/z* 619.23 and 582.16. Despite *m/z* 582.16 being ranked just below *m/z* 619.23, the difference in D Ave values was large (3.5-fold), making *m/z* 619.23 more likely to be a prominent and dependable acute wound healing feature. All four features of interest had a p-value <0.000001 indicative of a significant difference in intensity between the healthy tissue and all six selected time points. The most significant metabolites based on PKW, *m/z* 884.01 and 910.11, ranked 8th and 29th out of 132 peaks respectively based on largest D Ave.

Interestingly, clear quantitative changes could be seen in all four metabolites of interest on the pseudo-gel of the metabolome over the 16-day study, where *m/z* 582.16 and 884.01 appeared amongst the most prominent (Figure 4.10 A). None of the newly revealed metabolites corresponded to the top three masses of interest revealed in the PCA-based analyses (*m/z* 772, 1940 and 1968), highlighting not only

how different approaches may reveal new masses of interest, but also the complexity of the biological metabolome.

The lipidomic features identified as the top-two ranked Kruskal-Wallis and D Ave were revealed to be m/z 886.10 and 611.14; and m/z 619.31 and 647.29 respectively. All four lipids had a p -value <0.000001 in the intensity changes observed across the healthy tissue and the six selected time points. Interestingly, lipid m/z 619.23, was also ranked first in the D Ave metabolomic analysis. The fact that this lipid has been highlighted independently across two separate, 'omic' base analyses emphasises its potential importance and relevance as a feature of the acute wound healing process. Also of interest is that the lipid showing the second largest D Ave value, m/z 647.29, was highlighted in the peak statistics of the PCA data analysis in the previous section (Section 4.3.4.1) as showing great potential as a healing feature. Lipid m/z 647.29 was initially shown to be one of the most variant features in the loading plot analysis (Section 4.3.2.2; and Figure 4.8). As m/z 647.29 was highlighted as a lipid feature of interest by two independent statistical approaches, its potential as a reliable feature of acute wound healing was reaffirmed.

The most significant lipids based on PKW, m/z 886.10 and 611.14 ranked 7th and 8th out of 88 peaks respectively based on largest D Ave, further highlighting the potential of these features in wound healing. The lipidomic pseudo-gel (Figure 4.10 B) showed relatively large intensity changes in m/z 619.31 across the healthy tissue and the six selected time points. In addition, lipid m/z 886.1 fell within the m/z 880 – 960 mass range where some of the largest relative quantitative increases were seen on Day 5 on the gel.

Following the feature statistics analyses of the kinetic acute wound metabolome and lipidome, three additional features of interest per 'omic' base were revealed and added to the list of potential healing features for future investigation. The features of interest are metabolites: m/z 582, 772, 884, 910, 1940 and 1968; and lipids: m/z 611, 619, 647, 671, 687 and 886.

4.3.5 2D Peak distributions

The analyses performed up until this point have involved the average intensity of features, rather than each individual feature. 2D peak distribution analysis shows the features from all spectra collected and not just the averaged quantitative values. This allows visualisation of the distribution of data points from the healthy tissue and wound Days 3, 5, 8, 10 12 and 16, based on two selected features of interest falling on the x and y axes. The 2D distribution plot provides a basis for the assessment of features best differentiating the healthy tissue and the six selected time points from one another.

As explained in Chapter 3, the abilities of Kruskal-Wallis ranked values, PCA, D Ave ranked values and pseudo-gels to best differentiate the features of the kinetic changes in the acute wound (six selected time points) and healthy tissue were tested and compared using 2D peak distribution plots (see Section 3.3.5).

The Kruskal-Wallis ranked probability values and the D Ave assessment values reported in Table 4.5 were used. For the Kruskal-Wallis plots features m/z 884 vs 910 and m/z 886 vs 611 were used for the metabolome and lipidome respectively; and for the D Ave plots features m/z 619 vs 582 and m/z 619 vs 647 were used for the metabolome and lipidome respectively. For the PCA-based distribution plots, the three metabolites and lipids showing the greatest variance as already revealed in the loading plots of Section 4.3.2.2 were used in further calculations of contribution to total variance. To determine the two metabolites and lipids contributing most to variance during the healing process based on PCA calculations, feature contribution calculations were performed. Each of the three features were assigned three loading values directly corresponding to the first three PCs. According to the variance plots of the respective data, the metabolomic and lipidomic PC 1, 2 and 3 were responsible for explaining 25%, 17% and 12%; and 34%, 22% and 11% of the variance respectively. As explained in Section 3.3.5, the respective SC and TC values were calculated for each feature where the two features per 'omic' base with the highest absolute TC value were deemed as the most significant for use in the 2D distribution plots. These features were revealed to be metabolites m/z 1968 and 772, and lipids m/z 647 and 671 (Table 4.6).

To reveal features of interest highlighted through quantitative changes seen on pseudo-gels (Figure 4.10), mass region m/z 790 – 800 was selected for the metabolome and m/z 880 – 920 for the lipidome, where significant quantitative changes could be seen between the spectra of healthy tissue and the six selected time points during wound healing. The two features showing the greatest significant D Ave within these mass ranges according to the metabolomic and lipidomic peak statistic reports, were selected for the 2D distribution analysis. These features were m/z 791 vs 796 (metabolome) and m/z 886 vs 912 (lipidome). The m/z 886 is the same lipid utilised in the separate Kruskal-Wallis distribution plot. The 2D peak distribution plots differentiating kinetic acute wound healing in the metabolome and lipidome are illustrated in Figures 4.11 and 4.12 respectively.

Table 4.6: Variance contribution of differential PCA features of interest across metabolomic and lipidomic bases

Mass (m/z)	METABOLITES						LIPIDS					
	771.91	SC	1967.91	SC	1939.72	SC	647.29	SC	671.45	SC	687.42	SC
Loading 1	0.078	1.950	-0.016	-0.400	-0.006	-0.150	0.219	7.446	0.070	2.380	-0.012	-0.408
Loading 2	-0.002	-0.034	0.585	9.945	0.222	3.774	-0.135	-2.970	-0.078	-1.716	-0.039	-0.858
Loading 3	-0.365	-4.380	0.183	2.196	0.089	1.068	-0.163	-1.793	0.373	4.103	0.190	2.090
TC		6.364		12.541		4.992		12.209		19.482		3.356

Negative values only indicate the direction of variance scattering on the loading plots. SC: Single contribution; TC: Total contribution. SC= loading value x PC variance explained (%). TC= sum of SC values. Metabolites PC1= 25%; PC2=17%; PC3=12%; Lipids: PC1= 34% PC2=22% PC3=11%.

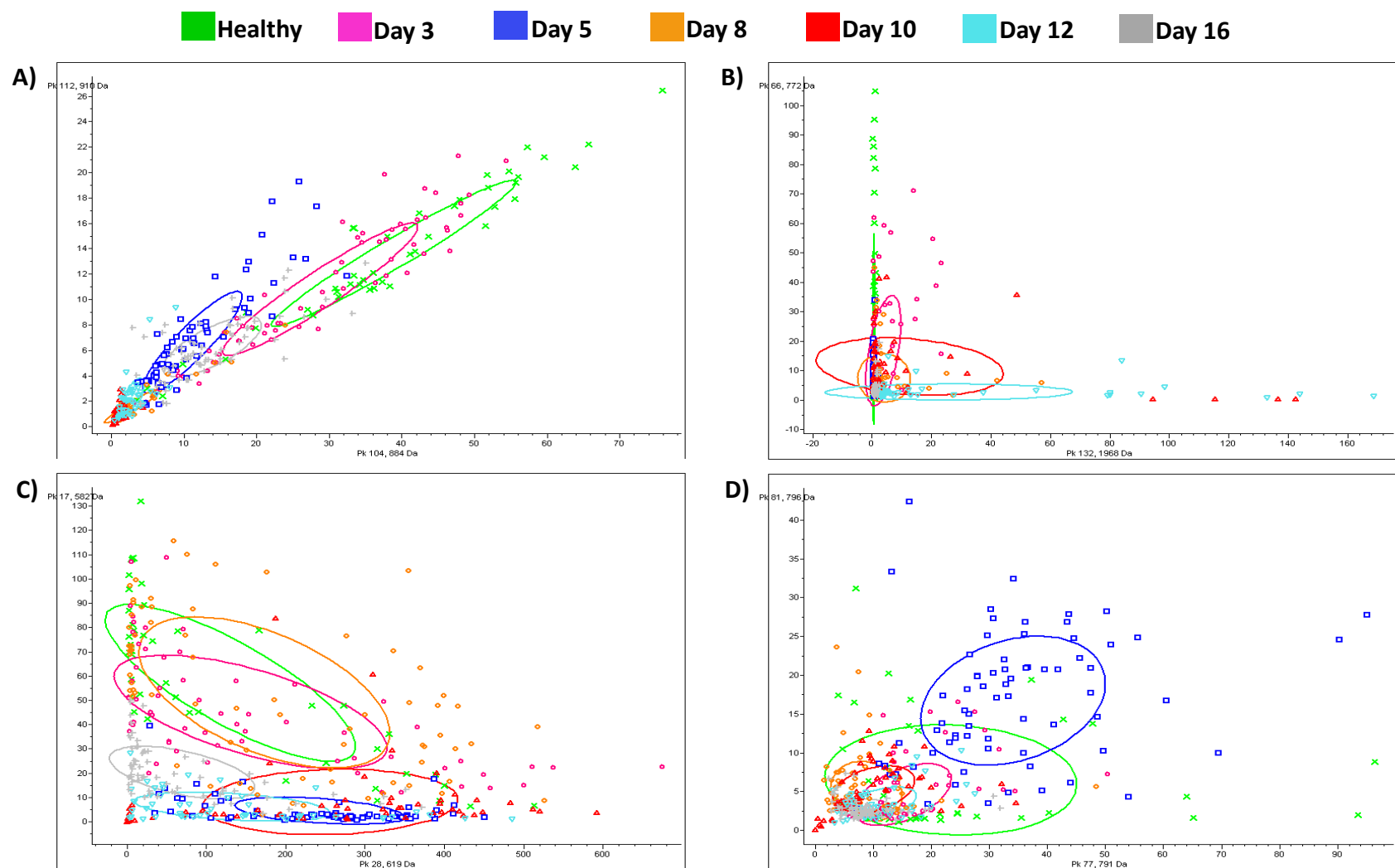


Figure 4.11: 2D peak distribution plots of metabolites of interest. 2D peak distributions of the two top-ranked features by (A) PKW value: m/z 884 vs 910; (B) PCA value: m/z 1 968 vs 772; (C) D Ave value: m/z 619 vs 582; and (D) pseudo-gel quantitative change: m/z 791 vs 796. The healthy tissue and six selected time points are shown according to the colour key. Ellipses represent the standard deviation of the time point average of the feature intensities. Axes measurements are in arbitrary units chosen automatically to optimally fit the plot in the plane.

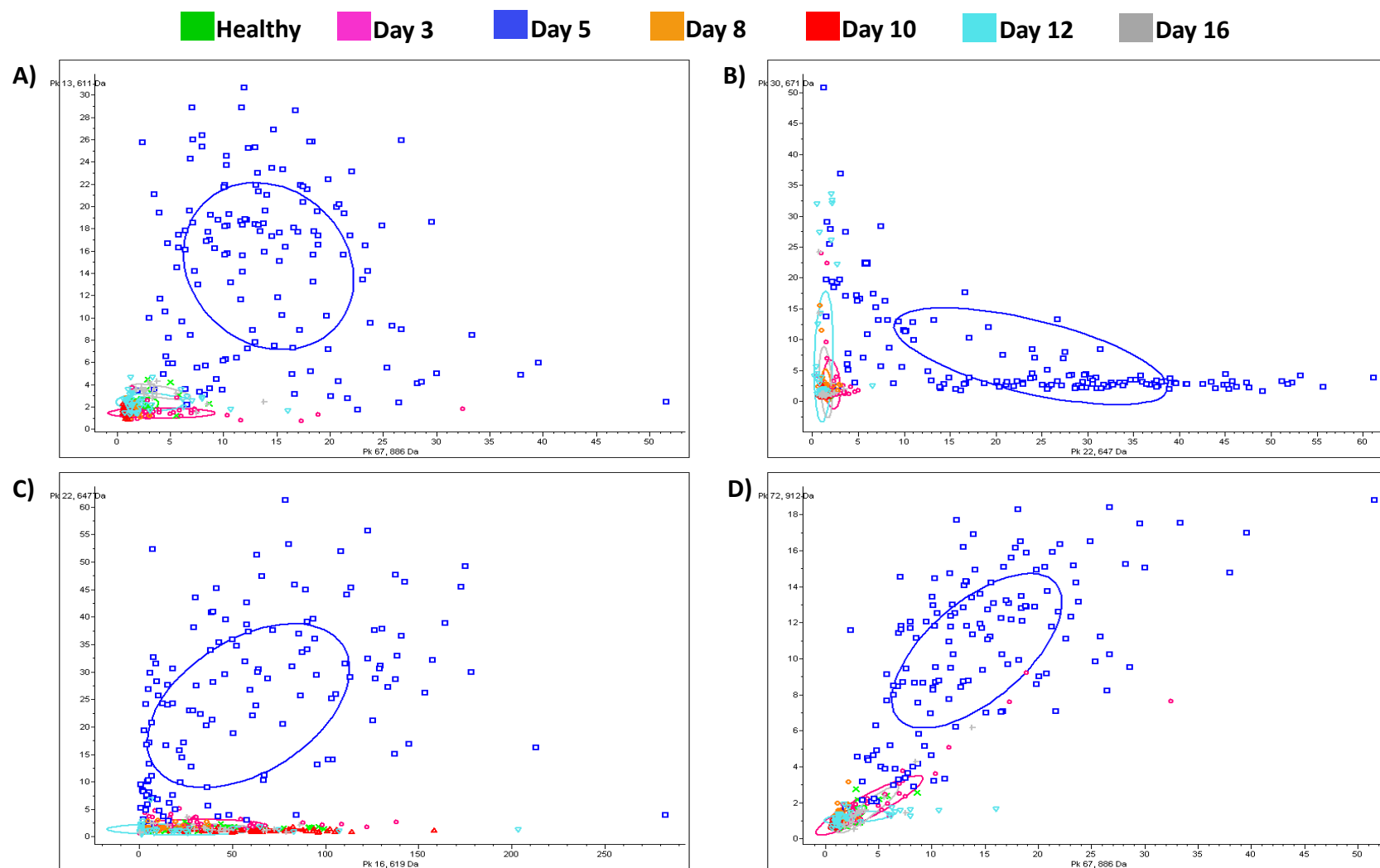


Figure 4.12: 2D peak distribution plots of lipids of interest. 2D peak distributions of the two top-ranked features by (A) PKW value: m/z 886 vs 611; (B) PCA value: m/z 647 vs 671; (C) D Ave value: m/z 619 vs 647; and (D) pseudo-gel quantitative change: m/z 886 vs 912. The healthy tissue and six selected time points are shown according to the colour key. Ellipses represent the standard deviation of the time point average of the feature intensities. Axes measurements are in arbitrary units chosen automatically to fit the plot optimally in the plane.

2D peak distribution was used to assess which metabolites and lipids best distinguish healthy tissue from that of wounded tissue and the different time points of healing using feature intensity changes. Feature candidates were selected based on three statistical rankings (PKW, PCA and D Ave) as well as relative quantitative changes observed in pseudo-gels.

The PKW-based metabolomic plot (Figure 4.11 A) revealed that from the six wound tissue time points only Days 3 and 5 were differentiable. Day 3 features however, showed the greatest similarity to the healthy tissue. This supports the association seen between Day 3 and healthy tissue data points in the PCA plots (Figure 4.5). Of the remaining wound time points, which showed no overlap with healthy tissue features, Day 5 features showed the largest amount of variance. Relatively high intensities of metabolites m/z 884 and 910 were seen in the healthy tissue, suggesting that lower levels of both metabolites are associated with wound healing. The lipidomic PKW-based plot (Figure 4.12 A) showed significant overlap and minor intensity variance in wound features from Days 3, 8, 10, 12 and 16 and healthy tissue, with Day 5 being the only time point that showed major differentiation and variance in both lipids m/z 886 and 611. These lipids could be used as reliable features to discern non-wounded, healthy tissue from wounded tissue within the inflammatory phase, due to the relatively large intensity differences between the two tissue groups.

Figure 4.11 B, the metabolomic plot based on PCA, showed significant overlap between all six wound tissue time points and healthy tissue. The outliers present on Days 3, 10 and 12 and in the healthy tissue were responsible for the majority of observed variance in the dataset. Day 3 showed the closest correlation to healthy tissue relative to the remaining wound time points. In the lipidomic PCA-based distribution plot (Figure 4.12 B), significant feature overlap and minor intensity variance were seen in the healthy tissue and wound Days 3, 8, 10 and 16 in both lipids. Lipid m/z 671 showed a relative increase on Days 5 and 12, differentiating these two wound time points from remaining samples. Lipid m/z 647 showed greatest variation and differentiation in the wound tissue of Day 5 highlighting this lipid as a potentially useful feature of acute wound healing, and possibly of the inflammatory phase. Interestingly, m/z 647 showed a relatively large D Ave revealed in the peak statistics due to a clear intensity increase on Day 5 (Table 4.4).

The third statistical ranking by maximal D Ave showed significant overlap between all features in the metabolic plot, where wound time points could not be differentiated from one another or from the healthy tissue (Figure 4.11 C). It appeared that in general, higher amounts of metabolite m/z 619 (x-axis) were found in the earlier phases of acute wound healing on Days 3, 5, 8 and 10 and as healing progressed metabolic levels started decreasing from Day 10 to Day 12, being substantially decreased by Day 16. Feature m/z 619 was also found in the lipidomic distribution plot (x-axis) (Figure 4.12 C) where on Day 5 relatively extensive quantitative variance was seen with outlier intensities spanning a 250-fold range. The higher intensity features were easily differentiated from remaining wound time points and healthy tissue. Almost all Day 5 m/z 647 features (y-axis) however, could be clearly differentiated from remaining wound days and the healthy tissue, which otherwise showed significant feature overlap. As described above for the PCA-based plot where m/z 647 was also utilised, this lipid showed substantial variation on Day 5, most likely increasing due to involvement in inflammatory-related processes.

The last metabolomic 2D peak distribution plot (Figure 4.11 D), the pseudo-gel analysis, revealed Day 5 features as the most differentiable wound time point with all remaining wound tissues and the healthy tissue showing significant overlap of features. This suggests that these Day 5 metabolites may have potential as phase-specific features in the acute wound healing process. While variation at this wound time point was seen in both metabolites of the plot, the variation in m/z 791 was significantly larger than in m/z 796 and it was therefore decided to only add the former to the list of potential acute wound healing metabolomic features. The lipidomic pseudo-gel 2D distribution plot (Figure 4.12 D) displayed a similar feature distribution to that of the metabolome, where only Day 5 features showed a high enough variance to be differentiable from the remaining five wound time points and healthy tissue. The variance on Day 5 highlighted lipids m/z 886 and 912 as potential inflammatory features of acute wound healing. While both lipids showed relatively increased intensities on Day 5, m/z 886 did so to a larger extent and this lipid was therefore added to the list of potential acute wound healing features.

Considering these outcomes, metabolomic features ranked according to the most significant PKW value presented the most favourable distinguishing features of acute wound healing. The PKW-based plot was the only plot providing definitive time point

differentiation (Day 5) of the statistical techniques and showed the least similarity between the healthy tissue and six selected time points. Mid to late wound healing tissue could be differentiated from healthy tissue, and while attributing unknown wound samples to exact time points or phases would not be possible, association with the earlier (up until Day 5) or later (Day 8 – 16) phases of healing could be determined. This may be applicable in the assessment of chronic wounds, where potentially being trapped in the inflammatory phase may allow for classification through association with the earlier phases of wound healing.

With respect to the lipidomic data, all four 2D peak distribution plots showed comparable results and clearly differentiated Day 5 from remaining wound time points and healthy tissue. While none of the approaches differentiated any of the remaining healing points from one another or from the healthy tissue, it is Day 5 that has been found to be the peak of acute wound healing activity (in this study) and arguably the most representative of the advancing healing process. The substantial variation of these lipids in Day 5 wound tissue may be useful in inflammatory phase modelling.

Comparing the results of the proteomic (Section 3.3.5), metabolomic and lipidomic 2D peak distribution plots revealed that the proteomic distributions highlighted Day 8 and both the metabolomic and lipidomic distributions highlighted Day 5, as the healing time points with the most variance as well as the most differentiation from non-wounded, healthy tissue. This confirms Days 5 and 8 as the peaks of healing activity across the three 'omic' bases, as highlighted throughout the multiple analyses of Chapters 3 and 4. The proteomic distribution found PCA to be the most distinguishing tool to reveal potential healing features for acute wound classification, while the PKW-based plot was the preferred tool in the metabolome. No specific distribution plot proved to be a superior differentiating tool in the lipidome. Comparing the best distinguishing plots suggested that the wound proteome and lipidome showed higher potential in revealing acute wound features for classification, as wound time points at the peak of healing activity (Day 8 for proteins and Day 5 for lipids) showed greater relative variance and differentiation compared to that of the metabolome. The first three statistically based 2D distribution plots of the metabolome (Figure 4.11 A-C) showed significantly greater correlation between the healthy tissue and six selected time points compared to the proteome and lipidome

(Figures 3.17 A-C and 4.12 A-C respectively). Of the three 'omic' bases, proteomic features showed superior potential for differentiation of the healthy tissue and each of the six wound points in the PKW-based plot. If a single 'omic' base were to be selected for development of an acute wound modelling tool through 2D peak distribution, the wound proteome presented with the most potential.

4.3.6 Spectral analysis

All MALDI-IMS analysis up until this point was based on the ROIs selected, being the wound bed, wound edges and a region of non-wounded healthy tissue (Section 4.3.1). This approach may be influenced by human error or bias and therefore spectral analysis, a reversed approach, was performed (see Section 3.3.6 in Chapter 3). This was done to reveal wound-associated features that may have been missed in previous statistical analyses, which could act as potential acute wound healing markers (Figure 4.13 and 4.14).

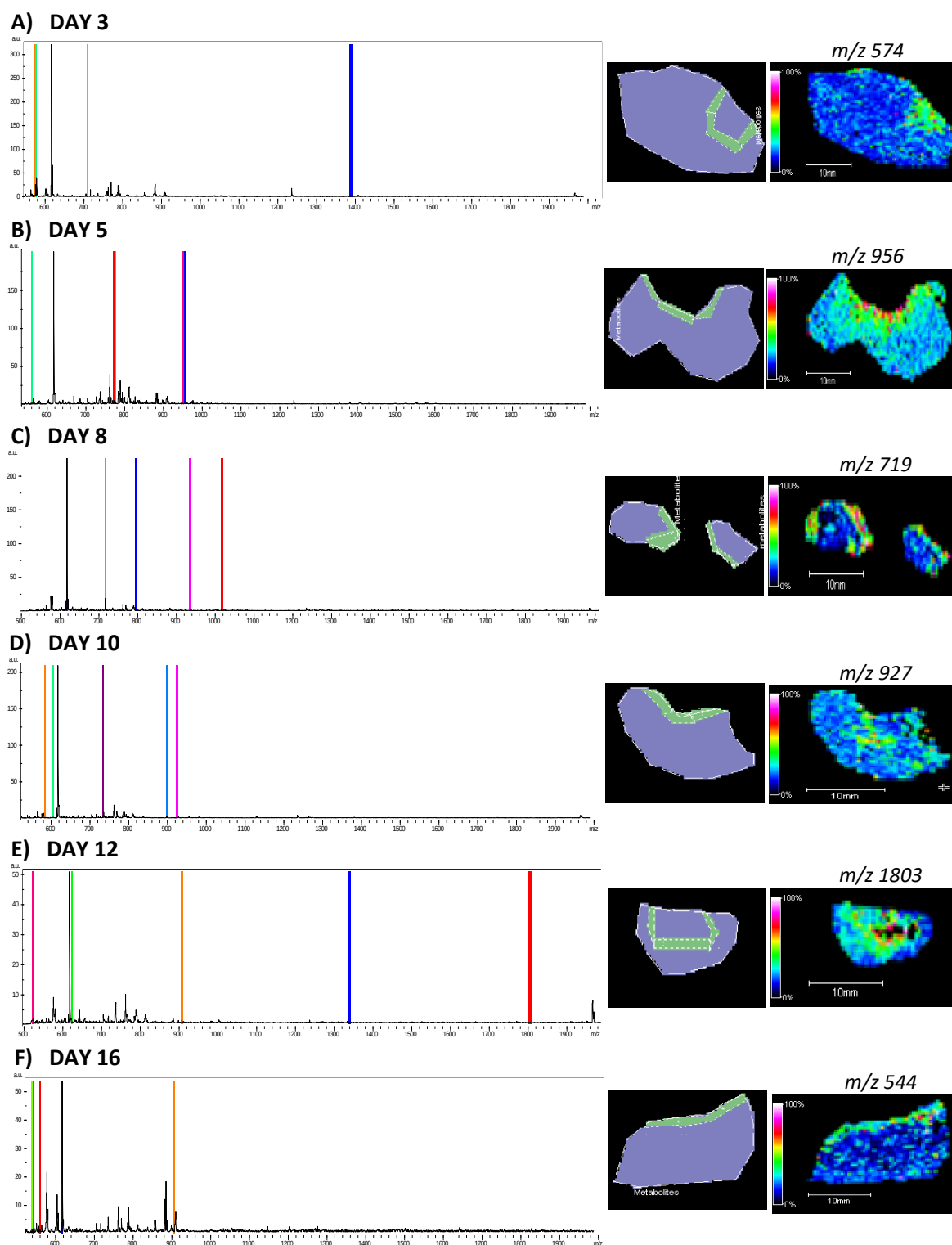


Figure 4.13: Selected wound-associated metabolomic features through spatial quantitation. Metabolites showing relative quantitative change in the wound region were found on Days 3, 5, 8, 10, 12, and 16 (A-F). Image sequence in A-F: Average mass spectrum of sample with coloured vertical lines indicating the masses with large quantitative changes within the wound site; grey areas show IMS scan template with the wound ROIs in green and IMS image showing the relative intensity of the metabolite with the highest association of the wound region. Colour intensity scale measures quantitation in arb. u.

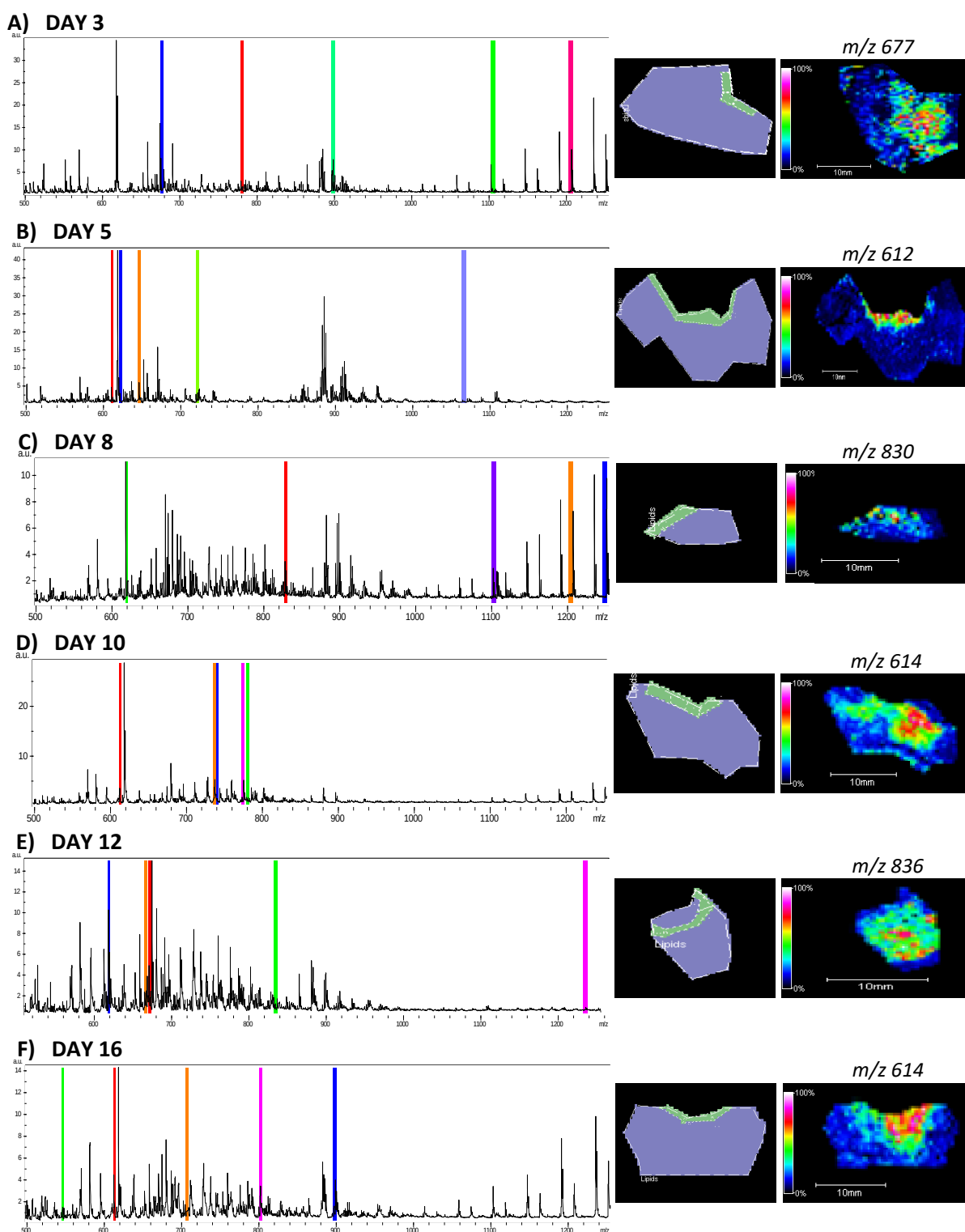


Figure 4.14: Selected wound-associated lipidomic features through spatial quantitation. Lipids showing relative quantitative change in the wound region were found on Days 3, 5, 8, 10, 12, and 16 (A-F). Image sequence in A-F: Average mass spectrum of sample with coloured vertical lines indicating the masses with large quantitative changes within the wound site; grey areas show IMS scan template with the wound ROIs in green and IMS image showing the relative intensity of the lipid with the highest association of the wound region. Colour intensity scale measures quantitation in arb. u.

Following analysis of the average mass spectra of the acute wound from Days 3, 5, 8, 10, 12 and 16, between two to five metabolites and four to five lipids were highlighted per time point as having quantitative differences within the wound region compared to the surrounding, non-wounded tissue. None of the time points displayed features completely unique to wound tissue in either 'omic' base. Only the features showing the greatest relative quantitative change in the wound area were illustrated in Figures 4.13 and 4.14.

In the metabolomic analysis Day 3 displayed the smallest relative difference between the wound and surrounding healthy tissue of the six selected time points, supporting what was generally seen in the statistical IMS analysis. Metabolite m/z 574 showed the largest visual difference with higher intensities throughout the wound region (Figure 4.13 A). Metabolite m/z 956 showed the greatest spatial association to the wound on Day 5, with the highest intensities seen within the centre and deepest point of the wound bed, decreasing moving further away from the wound site (Figure 4.13 B). This distribution is similar to that of the inflammatory cells observed in the Day 5 histology reported in Chapter 2 (Figure 2 E – F) and suggests that metabolite m/z 956 may be a promising feature of the inflammatory phase of healing. Interestingly, this metabolite falls into a mass range (940 – 1000 Da) already highlighted as showing relative increase on Day 5 on the pseudo-gel of the acute wound metabolome (Figure 4.10).

Day 8 showed a distinct increase in feature m/z 719 along the wound edges and bed (Figure 4.13 C), as well as along the outer edges of where the wound was excised from the pig (Figure 4.13 C). This suggests a feature association with blood where the increased intensity observed in the wound may be due to the presence of a large scab as seen photographically and histologically (Figures 2.4 and 2.8 I & K respectively). Interestingly, clear intensity differences could be seen in the features of the wound tissue (wound bed and both edges) compared to the healthy tissue on Day 8 at this mass in the ROI mass spectral analysis (Figure 4.1). The metabolite that showed the largest association with wound tissue on Day 10, m/z 927, was increased in the centre (deepest point) of the wound bed penetrating into the newly deposited granulation tissue (Figure 4.13 D). This suggested that this feature has an association with the proliferative phase of healing and new tissue deposition.

A similar distribution pattern was seen on Day 12 with metabolite m/z 1803, which was increased in the newly deposited granulation tissue of the wound (Figure 4.13 E). This feature intensity tissue distribution implied a role in proliferation or tissue remodelling. The occurrence of the proliferative and remodelling healing phases at this time was confirmed histologically where new collagen deposition in the wound space was seen on Day 8 that progressed significantly into the start of remodelling by Day 12 (Figure 2.8). On Day 16, metabolite m/z 544 was increased in the limited areas remaining of the wound bed and edges (Figure 4.13 F). This suggested that this feature may be involved in active proliferation and remodelling processes, especially with that of re-epithelialisation.

This reversed approach of scanning metabolomic mass spectra for spatial association with the wound, revealed additional masses of interest as potential wound markers. Metabolite m/z 956 on Day 5 showed promise as a phase marker of inflammation. Metabolite m/z 1803 showed a distinctive association with newly deposited tissue in the wound space and may be a phase feature of proliferation and remodelling. Both of these metabolites would be interesting to study in non-healing chronic wounds since these wounds tend to remain associated with inflammation and do not progress to the proliferative and remodelling phases successfully. These metabolites are also potential features for the two healing stages that have previously been described as the most critical transitional phases for successful wound healing (Landén *et al.*, 2016). Both m/z 956 and 1803 were added to the list of metabolites showing the greatest potential as acute wound healing features.

Interestingly, the wound lipidome visualised the greatest relative intensity differences between wound and surrounding healthy tissue overall when compared to the wound proteome and metabolome (Figures 3.18 and 4.13). The wound lipidome showed relatively comparative degrees of quantitative change between wound and healthy tissue over all six selected time points (Figure 4.14).

On Day 3 lipid m/z 677 was most associated with the wound, where increased intensities were seen extending into the region beneath the wound bed (Figure 4.14 A). Interestingly, this lipid falls within the range of m/z 650 – 700 previously highlighted as showing wound-association in the ROI mass spectral analysis (Section 4.3.1, Figure 4.2). Lipid m/z 612 on Day 5 showed a distinct spatial

association with wound tissue, where relatively high amounts were seen in the wound bed, with little to none of this feature being found in the wound edges and surrounding healthy tissue (Figure 4.14 B). The restricted spatial distribution of this feature's increase in the wound bed suggests a possible association with the very early phases of proliferation, which theoretically may occur as early as 3 – 4 days after wound formation (Broughton *et al.*, 2006, Velnar *et al.*, 2009).

On Day 8 *m/z* 830 was found to be the lipid most associated with the wound with obvious increases in the wound edge spreading into immediate surrounding tissue (Figure 4.14 C). Feature association with the wound at this time point is suggestive of proliferative or possibly inflammatory involvement of *m/z* 830 in healing, based on histology (Figure 2.8 I – L).

When applying a mass error of $\pm 0.25\%$, lipid *m/z* 614, showing the greatest spatial association with the wound tissues of Days 10 and 16 (Figure 4.14 D and F), may be the same feature as *m/z* 612 of Day 5. This observation supports the idea of *m/z* 612 being associated with proliferation on Day 5. Lipid *m/z* 614 was found to be increased in the newly deposited granulation tissue of the wound on both Days 10 and 16. Interestingly, this lipid was highlighted as a feature of interest on the pseudo-gel of the acute wound lipidome (Figure 4.10 B) where increases in intensity were clearly seen on Days 5, 10 and 16 relative to healthy tissue. Lipid *m/z* 836 showed the largest intensity change between wound and healthy tissue on Day 12 and also showed a possible association with newly deposited granulation tissue that had clearly filled the wound space (Figure 4.14 E). Scanning lipidomic mass spectra for spatial association with wound tissue over 16 days revealed lipids *m/z* 614, 677, 830 and 836 as potential healing features.

Spectral assessment across the three 'omic' bases revealed both protein (Section 3.3.6) and lipid features previously highlighted in prior analyses of this study through spatial association with wound tissue but did not present as a confirmatory tool in the wound metabolome. This observation highlights the complexity of the acute wound metabolome and supports what was observed in the 2D peak distribution analysis (Section 4.3.5) where the wound metabolome shows multiple minor intensity changes rather than the more distinctive changes observed in the proteome and lipidome. PCA-based influence plots also visualised the wound metabolome to be

substantially less variable than its proteomic and lipidomic counterparts (Section 4.3.2.3). This suggests that the acute wound proteome and lipidome kinetics may offer more potential in revealing statistically significant wound features through visual spatial association than the wound metabolome.

4.3.7 PCA and peak statistics as treatment screening tools

As described in Chapter 1 (Section 1.1.12) the animal study was done in collaboration with another study utilising the same porcine model where in addition to the untreated, control wounds, three different wound treatments were assessed (see Figure 2.1). Due to the blinding of the treatments, these three wound treatments will be referred to as the 'green', 'red' and 'orange' treatments.

The ability of MALDI-IMS followed by PCA and peak statistics to assess large datasets has been shown in Chapters 3 and 4. PCA and peak statistics assays were applied as screening tools to a limited number of treated wounds to determine whether wound treatments demonstrated any measurable effects relative to untreated control wounds. These tests were performed across all three 'omic' bases on whole wound tissue collected on Day 5 and Day 8, as these time points were revealed as the peak of acute wound healing activity in the metabolome and lipidome, and proteome respectively. Whole wound tissue, comprising the wound bed and both edges, was selected as ROIs. The PCA results are shown below in Figure 4.15.

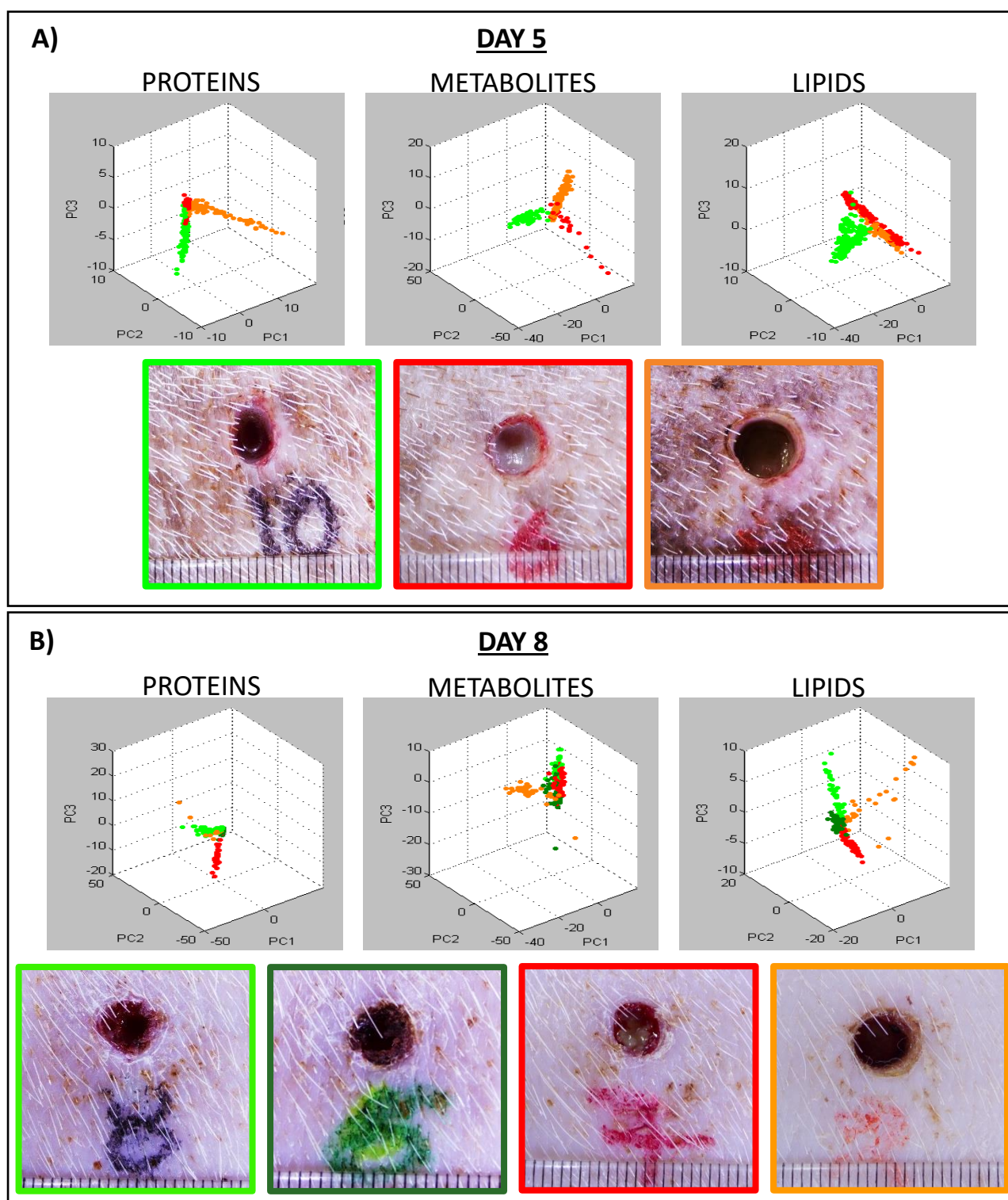


Figure 4.15: PCA as a screening tool for assessing multiple wound treatments. PCA was used as a screening tool to assess the physiological effects of treatments on acute wound healing relative to an untreated control wound on (A) Day 5 and (B) Day 8 across proteomic, metabolomic and lipidomic bases. Photographs of the control and treated wounds prior to IMS analysis are shown. The untreated control is shown in light green with remaining colours each representing an active wound treatment.

PCA was performed to determine its potential as a treatment screening tool, as well as to establish whether prominent visual wound differences translated into significantly different wound healing physiologies. On Day 5, the control and alternatively-treated wounds (red or orange treatments) all appeared different photographically. Significant differences between the control and treated wounds were visualised in the PCA plots across proteomic, metabolomic and lipidomic bases (Figure 4.15 A). The proteomic 3D PCA plot showed substantial differences between the control and orange treatment where features extended in independent directions with no significant correlation. The red treatment features could be differentiated from the control and orange treatment features, however showed considerably less variation. The wound metabolome PCA plot visualised the three tissue groups as almost completely separate entities where apart from sharing a common central point, each wound appeared to extend along opposing x, y and z axes. In the lipidomic PCA plot, the orange and red treatment features showed significant overlap where the untreated control wound appeared almost completely differentiated. The Day 5 metabolomic 3D PCA plot proved to be the most useful tool for the differentiation of the three alternatively treated wounds. While on a proteomic level the orange treatment could be differentiated from the control, and on a lipidomic level both treatments could be differentiated from the control, the metabolic plot was the only 'omic' base differentiating each wound sample.

The Day 8 3D PCA plots included an additional wound treatment group, shown in dark green, as this wound appeared visually different to the eye compared to the control wound (Figure 4.15 B). Across the proteome and metabolome, only a single treatment type in each PCA plot could clearly be differentiated, being the red and orange treatments respectively. The lipidomic PCA plot however, differentiated all four of the wound sample treatments and control. The control, red and orange treatments appeared to share a common central point, being the features of the dark green treatment, and then extended in highly differentiable and independent directions on the plot. Even though significantly less relative variation was seen in the dark green sample features, it could still be isolated from the remaining control and treatment features. The Day 8 wound lipidome served as the best tool to differentiate wound treatments on a 3D PCA plot.

PCA proved to be a resourceful and straight forward screening tool for the assessment of wound treatments and their relative abilities to incur significant change in acute wound healing physiology. PCA successfully highlighted the extent to which wound treatments affect the healing process as well as the potential correlation between visual wound appearance and actual significant molecular change. A single 'omic' base could not be confirmed as the most reliable option for such a screening tool (metabolome on Day 5 and lipidome on Day 8) and therefore a triple 'omic' analysis may need to be performed for each unique wound treatment analysis. PCA offers a relatively simple and timely statistical method to be used as an initial screening tool for the assessment of novel wound treatments, potentially saving funds, time and resources on further analyses such as histology, immunohistochemistry and LC-MS/MS.

The ability of peak statistics to highlight potential acute wound healing features and future drug targets through wound appearance was assessed. In the photographs of Figure 4.15 distinct differences between the different wound tissues can be seen. Briefly, on Day 5 both the red and orange treatments seemed to have a negative impact on healing compared to the control wound. While the control presented with constricted wound edges, both treatment wounds appeared larger in size, suggestive of hindered healing. In addition, the red treatment had caused the appearance of a white coloured sheath lining the wound, while the orange treatment had caused a yellow-like puss in the wound bed, indicative of an infection or cellular necrosis. On Day 8, the dark green treatment appeared to have aided wound healing progression relative to the control, while the red treatment contained puss in the wound bed and the orange treatment lagged in wound progression based on relative wound circumference and depth. Due to the fact that this was only an initial assessment of peak statistics as a potential screening tool for acute wound features, only the results of the top-two most differential PCA plots (Figure 4.15) will be shown *i.e.* the metabolome on Day 5 and the lipidome on Day 8. Two-dimensional peak distributions were created on the two metabolites or lipids from Days 5 and 8 respectively, showing the most significant PKW as determined by the peak statistic reports (Figure 4.16).

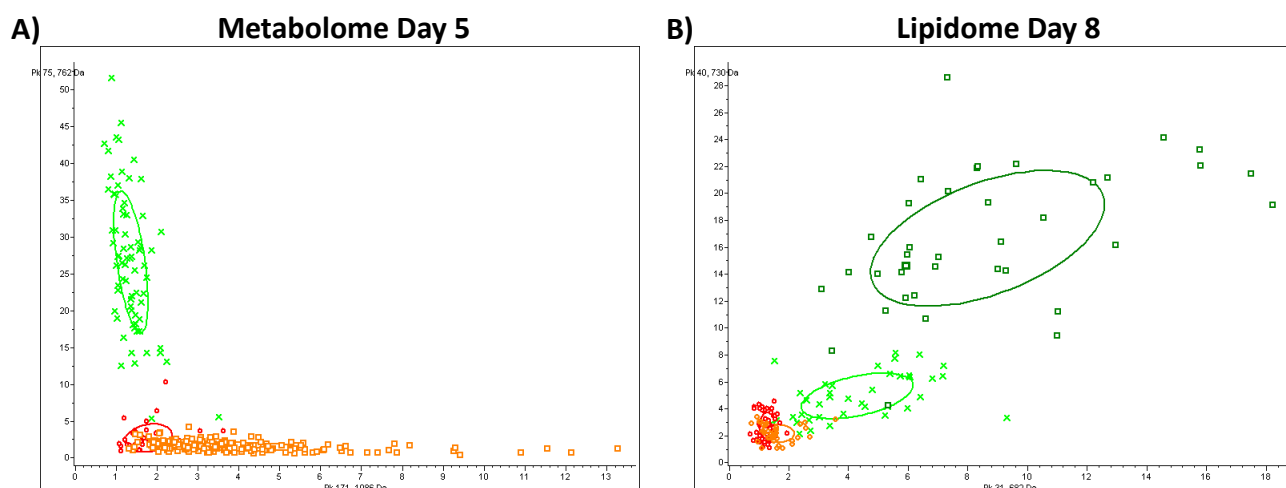


Figure 4.16: 2D peak distribution plots of potential metabolite and lipid healing features.

2D peak distributions of the two top-ranked features based on PKW value with (A) metabolites m/z 1086 vs 762 and (B) lipids m/z 682 vs 730. Control wound features are shown in light green with the various treatments in red, orange and dark green. Ellipses represent the standard deviation of the average of the feature intensities. Axes measurements are in arbitrary units chosen automatically to fit the plot optimally in the plane.

Peak statistics revealed metabolites m/z 1086 and 762, and lipids m/z 682 and 730 to be the top-ranking PKW features. The intensities of each feature in the wound tissue were plotted onto metabolomic and lipidomic 2D distribution plots for assessment. In the metabolomic Day 5 plot the control wound could clearly be differentiated from the treated wound samples (Figure 4.16 A). The plot revealed m/z 1086 (x-axis) to be highly variable and increased in the orange-treated wound, while metabolite m/z 762 (y-axis) was 7-fold higher in the control wound compared to both treated wounds. This suggests that feature m/z 1086 may be associated with the potential infection and/or necrosis seen in the orange treatment (Figure 4.15 A) and may be useful as a potential feature of impaired healing. Metabolite m/z 762 on the other hand shows potential as a feature of normal wound healing, where decreased levels were found in the healing-impaired treated wounds.

In the lipidomic Day 8 plot, the control and dark green treatment could clearly be differentiated from the orange and red treatments, which showed substantial feature overlap (Figure 4.16 B). Higher levels of both lipids m/z 682 and 730 were found to be associated with the dark green treatment relative to the control, while minimal

levels were seen in the orange and red treatment groups. Based on the wound photographs in Figure 4.16 B, these results suggest that both these lipids may be features of acute wound healing progression where the dark green-treated wound appeared to be healed to the greatest extent, closely followed by the control, with the red and orange treatments lagging behind in healing progression.

Once features of interest are revealed through initial screening of alternatively treated wounds using 2D peak distribution plots, further histological and mass spectrometric analyses could to be performed to confirm whether each treatment did in fact have a positive or negative influence on the healing process as suggested photographically.

Peak statistics combined with 2D peak distribution plots were shown to be useful, time efficient and relatively simple tools in revealing potential features of wound healing enhancement or disruption. While PCA highlighted the occurrence of any statistically significant changes between alternatively treated wounds, peak statistics and distribution plots revealed masses of features possibly responsible for these discernible changes. These features in turn can be targeted in further studies for their viability in a wound model setting or as future drug targets.

4.4 Summary

Through MALDI-IMS analysis the acute wound kinetic (time-based) changes were selectively analysed across three major molecular bases being the proteome (Chapter 3), metabolome and lipidome, where features within selected wound ROIs (wound bed and wound edges) were tracked independently and relative to non-wounded healthy tissue on Days 3, 5, 8, 10, 12 and 16 post-wounding. MALDI-IMS successfully monitored features over the 16-day acute wound healing across multiple 'omic' bases through as yet unidentified feature kinetic changes within concise combined single mass spectral images.

PCA was an indispensable tool in the analysis of large amounts of metabolomic and lipidomic data. Resultant PCA plots provided optimised reduced profiles of the acute wound metabolome and lipidome without requiring feature identification and could be further analysed for potential clinical applications. PCA revealed Day 5 to be the

height of metabolomic and lipidomic wound healing activity based on feature intensity differentiation relative to the control and healing Days 3, 8, 10, 12 and 16. This derived information could be supported by pseudo-gel analysis and through 2D peak distribution analysis.

In Chapter 3, the peak of proteomic acute wound healing activity was found to be on Day 8 however, Day 5 followed very closely. If a single time point were to be focussed upon for further 'omic' studies of the acute wound healing process in the porcine model, Day 5 would be the most significant healing time point across all three 'omic' bases, potentially offering the greatest wealth of information for modelling and feature discovery.

Non-healing chronic wounds are thought to be unable to progress successfully beyond the inflammatory phase of healing. Based on literature (Broughton *et al.*, 2006, Landén *et al.*, 2016) and confirmed by the histological analysis of this study (Section 2.3.4) Day 5 represents the most active state of inflammation in the wound tissue. This adds further interest and an increased dimension of applicability for using this healing time not only for further 'omic' modelling analysis of the acute wound environment but also for comparative chronic wound research.

Based on PCA calculations, loading plots, peak statistics and spatial wound association, nine averaged metabolite masses and eight averaged lipid masses were revealed that could potentially be used as unique features defining acute wound healing phases in the porcine model being metabolites with m/z 582, 772, 791, 884, 910, 956, 1803, 1940 and 1968; and lipids with m/z 611, 614, 619, 647, 671, 677, 687 and 886.

Repeated experimentation with several replicates from sequential histological slices would need to be performed to confirm these features as reliable and robust markers of acute wound healing. In future studies, identification of these metabolites and lipids (through application of MS/MS data collection directly from the tissue samples during imaging) would also contribute in defining their appropriateness as healing markers. Revealing markers of acute wound healing across three independent 'omic' bases offers great opportunity for application in various healing studies and developments for the discovery of new drug targets.

PCA and peak statistics combined with 2D peak distribution analysis were successfully demonstrated as initial screening tools for wound treatment evaluation. With minimal sample preparation, method development and cost, MALDI-IMS combined with PCA highlights statistically significant effects of healing treatments on wound physiology, relative to a control region. Peaks statistics with 2D peak distribution plots revealed the most likely features indicating healing effects, which hold potential as new healing targets. These statistical tools offer the ability to screen multiple treatments for significant effects saving time, resources and funding prior to investing in further experimentation such as microscopy, immunohistochemistry and LC-MS/MS. This initial screening approach holds promise for a faster, targeted and more efficient assessment of potential healing agents.

Chapter 5: Kinetic proteome assessment by LC-MS/MS analysis of acute wound tissue

5.1 Introduction

Several limitations were identified in the initial proteomic data collected using the MALDI-IMS assays, where the two most notable limitations were the low mass range that can be measured during imaging and secondly that peptide fragmentation could not be performed while imaging, precluding peptide sequence data collection required for protein identification. As the relative quantitative changes of specific proteins is important in determining which biological pathways may be activated or shut down during wound healing, specific protein levels need to be assessed. To be able to perform a comparison with the data already collected, the same sample types used during the MALDI-IMS assays were analysed using classic ESI-based LC-MS/MS proteomic techniques. Sequential histological sections from the same wound samples assayed by MALDI-IMS were initially treated differently to optimise the sample preparation steps and to use semi-automated sample preparation methods that can reduce technical variability in the data from sample collection through to analysis of identified proteins. The continuous change in wound size over time and different volume of surrounding healthy tissue per biopsy sample makes absolute quantitation for comparative purposes futile.

Extracellular matrix (ECM) proteins are dominant in the skin, where proteins such as collagen and fibronectin are expected to be present in wound tissue and in the surrounding healthy tissue. Due to this consistent abundance, no potential is offered in using these proteins as proteomic features for the progression of acute wound healing. Furthermore, ECM proteins will dominate label-free relative quantitation obscuring the detection and identification of less abundant proteins. For this reason, protein extraction techniques from the tissue sections attempted to exclude the dominant, low solubility ECM proteins. This chapter discusses the various sample preparation and processing techniques used for optimal proteomic profiling of wound tissue for healing phase proteomic feature analysis by ESI-LC-MS/MS.

Although all samples are histological sections from snap frozen wound tissue created using a cryotome, there are variable textures and cell densities, and areas of

both healthy and repairing tissue that, due to the homogenising effects of the tissue preparation, cannot be divided into regions of interest as performed with the MALDI-IMS data collected. Several sample processing adaptations were tested and compared to the in-gel digestion technique which has been routinely used in the laboratory.

5.2 Materials and Methods

5.2.1 In-solution analysis

5.2.1.1 Tissue protein extraction

Reagents

i. Phosphate buffered saline (PBS)

FTA Hemagglutination buffer was purchased from BD Biosciences (Sparks, USA). A solution was made up at 9.23 g/l as per product instructions, with deionised water. The solution was stored at 4°C until used.

ii. Extraction buffer

The extraction buffer solution contained 4% SDS and 50 mM Tris-HCl (pH 7.6) (both from Sigma-Aldrich, St Louis, USA), in deionised water containing a protease inhibitor cocktail used for the inhibition of serine, cysteine and metalloproteases in mammalian extracts (cOmplete Protease Inhibitor Cocktail Tablets, Roche, Basel, Switzerland). The protease inhibitor solution was made fresh according to manufacturer instructions (1 tablet per 50 ml solution) for use in the extraction buffer. The final buffer solution was stored at 4 °C and used within 1 week.

iii. Delipidation solution

The delipidation solution contained chloroform and methanol (both from Merck, Darmstadt, Germany) in a 2:1 ratio, based on the methods of Folch *et al.* (Folch *et al.*, 1957).

Methods

Fresh, frozen wound tissue was sectioned as described for MALDI-IMS in Chapter 3 (Section 3.2.1) except that 60 μm thick sections were sectioned and collected into Eppendorf Protein LoBind Tubes (Hamburg, Germany) that were stored at -80°C until just prior to extraction. Seven different protein Extraction Methods were then performed in duplicate using individual tissue sections as described below.

Extraction Method 1:

A volume of 150 μl of the 4% SDS-containing extraction buffer was added to each tissue slice, followed by homogenisation using a 3000 W ultrasonic homogeniser with a 2 mm titanium tip (BioLogics, Cary, NC, USA). Tissue was homogenised at 30% power until a uniform suspension was acquired, where tubes were placed on ice after every 10 ultrasonic pulses (30 pulses total) to prevent sample heating and protein degradation. Tubes were then centrifuged at $16\,000 \times g$ for 10 minutes and the resultant supernatant collected into a new tube and any remaining insoluble matter discarded.

Extraction Method 2:

Prior to extraction, tissue sections were first washed with cold PBS. Sample tubes were filled with the PBS, sonicated in a 120 W ultrasonic water bath (Branson Ultrasonics, Danbury, USA) at ambient temperature (22°C) for 5 minutes, vortex mixed for 1 minute and centrifuged at $16\,000 \times g$ for 10 minutes. The resulting supernatant was discarded, with the remaining tissue section now following the protocol of Extraction Method 1 as described above.

Extraction Method 3:

This Extraction Method consisted of removing excess lipid from tissue slices prior to protein extraction. To achieve this, 400 μl of delipidation solution was added to each tissue slice after which tubes were placed on a shaker for 20 minutes at room temperature, sonicated for 5 minutes and vortex mixed for 1 minute. Samples were then centrifuged for 10 minutes at $16\,000 \times g$ and all the supernatant carefully removed and discarded. The tubes were left open to dry for an hour in a laminar flow

cabinet before the delipidated tissue sections were treated according to Extraction Method 1 as described above.

Extraction Methods 4 and 5:

These two methods consisted of a combination of Extraction Methods 2 and 3 that included the combination of tissue sample washing and delipidation prior to the commencement of protein extraction (as described in Extraction Method 1). Extraction Method 4 consisted of tissue delipidation followed by washing with PBS, whereas Extraction Method 5 reversed the order of the processes and consisted of first washing and then delipidating samples. Delipidation and washing procedures followed the same methods as described respectively above.

Extraction Methods 6 and 7:

Extraction Methods 6 and 7 were performed to assess the effects of varying degrees of heat on tissue protein extraction efficiency. For these methods tissue sections were prepared according to Extraction Method 1 as described above. The resultant extracted sample volumes were then split equally into two tubes, whereby one tube was submerged in a pre-heated water bath at 50°C and the other at 90°C for 5 minutes. Samples were then immediately placed on ice for cooling.

Following the extraction procedures, centrifugation-clarified extracts were assayed using the BCA protein assay to determine protein concentration (as described below in Section 5.2.1.2). Samples were then aliquoted for SDS PAGE or HILIC in-solution digestion and subsequent LC-MS/MS analysis. Laemmli sample buffer (2x) was added to the designated SDS PAGE samples and boiled at 100°C for 10 minutes (as described in Section 5.2.2.1) after which all samples were stored at -80°C until use.

5.2.1.2 Bicinchoninic acid (BCA) protein assay

Reagents

i. Reagent A

A volume of 100 ml solution in deionised water was made up by dissolving 1 g bicinchoninic acid (BCA) disodium salt hydrate purchased from Sigma-Aldrich

(St Louis, USA), 5.4 g sodium carbonate decahydrate ($\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$) purchased from UniLab (Krugersdorp, RSA), 0.21 g disodium tartrate dihydrate ($\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O}$), 0.40 g sodium hydroxide (NaOH) both purchased from Merck (Darmstadt, Germany) and 0.95 g sodium bicarbonate (NaHCO_3) purchased from Sigma-Aldrich (St Louis, USA). The pH of the solution was adjusted to 11.25 with 10 M NaOH. Reagent A was stored at 4°C until use.

ii. **Reagent B**

An amount of 0.26 g cupric sulfate (CuSO_4) purchased from Rochelle Chemicals (Johannesburg, RSA) was dissolved and made up to 10 ml with deionised water. Reagent B was stored at 4°C until used.

iii. **Phosphate buffered saline (PBS)**

FTA Hemagglutination buffer was purchased from BD Biosciences (Sparks, USA). A solution was made up at 9.23 g/l as per product instructions, with deionised water. The solution was stored at 4°C until used.

iv. **Protein standards**

Bovine serum albumin (BSA) was purchased from Santa Cruz Biotechnology, Inc, (Dallas, USA). A 2.5 mg/ml (37.6 μM) BSA stock solution was prepared by adding 7.5 mg of BSA into 3.0 ml of PBS and dissolving by rotation on a mixing wheel. A range of BSA protein standards of varying concentrations: 2.5 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml, 0.4 mg/ml, 0.25 mg/ml, 0.2 mg/ml and 0.1 mg/ml were prepared individually from the 2.5 mg/ml BSA stock solution to a volume of 1 ml using PBS. A quality control BSA protein standard was also prepared in PBS independently of the stock solution at 1.25 mg/ml. Protein standards were made up fresh.

Methods

The bicinchoninic acid (BCA) protein assay as first described by Smith *et al.* (1985) was used to determine the protein concentrations of the wound tissue extractions prepared by each of the methods described above. Briefly, a standard working reagent (SWR) was made by mixing 50 volumes of reagent A with 1 volume of reagent B. Using a round-bottomed 96-well plate, 250 μl of SWR and 5 μl of each BSA protein standard (or quality control) or extraction sample were added to triplicate wells on the plate. The plate was shaken gently for 10 minutes and was

then incubated at 60°C for 30 minutes. The samples were cooled to room temperature and the absorbance, at a single wavelength of 562 nm, of the wells were measured using an ELx800 UV universal microplate reader (Bio-Tek Instruments Inc., Vermont, USA).

Using GraphPad Prism Version 5.00 (GraphPad Software, San Diego, USA) the absorbance values of the BSA protein standards were used to plot a calibration curve of absorbance (A_{562}) versus protein concentration (mg/ml). The protein concentrations of the tissue extract solutions were determined from the linear regression of the BSA standard curve (mg/ml).

5.2.1.3 HILIC in-solution digestion and clean-up

Reagents

i. **DTT solution**

A 1 M DTT solution was made up by adding 154 mg DTT to 1 ml MS-grade water. This solution was freshly prepared just before use.

ii. **IAA solution**

A 1M IAA solution was made by adding 18.5 mg IAA to 100 μ l of 50 mM ammonium bicarbonate. This solution was freshly prepared just before use and was stored in a dark container.

iii. **SDS solution**

A 1% SDS solution was made by adding 25 μ l of a 40% SDS stock solution to 975 μ l MS-grade water.

iv. **Binding buffer**

Binding buffer consisted of 200 mM ammonium acetate in a 30% MS-grade acetonitrile solution.

v. **Equilibration buffer I**

Equilibration buffer consisted of 100 mM ammonium acetate in a 15% MS-grade acetonitrile solution and was made by diluting the binding buffer 1:1 with MS-grade water.

vi. **Equilibration buffer II**

The second equilibration buffer consisted of 20 mM ammonium formate (pH 2.5) in 90% acetonitrile which was prepared from 400 mM ammonium formate stock solution diluted with acetonitrile and MS-grade water in a ratio of 5:90:5.

vii. **Wash buffer**

Wash buffer consisted of 95% acetonitrile prepared from acetonitrile stock diluted with MS-grade water.

viii. **Trypsin digest working solution**

Sequencing Grade Modified Trypsin was purchased from Promega (Madison, USA). Lyophilised trypsin was dissolved in a buffer of 40 mM ammonium bicarbonate/acetonitrile (9:1). Aliquots (20 µl) of 0.1 µg/µl trypsin solution were prepared and stored at -20°C until use.

Methods

A semi-automated protocol for in-solution protein clean-up and trypsin digestion as preparation for nano-LC-MS/MS analysis was performed using MagReSyn HILIC (hydrophilic interaction liquid chromatography) Beads. These beads selectively bind protein allowing effective sample clean-up for the removal of common contaminants or additives that may interfere with sample solubilisation and denaturation, prior to protein digestion. By using automated clean-up and digestion techniques, sample handling and ultimately experimental variability is reduced, resulting in more reliable and robust MS results.

All chemicals and reagents used were of MS-grade from reputable chemical companies.

All tissue extract samples were aliquoted into 100 µl volumes (using Eppendorf Protein LoBind Tubes) prior to the commencement of the protocol. All experimentation was performed in duplicate.

Before sample clean-up and digestion, alkylation and reduction of proteins was performed. To do this DTT was added to each sample extract to a final concentration of 10 mM and incubated at 23°C (using a heat block) for 30 minutes. This reducing step was followed by alkylation where IAA was added to each sample to a final concentration of 40 mM, which was again placed into the heating block for 45

minutes (foil was placed over the heating block to ensure light exclusion as this reagent is light sensitive). Each well per row of an Eppendorf 96-well Protein LoBind Deepwell Plate (Hamburg, Germany) was prepared for the automated in-solution clean-up and digestion protocol as follows:

Row A: 137.5 µl of 50 mM ammonium bicarbonate and 12.5 µl of 0.1 µg/µl trypsin solution.

Row B: Left empty.

Row C: 200 µl 95% acetonitrile (wash 2).

Row D: 200 µl 95% acetonitrile (wash 1).

Row E: 50 µl binding buffer and 50 µl sample (25 µg protein sample made up to volume, if required, with MS-grade water).

Row F: 500 µl of equilibration buffer I (to equilibrate beads).

Row G: 25 µl MagReSyn HILIC bead suspension (20 mg/ml) topped up to a total volume of 175 µl with Equilibration buffer I (to dilute storage buffer of beads).

Automated sample clean-up and digestion of the prepared sample plate was performed on a Thermo Scientific KingFisher™ Duo magnetic handling station (Massachusetts, USA) using a pre-programmed protocol processing all 12 sample wells in parallel. The system utilises a 12-pin robotic magnet head with a disposable plastic comb to prevent sample cross-contamination during binding, mixing and transfer steps between wells. The comb was initially placed in Row H moving sequentially from Row H through to Row A. Washed HILIC beads were transferred from Row G to Row F for equilibration (one minute) after which they were transferred to Row E for protein binding (30 minutes) where protein samples were already pre-mixed with binding buffer. Contaminants were removed with two successive washes in Rows D and C (one minute each), after which protein digestion proceeded for four hours at 37°C in Row A using a KingFisher™ Duo Peltier heating block. HILIC microparticles were then removed from the digested protein sample mixture back to Row G for storage (Figure 5.1). Sample digests were manually transferred from Row A to new Eppendorf Protein LoBind Tubes and vacuum dried using a Labconco CentriVap (Missouri, USA) at room temperature. Dried samples were stored at -80 °C until peptide clean-up.

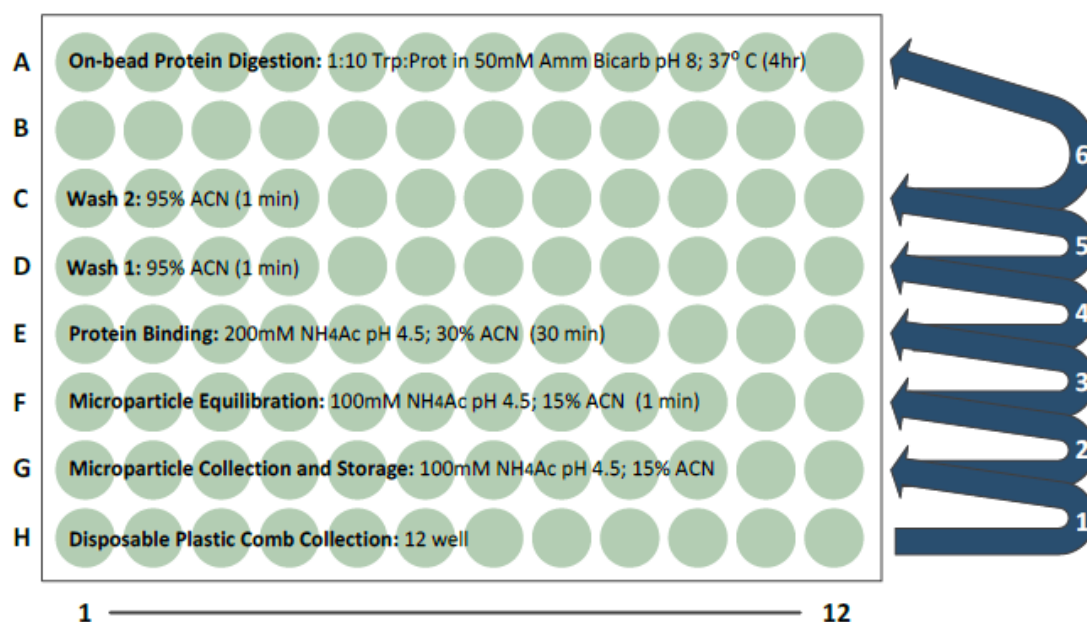


Figure 5.1: Plate setup for HILIC in-solution protein sample clean-up and digestion. Semi-automated in-solution protein clean-up and trypsin digestion was performed using MagReSyn HILIC Beads and a KingFisher™ Duo magnetic handling station (Stoychev *et al.*, 2012) (with permission).

Due to the nature and complexity of biological tissue samples, an additional HILIC clean-up of sample peptides post-protein digestion and before LC-MS/MS analysis was done. Briefly, the method was performed as described below.

A volume of 10 μ l of MagReSyn HILIC beads (20 mg/ml suspension) per sample were prepared for clean-up. This was done using a magnetic separator and removing the shipping solution by pipette aspiration. The microparticles were then washed and equilibrated with 500 μ l equilibration buffer II, with gentle agitation for 30 seconds. Using the magnetic separator, the equilibration buffer was removed and discarded. This wash step was repeated once more. Dried samples were resuspended and vortex mixed in 50 μ l of equilibration buffer II, and then added to the pre-equilibrated beads. Peptides were allowed to bind to the beads for 30 minutes while mixing gently on a shaker to ensure sufficient sample-bead interaction during the binding procedure. Tubes were placed on the magnetic separator allowing microparticles to clear, and the supernatants were removed and discarded by pipette aspiration. The beads were resuspended in 200 μ l of a wash buffer (95% acetonitrile) and mixed gently on a shaker for 1 minute, after which the beads were recovered on the magnetic separator and the supernatants removed and discarded.

Peptides were eluted with 20 µl of elution buffer (50 mM ammonium bicarbonate pH 8) for 15 minutes gently agitated on a shaker. Beads were recovered on the magnetic separator and the supernatants containing the peptides were transferred to new Eppendorf Protein LoBind Tubes. This elution process was repeated once more for maximum peptide yield. A solution of acetonitrile and formic acid to final concentrations of 2% and 0.2% respectively was added to the peptide samples. The tubes were centrifuged at 14 000 x g for 10 minutes (done in case of any bead transfer), after which the supernatants were transferred to new Eppendorf Protein LoBind Tubes and stored at -80°C until LC-MS/MS analysis.

5.2.1.4 Nano LC-MS/MS analysis

Reagents

i. Acetonitrile/formic acid solutions

The acetonitrile, formic acid and water were all of MS-grade and were purchased from Fluka Analytical (Basel, Switzerland).

Methods

Approximately 1 µg of tryptic digest peptides per sample were analysed using a Dionex Ultimate 3000 RSLC system coupled to a SCIEX 6600 TripleTOF mass spectrometer. Injected peptides were inline de-salted using an Acclaim PepMap C18 trapping column (75 µm × 2 cm) for 2 minutes at 5 µl/minute using 2% acetonitrile/0.2% formic acid. Trapped peptides were gradient eluted and separated on an Acclaim PepMap C18 RSLC column (75 µm × 15 cm, 2 µm particle size) at a flow rate of 0.5 µl/minute with a linear gradient of 4 - 50% B over 60 minutes (A: 0.1% formic acid; B: 80% acetonitrile/0.1% formic acid). A final wash step was performed for 10 minutes with 99% B, followed by a 10-minute re-equilibration after returning to starting conditions. The 6600 TripleTOF mass spectrometer was operated in positive ion mode. For data-dependent acquisition (DDA) runs, precursor (MS) scans were acquired from *m/z* 400-1500 (2⁺ - 5⁺ charge states) using an accumulation time of 250 milliseconds (ms) followed by 80 fragment ion (MS/MS) scans, acquired from *m/z* 100-1800 with 25 ms accumulation time each. The raw *.wiff files generated by the mass spectrometer were processed using bioinformatics software tools (Section 5.2.4).

5.2.2 In-gel analysis

5.2.2.1 Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE)

Reagents

All reagents were of analytical grade or better purchased from reputable chemical companies and handled with gloved hands and clean equipment to avoid any protein contamination.

i. 10 x Running buffer

An amount of 5 g SDS, 15 g tris(hydroxymethyl)aminomethane and 72 g glycine were added to a volumetric flask with 400 ml deionised water. The flask was sonicated for 5 minutes to produce a homogenous stock solution. Finally, the volume was adjusted to the 500 ml mark and mixed well.

ii. 2 x Laemmli sample buffer

A 2 x Laemmli sample buffer stock solution (0.125 M Tris-HCl (pH 6.8) 4% SDS, 20% glycerol and 0.004% bromophenol blue) was purchased from Bio-Rad Laboratories, Inc. (Berkeley, USA). A volume of 50 µl of the reducing agent β-Mercaptoethanol per 950 µl sample buffer was added just prior to use.

iii. Protein mass standards

Precision Plus Protein Standards Unstained were purchased from Bio-Rad Laboratories, Inc. (Berkeley, USA) covering a reference mass range from 10 kilodaltons (kDa) to 250 kDa.

iv. Precast gels

For all SDS PAGE Mini-PROTEAN TGX Stain-Free™ Gels of 4-20% linear acrylamide gradient, 10x, 50 µl well precast gels purchased from Bio-Rad Laboratories, Inc. (Berkeley, USA) were used.

v. Fixing solution

The fixing solution contained 50% methanol, 10% glacial acetic acid and 40% deionised water.

vi. **Coomassie Blue**

Aqua Colloidal Coomassie Blue stain was purchased from Vacutec (Johannesburg, RSA).

Methods

To prepare tissue extraction samples for SDS PAGE, samples prepared as described in Section 5.2.1.1 were diluted 1:1 with Laemmli sample buffer and heated for 10 minutes at 100°C in a water bath. Once removed from the heat and cooled to room temperature, samples were centrifuged for 5 minutes at 16 000 x *g* to remove any insoluble products. Samples were stored at -80°C until the following day.

Samples were removed from the -80°C freezer and thawed at room temperature. A total of 5, 10 or 15 µg of protein from each of the prepared tissue extracted samples was loaded onto the gels. SDS PAGE was performed using a Mini-PROTEAN Tetra cell (Bio-Rad Laboratories, Inc., Berkeley, USA) vertical electrophoresis tank. The gel was run at 60 volts (V) for the first 15 minutes and then increased to 200 V for the remainder of the run. Upon completion gels were scanned using the Stain-Free Sample Tray in the Gel Doc EZ Imager from Bio-Rad Laboratories then placed in fixing solution overnight, rinsed and stained with Aqua Colloidal Coomassie Blue overnight and briefly soaked in distilled water to improve band colour. Gels were again visualised using a Gel Doc EZ Imager from Bio-Rad Laboratories and imaged for mass distribution analysis of the protein bands.

5.2.2.2 In-gel digestion of protein bands

Reagents

i. **Ammonium bicarbonate (NH₄HCO₃) solution**

A 50 mM solution was made by dissolving 200 mg of NH₄HCO₃ in 50 ml of deionised water. The solution was stored at 4°C and was used within 2 weeks.

ii. **NH₄HCO₃/50% methanol solution**

A 50 mM NH₄HCO₃ solution was made by dissolving 200 mg of NH₄HCO₃ in 25 ml of deionised water and 25 ml of 100% mass spectral grade methanol. The solution was stored at 4°C and was used within 2 weeks.

iii. **NH₄HCO₃/50% acetonitrile solution**

A 25 mM NH₄HCO₃ solution was made by dissolving 1 ml of the 50 mM aqueous NH₄HCO₃ solution as described above (i) in 1 ml of 100% mass spectral grade acetonitrile. The solution was sealed, stored at 4°C and was made up fresh.

iv. **Acetonitrile solution**

A 75% acetonitrile solution was made by adding deionised water to 7.5 ml of 100% acetonitrile to exactly 10 ml. The solution was sealed to avoid evaporation, stored at 4°C and was used within 2 weeks.

v. **Trypsin digest working solution**

Prepared as described in Section 5.2.1.3 (viii).

vi. **Dithiothreitol (DTT) stock solution**

A 1 M DTT stock solution was made by dissolving 154 mg of DTT in 1 ml of deionised water. This solution was freshly prepared just before use.

vii. **DTT/ NH₄HCO₃ solution**

Ten microlitres of the 1 M DTT stock solution described above (vi) was added to a solution which contained 495 µl of the 50 mM NH₄HCO₃ solution as described in (i) and 495 µl of deionised water. This resulted in a 10 mM DTT/25 mM NH₄HCO₃ solution. This solution was freshly prepared just before use.

viii. **Iodoacetamide (IAA)/NH₄HCO₃ solution**

An amount of 10.2 mg of IAA was dissolved in a solution made up of 500 µl of 50 mM NH₄HCO₃ solution as described in (i) and 500 µl of deionised water. This resulted in a 55 mM IAA/25 mM NH₄HCO₃ solution. This solution was freshly prepared just before use and was stored in a dark container.

Methods

In-gel trypsinisation of protein bands was based on the methods of Shevchenko *et al.* (2007).

Gel manipulations were carried out with all necessary precaution so as to avoid contamination with extraneous protein (sterilised glass and plasticware, gloved hands, no woollen clothing, hair covered). Following Coomassie-staining and scanning the SDS PAGE gel, prominent protein bands were carefully cut from the gel using a new sterile disposable scalpel blade per band, the removed gel section

diced into small pieces (approximately 1 mm x 1 mm) and placed in a 1.5 ml Eppendorf Protein LoBind Tube. Approximately 200 µl (or enough to cover the gel pieces) of 50 mM NH_4HCO_3 /50% methanol was added to each tube, which was then vortex mixed for 20 minutes to destain the protein. The resulting supernatant was discarded and the wash step was repeated until the bands showed no blue colour. After discarding the supernatant approximately 100 µl (or enough to cover the gel pieces) of 75% acetonitrile was added to each tube, which was again vortex mixed for 20 minutes. The supernatant was once again removed and discarded. The gel pieces were then dried using a Labconco CentriVap (Missouri, USA) at 37°C for 20 minutes.

Approximately 25 µl (or enough to cover the gel pieces) of freshly prepared 10 mM DTT in 25 mM NH_4HCO_3 was added to the dry gel pieces. Each tube was vortex mixed and briefly centrifuged (75 x *g*, 5 minutes) to collect the gel at the bottom of the tube. To allow the reduction reaction to proceed, the tubes were incubated in a water bath at 60°C for 1 hour, after which the tubes were cooled to room temperature (22-23°C). Following the addition of 500 µl acetonitrile, the tubes were incubated for 10 minutes at room temperature. The supernatant was then removed, discarded and approximately 25 µl (or enough to cover the gel pieces) of 55 mM IAA in 25 mM NH_4HCO_3 was added to the tubes. Each tube was vortex mixed and centrifuged briefly, and the alkylation reaction was allowed to proceed in the dark for 20 minutes at room temperature. After the incubation period the supernatant was removed, discarded and the gel pieces were washed with 100 µl of 25 mM NH_4HCO_3 in deionised water. The tubes were vortex mixed for 10 minutes and centrifuged for 5 minutes at 75 x *g*, after which the resulting supernatant was removed and discarded. The gel pieces were then washed with the addition of approximately 100 µl (or enough to cover the gel pieces) of 25 mM NH_4HCO_3 in 50% acetonitrile, the tubes vortex mixed for 5 minutes, centrifuged for 5 minutes at 75 x *g* and the supernatant removed and discarded. The wash step was repeated once again, after which the gel pieces were dried completely at 37°C for 20 minutes in the CentriVap.

The Eppendorf Protein LoBind Tubes were placed on ice, and each gel plug was covered with trypsin solution (approximately 10-50 µl). After 30 minutes it was checked whether all the trypsin solution had been absorbed and more solution was added if necessary, as gel pieces needed to be covered completely with trypsin

solution. The gel pieces were rehydrated on ice for another hour and were then centrifuged briefly (1 minute at 75 x *g*). A 25 mM NH₄HCO₃ solution in deionised water was then added as needed to cover the gel pieces and keep them wet during enzyme cleavage. The tubes were spun briefly to collect the contents at the bottom of the tube and were incubated at 37°C in a water bath for 16-18 hours.

The following morning the tubes were centrifuged briefly (1 minute at 75 x *g*) and the digest solutions (the aqueous extraction) were transferred into a clean marked Eppendorf Protein LoBind Tubes. Enough of a 50% acetonitrile/5% formic acid solution to cover the gel pieces (±50 µl) was added to the remaining digested gel pieces. These tubes were then vortex mixed for 25 minutes, sonicated for 5 minutes and briefly centrifuged. The new extract (the supernatant) was added to the first extraction. The extraction process was repeated once more to allow for maximum peptide extraction from the gel pieces. The gel pieces were then discarded and the combined extracts completely dried using the CentriVap for approximately 3 hours at room temperature. The dried digests were stored at -20°C until LC-MS/MS was performed.

5.2.2.3 Nano LC-MS/MS

To prepare in-gel digests for analysis, dried samples were resuspended in 40 µl of a 2% acetonitrile/0.2% formic acid solution and were vortex mixed for 1 minute. The tubes were sonicated on ice for 5 minutes and centrifuged at 125 x *g* for 10 minutes. Individual samples of 35 µl were transferred to 0.5 ml Eppendorf Protein LoBind Tubes for mass spectrometric analysis. LC-MS/MS was performed as described in Section 5.2.1.4.

5.2.3 On-tissue analysis

Reagents

i. Trypsin digest working solution

Prepared as described in Section 5.2.1.3 (viii).

All reagents used in the analyses were of mass spectrometry grade and purchased from Sigma-Aldrich (St Louis, USA).

5.2.3.1 On-tissue digestion and extraction

Methods

For on-tissue digestion and peptide extraction, wounds were sectioned as described in Chapter 3 (Section 3.2.1). Sections were thaw-mounted onto pre-cooled microscope slides and stored at -80°C until analysis.

Tissue sections were removed from -80°C and desiccated under vacuum for 30 minutes before the slides were washed with 70% ethanol and fixed with 95% ethanol for 2 minutes each. A water wash at 40°C was done to remove any potential residual embedding (OCT) medium followed by a repeat of the ethanol wash steps. Slides were briefly air dried and desiccated for one hour prior to deposition of an evenly sprayed layer of 0.1 µg/ul trypsin using an ImagePrep Station (Bruker Daltonics, Bremen, Germany) where a pre-installed standard spraying protocol was followed. Following trypsin deposition, the slides were placed into a high humidity airtight container and incubated at 37°C for 90 minutes. Peptides were then extracted (twice) with 10 µl of 10% acetonitrile and 10 µl of 30% acetonitrile with 0.1% trifluoroacetic acid respectively. This was achieved by pipetting the first acetonitrile solution onto an area of the tissue section, which was then aspirated and dispensed in quick succession four times (after each aspiration the solution was dispensed onto an adjacent area of the tissue and aspirated again, covering the whole tissue section methodically). The process was repeated with the acetonitrile/trifluoroacetic acid solution. These steps were performed as quickly as possible in order to prevent evaporation. The combined peptide extracts were transferred into Eppendorf Protein LoBind Tubes, dried in a Labconco CentriVap (Missouri, USA) and resuspended in 10 µl of 0.1% trifluoroacetic acid. Samples were then stored at -80 °C until LC-MS/MS analysis.

5.2.3.2 Nano LC-MS/MS of on-tissue digestion samples

Methods

Approximately 1 µg of tryptic peptides per sample were analysed using a Dionex Ultimate 3000 RSLC system coupled to a Thermo Scientific Orbitrap Fusion Mass Spectrometer equipped with a Nanospray Flex ionisation source. Samples were loaded from a temperature controlled autosampler set at 7°C onto the C18 trap

column (2 cm x 100 μ m) using loading solvent (2% acetonitrile/0.1% formic acid) at a flow rate of 15 μ l/minute for 5 minutes, before being eluted onto a Luna C18 analytical column (35 cm x 75 μ m, 5 μ m particle size) (Phenomenex, California, USA). Peptides were separated at 50°C with a flow rate of 350 nl/minute using mobile phases A (2% acetonitrile/ 0.1% formic acid) and B (100% acetonitrile). A non-linear gradient was used for separation as per Table 5.1 under the control of Chromeleon Chromatography Data System 6.0 software (Thermo Scientific, Massachusetts, USA). Column eluent was delivered directly to the mass spectrometer through a stainless steel nano-bore emitter.

Table 5.1: Mobile phase gradient settings

Time (minutes)	Flow rate (μl/minute)	Mobile phase A (%)	Mobile phase B (%)
0	0.35	95	5
60	0.35	70	30
70	0.35	50	50
80	0.35	20	80
85	0.35	95	5
110	0.35	95	5

The mass spectrometer was operated in positive mode with a spray voltage of 2 kV and ion transfer capillary set to 275°C. Spectra were internally calibrated using polysiloxane ions at m/z = 371.10024 and 445.12003. MS1 scans were performed using the Orbitrap detector (resolution of 120 000) over the scan range m/z 350 – 1650 with the automatic gain control (AGC) target set at 3e5 and a maximum ion injection time of 40 ms. Data was acquired in profile mode.

MS² acquisitions were performed using monoisotopic precursor selection for ions with 2⁺ - 6⁺ charge states with an error tolerance of $\pm$ 0.02 Da. Precursor ions were excluded from fragmentation once for a period of 30 seconds and were then selected for fragmentation in higher-energy collisional dissociation (HCD) mode using the quadrupole mass analyser with 32.5% HCD energy. Fragment ions were detected in the orbitrap mass analyser (resolution of 15 000). The AGC target was set to 1e4 and the maximum injection time to 45 ms. Data was acquired in centroid mode. The raw files generated by the mass spectrometer were processed using bioinformatics tools.

5.2.4 Data processing

Raw data files generated from both the SCIEX 6600 TripleTOF (.WIFF) and Thermo Scientific Orbitrap Fusion (.RAW) mass spectrometers were converted to mascot generic format (mgf) for peak list generation using MSConvert from ProteoWizard (Kessner *et al.*, 2008). Conversion was performed using a peak picking filter and a parameter threshold count of 150 of the most-intense peaks, in order to reduce the amount of data for interpretation. To match peptides to spectra, mgf files were searched against a reviewed UniProtKB/Swiss-Prot *Sus scrofa* canonical database (accessed: 13 May 2017 with 1419 sequences) including the sequences of common contaminating proteins, using the search engines X!Tandem, MS-GF+ and Comet using SearchGUI software (Version 3.2.20) (Vaudel *et al.*, 2011). From the resultant peptide to spectrum matches (PSMs), peptide and proteins were identified using PeptideShaker (Version 1.16.21) (Vaudel *et al.*, 2015). PSMs, peptides and proteins were validated at a 1.0% False Discovery Rate (FDR) estimated using the decoy hit distribution. Decoy sequences were created by reversing the target sequences in SearchGUI. The main identification settings used are shown in Table 5.2. PeptideShaker protein identification results were exported to Microsoft Excel and filtered. Only proteins identified with 100% confidence and at least 2 unique peptides were used (unless otherwise stated). Only filtered protein lists were used for further analyses of results.

Table 5.2: Summary protein identification settings for SearchGUI and PeptideShaker

Precursor (MS1) m/z tolerance	100.0 ppm
Fragment (MS2) m/z tolerance	0.1 Da
Precursor charge	2+ - 5+ (TOF)/2+ - 6+ (Orbitrap)
Precursor mass min. - max.	600 - 5000 Da
Min. fragment m/z mass	200
False discovery rate (FDR)	0.01
Max. missed cleavages	2
Enzyme	Trypsin
Fixed modifications	Carbamidomethylation (C)
Variable modifications	Oxidation (M)
	Sodium adduct (D, E)
	Deamidation (N, Q)
Search engines	X!Tandem
	MS-GF+
	Comet
Reference protein database	UniProtKB/Swiss-Prot <i>Sus scrofa</i>
MS/MS detector	TOF/Orbitrap
Fragmentation method	CID/HCD

5.2.5 Overview of methods

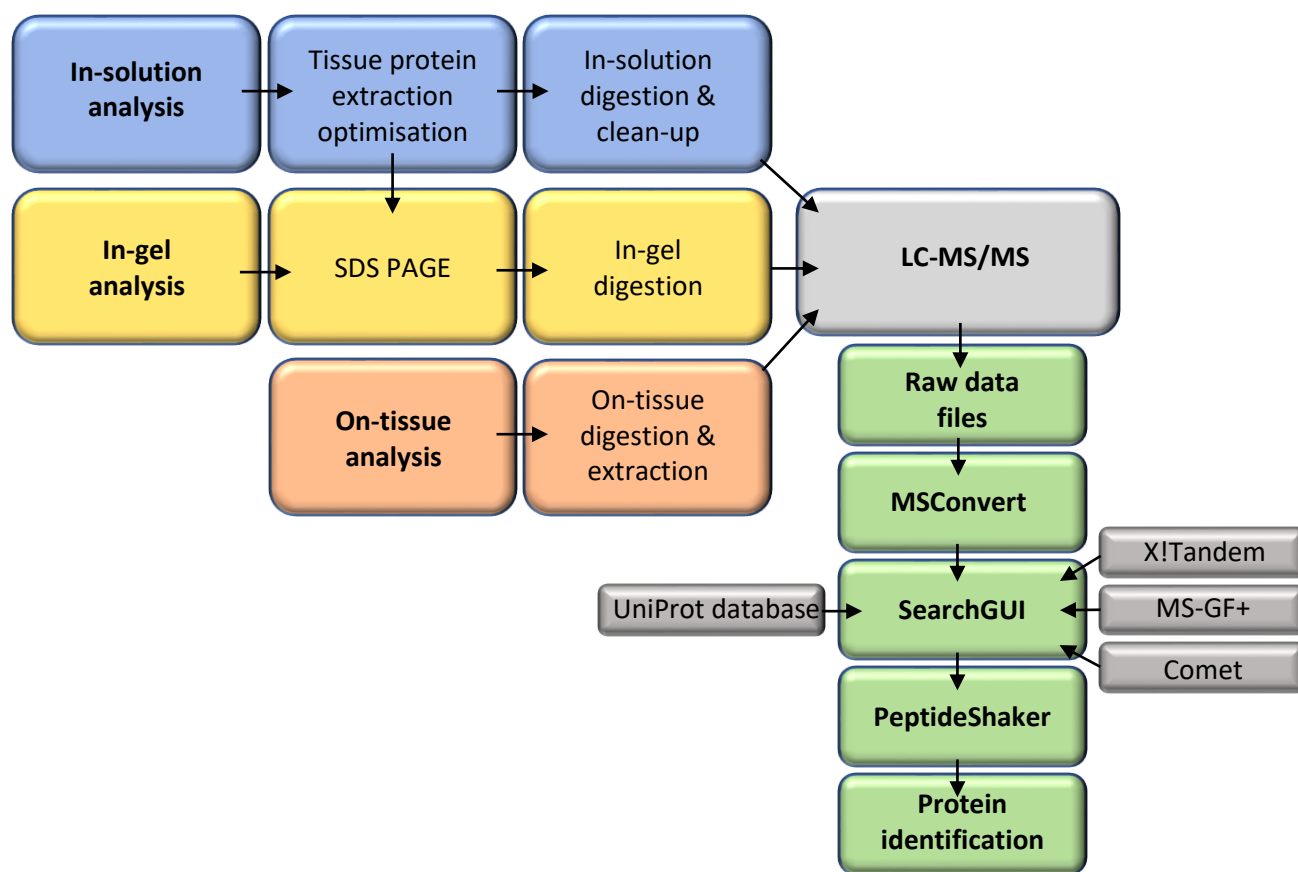


Figure 5.2: Overview and workflow of methods for LC-MS/MS and bioinformatics analyses. Wound tissue samples were prepared for mass spectrometric proteomic analyses using three different sample preparation approaches for comparison. Several protein extraction methods were assessed based on number of proteins identified after LC-MS/MS and bioinformatics analyses. Optimised protein extraction from tissue sections was used for in-solution and in-gel analysis pipelines followed either by HILIC in-solution digestion and clean-up or SDS PAGE and in-gel digestion, respectively. On-tissue tryptic digestion was performed directly on tissue sections. Protein samples were analysed using ESI-LC-MS/MS. Resultant raw files were filtered and converted to mgf format by MSConvert and peptides matched to spectra against a UniprotKB/Swiss-Prot *Sus scrofa* specific database including reverse decoy sequences using SearchGUI with the X!Tandem, MS-GF+ and Comet search engines. Protein analysis and identification were done using PeptideShaker.

5.3 Results and Discussion

5.3.1 Tissue protein extraction

As described in detail in Chapter 1, biological wound tissues are complex in nature consisting of a multitude of cell types and structural components. Proteins within these tissues play vital roles in various physiological processes, including membrane-bound proteins (MPs). MPs remain a challenge in proteomic studies as these hydrophobic molecules must be extracted and solubilised from the membranes in the tissue for successful MS analysis. Various strategies have been applied to optimise MP extraction, enrichment and solubilisation using detergents (Lee, 2007, Nagaraj *et al.*, 2008), salt buffers (Shevchenko *et al.*, 2012, Wu *et al.*, 2003) and delipidation techniques (Shevchenko *et al.*, 2010). To efficiently extract not only MPs but all proteins from wound tissue for a contaminant-free, clean and enriched protein sample for LC-MS/MS analysis, seven different protein extraction protocols were assessed in duplicate on comparable wound tissue sections. Assessment was based on the resultant number and nature of proteins identified in a wound healing context using LC-MS/MS and bioinformatics analyses. Resultant extracted protein concentrations using the BCA assay were also taken into account. Results comparing the efficiency of the various sample preparation techniques used for SDS PAGE and in-gel digestion, and in-solution digestion as set out in the methods section are summarised in Table 5.3.

Table 5.3: Protein extraction efficiency showing number of proteins identified and extracted protein concentration per wound tissue section

Extraction Method	Details	# Proteins identified	Ave. protein concentration (µg/µl)
1	Base method	280	2.3
2	PBS wash + Base method	137	1.4
3	Delipidation + Base method	22	1.8
4	Base method + Heat (50°C)	267	2.5
5	Base method + Heat (90°C)	231	2.5
6	Delipidation + PBS wash + Base method	227	2.2
7	PBS wash + Delipidation + Base method	134	1.4

Protein was extracted from wound tissue sections using the seven protein extraction protocols as listed in Table 5.3. Extraction Method 1 was set as the reference base method common in all subsequent protocols with additional procedures. It was found that between 196 – 350 µg of protein was extracted per single tissue section using the seven different protocols. Based on the number of proteins identified it was found that the base method, Extraction Method 1, was the most efficient extraction protocol, identifying an average of 280 proteins per tissue section. This result was closely followed by Extraction Methods 4 and 5, which utilised heating at 50°C and 90°C for protein extraction and which identified 267 and 231 proteins respectively. Interestingly, both Methods 4 and 5 extracted the highest amount of protein per tissue section both at 2.5 µg/µl (approximately 350 µg), with the base method (Extraction Method 1) extracting 2.3 µg/µl (approximately 322 µg). This suggested that although heat may have improved the overall extraction of protein from tissue, it also may have resulted in protein modification to such an extent that interference with successful downstream identification occurred. This was supported by the fact that more proteins were identified when extracted at 50°C compared to 90°C, where higher temperatures seemed to further interfere with protein identification. Higher temperatures may have supported certain proteolytic activity, contributed to additional modifications such as oxidation of methionine or caused cross-linking of certain amino acids. Approximately 8% of proteins identified in the heat-based Extraction Methods 4 and 5 were not found using Extraction Method 1. The majority

of these extra proteins identified showed no specific correlation to wound healing. Potential enrichment achieved with the addition of heat in protein extraction methods was deemed not to provide any benefit.

Extraction Method 6 (delipidation followed by PBS wash) was also relatively successful in tissue protein extraction, extracting 2.2 µg/µl (approximately 308 µg) of protein and identifying 227 proteins successfully. Approximately 10% of these proteins (22) were not identified using Extraction Method 1, with just two proteins showing correlation to wound healing through association to immune responses, being complement C1q subcomponent subunit A and leukocyte surface antigen CD47. Interestingly, when the process was reversed and tissue was first washed and then delipidated (Extraction Method 7) there was a 36% decrease in the amount of protein extracted. This suggested that washing fresh frozen wound tissue in PBS resulted in a significant loss of soluble proteins. This confirmed the results of Extraction Method 2 where samples were washed with PBS prior to extraction and the resultant protein concentration decreased to 1.4 µg/µl (approximately 96 µg) and less than half the number of proteins (137) could be identified.

Wound tissue was washed with PBS to assess whether the removal of dominant proteins, such as haemoglobin and serum proteins, would allow detection of otherwise obscured, less prominent proteins. Only seventeen proteins (12%) of the total proteins identified using the PBS wash method (Extraction Method 2) were not identified using the base method (Extraction Method 1). These proteins mainly consisted of enzymes, membrane transporters and ribosomal proteins, with no distinct association to the wound healing process. While the PBS wash method successfully decreased haemoglobin and the most dominant plasma protein, serum albumin by approximately 1.3-fold and 1.5-fold respectively (based on MS² quantitation), no specific wound tissue protein enrichment occurred. A negative outcome was that other plasma proteins with potential wound involvement were completely removed by the wash. Haptoglobin (linked to haemolysis, binds free plasma haemoglobin), fibrinogen (required for fibrin-based blood clots) and immunoglobulin lambda chain C region (obtained from various immunoglobulins) (TheUniProtConsortium., 2014) were found in the base method but were not present following the PBS wash. Additional proteins related to wound healing identified after using the base extraction method but not identified after including the PBS wash

were: macrophage migration inhibitory factor (pro-inflammatory cytokine involved in the innate immune response); c-reactive protein (associated with host defence and complement activation); arachidonate 15-lipoxygenase (regulates macrophage function and may play a role in epithelial wound healing); prothrombin (vital for blood coagulation) and plasminogen (precursor of plasmin that dissolves fibrin in blood clots) (TheUniProtConsortium., 2014). The failure to enrich wound-related proteins coupled to the loss of proteins other than the dominant plasma proteins, rendered the use of the PBS wash approach unproductive.

While sample delipidation, Extraction Method 3, did not result in as large a protein loss as caused by the PBS wash (1.8 µg/µl protein extracted) protein identification was substantially compromised with only 22 proteins being identified. The delipidation process significantly influenced the sample signal during mass spectrometric analysis resulting in limited results. The cause of such effects could be due to multiple factors (*e.g.* trypsin inhibition, protein denaturing, ion suppression, increased matrix effects, analyte derivatisation etc.) beyond the scope of this study. This extraction method provided no advantage to the study and was not used further.

Based on the results obtained from each of the extraction techniques, Extraction Method 1 was found to be the most efficient extraction protocol and was used for all further protein extraction in the study.

5.3.2 SDS PAGE

SDS PAGE was performed on wound tissue samples in order to assess the viability of gel-based proteomics in tracking the kinetics of the acute wound proteome. Proteins were independently extracted from three sequential wound tissue sections (using Extraction Method 1) prior to SDS PAGE. Only Day 3 wound tissue was used for initial assessment. The protein mass fingerprints resulting from gel loadings of 5 µg, 10 µg and 15 µg of extracted protein were visualised on the gels by Stain-Free™ technology (Figure 5.3) or Aqua Colloidal Coomassie staining (Figure 5.4).

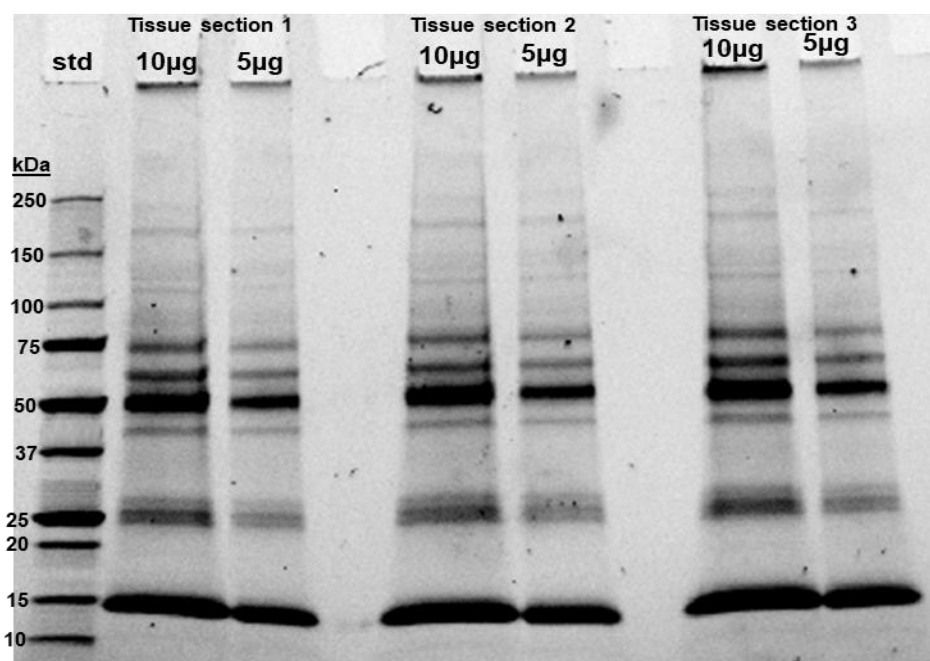


Figure 5.3: Stain-Free™ visualisation of extracted wound tissue protein bands separated by SDS PAGE. Proteins were independently extracted from three sequential wound tissue sections with 5 and 10 µg of protein from each sample loaded onto the gel. The standard (std) lane contained protein mass standards annotated in kDa. The stain-free technique relies on tryptophan residues in the proteins for detection, which in this case showed a separation of a limited number of bands with highly differential intensities.

The three wound tissue sections produced comparable, relatively simple protein mass fingerprints for both the 5 and 10 µg loading concentrations, highlighting the reproducibility of the tissue protein extraction protocol. The most abundant mass range protein bands were seen between 45 – 75 kDa, at 25 kDa and around 15 kDa. It was noted that although the more prominent protein bands were easily visualised fainter bands, especially around 80 – 250 kDa, were not easily identifiable in any of the lanes on the Stain-Free™ gel. Another limitation of the gel was that protein aggregates were found at the bottom of all six sample wells, being too large to move through the pores of the gel. The relative intensity of these proteins suggested that a significant amount of protein could not enter the gel for separation, resulting in an incomplete protein mass fingerprint of the wound proteome. This was further highlighted in the relatively simple protein mass fingerprint produced by the wound tissue samples. In addition, Stain-Free™ technology visualises proteins by incorporating a trihalo compound into tryptophan residues where resulting adducts

emit fluorescence upon excitation by ultraviolet (UV) irradiation (Gürtler *et al.*, 2013). Proteins with a low tryptophan content are therefore not easily visualised, limiting the sensitivity of the technique. The complexity contained within biological tissue proteomes was not accurately reflected in the Stain-Free™ resultant SDS PAGE fingerprint.

To overcome the sensitivity and selectivity limitation of Stain-Free™ technology and in preparation for in-gel digestion and LC-MS/MS analysis, SDS PAGE was performed on 15 µg of extracted wound tissue protein and the resultant gel stained with Colloidal Coomassie Blue. The most prominent protein bands were then excised, in-gel digested, analysed using LC-MS/MS and identified using bioinformatics tools (Figure 5.4).

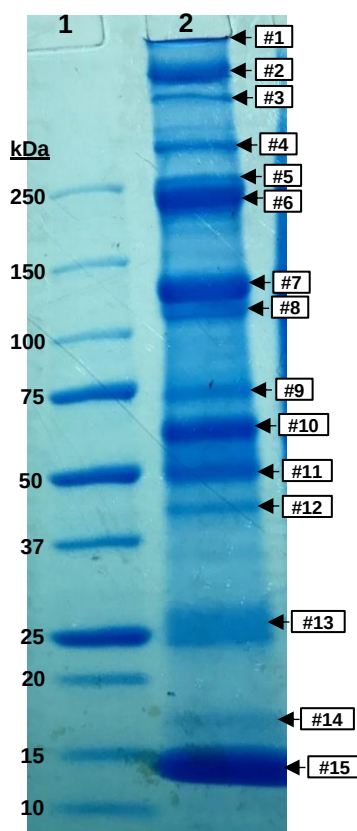


Figure 5.4: Coomassie Blue-stained gel of extracted wound tissue proteins. A total of 15 µg of extracted wound proteins from Day 3 tissue were separated by SDS PAGE (lane 2). Aqua Colloidal Coomassie Blue stain was used to visualise protein bands. Arrows #1 - 15 identify bands removed for in gel-digestion followed by sequencing by LC-MS/MS. Lane 1 contained mass standards annotated in kDa. Note the different band intensities and the higher number of intense protein bands visualised by Coomassie staining especially in the 100 – >250 kDa region compared to the Stain-Free™ technique.

Increasing the amount of wound protein sample to be run from 10 µg to 15 µg and staining the gel with Coomassie Blue resulted in the visualisation of additional protein bands not seen in the Stain-Free™ gel (Figure 5.3), especially above 250 kDa (out of the range of the mass standards) (Figure 5.4). The Coomassie Blue-stained gel still however provided a relatively simple protein mass fingerprint of the acute wound proteome. Based on these results it was determined that protein mass fingerprinting using SDS PAGE alone, although labour and time efficient and relatively cheap, was not a viable method for tracking acute wound tissue kinetics.

LC-MS/MS analysis was done on prominent bands from the gel to determine protein identity and assess the degree to which multiple proteins were contained within single bands. A total of 15 protein bands were excised from the gel for in-gel trypsinisation, LC-MS/MS analysis and protein identification. Results are displayed in Table 5.4 where “Band #.” follows the labelling shown in Figure 5.4.

Table 5.4: Summary of protein identities of in-gel trypsinised bands from SDS PAGE using LC-MS/MS and bioinformatics analyses

Band #.	Proteins Identified (100% confidence of ≥2 peptides per protein in addition to ≥2 unique peptides) (Repeat proteins are colour coded)	
1 (>250 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10) ▪ Keratin type II cytoskeletal (subtypes 1, 2, 5) ▪ Trypsin	▪ Haemoglobin subunits α and β ▪ Collagen type III α 1 chain
2 (>250 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10, 14) ▪ Keratin type II cytoskeletal (subtypes 1, 2, 5) ▪ Collagen type III α 1 chain	▪ Haemoglobin subunit α ▪ Vimentin ▪ Trypsin
3 (>250 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10) ▪ Keratin type II cytoskeletal (subtypes 1, 2) ▪ Collagen type III α 1 chain	▪ Haemoglobin subunit β ▪ Trypsin
4 (>250 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10) ▪ Keratin type II cytoskeletal (subtypes 1, 2) ▪ Collagen type III α 1 chain ▪ Trypsin	▪ Haemoglobin subunits α and β ▪ Serum albumin ▪ Actin (cytoplasmic 1)
5 (250 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10) ▪ Keratin type II cytoskeletal (subtypes 1, 2, 5) ▪ Collagen type III α 1 chain	▪ Haemoglobin subunits α and β ▪ Actin (cytoplasmic 1) ▪ Trypsin

Band #.	Proteins Identified (100% confidence of ≥2 peptides per protein in addition to ≥2 unique peptides) (Repeat proteins are colour coded)	
6 (210-240 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10, 16) ▪ Keratin type II cytoskeletal (subtypes 1, 2, 5) ▪ Collagen type III α 1 chain ▪ Transitional endoplasmic reticulum ATPase ▪ Actin (cytoplasmic 1) ▪ Trypsin	▪ Haemoglobin subunits α and β ▪ Serum albumin ▪ Inter-α-trypsin inhibitor (heavy chain H2) ▪ Vimentin ▪ Phosphatidylinositol 3-kinase catalytic subunit type 3
7 (120-140 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10, 24) ▪ Keratin type II cytoskeletal (subtypes 1, 2, 5) ▪ Collagen type III α 1 chain ▪ Actin (cytoplasmic 1) ▪ Decorin	▪ Haemoglobin subunits α and β ▪ Complement C3 ▪ Serum albumin ▪ Vimentin ▪ Trypsin
8 (105-120 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10, 14) ▪ Keratin type II cytoskeletal (subtypes 1, 2, 5) ▪ Complement C3 ▪ Collagen type III α 1 chain ▪ Decorin ▪ Actin (cytoplasmic 1) ▪ Annexin A1	▪ Haemoglobin subunits α and β ▪ Inter-α-trypsin inhibitor (heavy chain H4) ▪ Serum albumin ▪ Vinculin ▪ Vimentin ▪ Serotransferrin ▪ Trypsin
9 (73 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10, 24) ▪ Keratin type II cytoskeletal (subtypes 1, 2, 5) ▪ Serotransferrin ▪ Moesin ▪ Lactotransferrin ▪ Collagen type III α 1 chain ▪ Decorin ▪ Actin (cytoplasmic 1) ▪ 78 kDa glucose-regulated protein (fragment) ▪ Trypsin	▪ Haemoglobin subunits α and β ▪ Serum albumin ▪ Prelamin-A/C ▪ Heat shock protein HSP 90-α ▪ Inhibitor of carbonic anhydrase ▪ Fibrinogen α chain precursor ▪ Heat shock 70 kDa protein 1-like ▪ Annexin A1 ▪ Ig λ chain C region
10 (60-67 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10, 14) ▪ Keratin type II cytoskeletal (subtypes 1, 2, 5) ▪ Complement C3 ▪ Actin (cytoplasmic 1) ▪ Leukocyte elastase inhibitor	▪ Haemoglobin subunits α and β ▪ Serum albumin ▪ Prelamin-A/C ▪ TGF-β induced protein ig-h3 ▪ Catalase

Band #.	Proteins Identified (100% confidence of ≥ 2 peptides per protein in addition to ≥ 2 unique peptides) (Repeat proteins are colour coded)	
10 (60-67 kDa)	▪ Hemopexin ▪ Alpha-2-HS-glycoprotein (fragment) ▪ Trypsin	▪ α-1-antitrypsin ▪ Vimentin
11 (50 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10) ▪ Keratin type II cytoskeletal (subtypes 1, 2) ▪ Actin (cytoplasmic 1) ▪ Tubulin β chain ▪ β-enolase ▪ Trypsin	▪ Haemoglobin subunits α and β ▪ Vimentin ▪ Serum albumin ▪ Tubulin α-1B chain ▪ ATP synthase subunit α (mitochondrial)
12 (44 kDa)	▪ Keratin type I cytoskeletal (subtypes 9, 10) ▪ Keratin type II cytoskeletal (subtypes 1, 2) ▪ Actin (cytoplasmic 1) ▪ Actin (α skeletal muscle) ▪ Phosphoglycerate kinase 1 ▪ 40S ribosomal protein SA	▪ Haemoglobin subunits α and β ▪ Vimentin ▪ Serum albumin ▪ Haptoglobin ▪ Isocitrate dehydrogenase [NADP] ▪ Trypsin
13 (25 kDa)	▪ Keratin type I cytoskeletal (9, 10) ▪ Keratin type II cytoskeletal (1, 2) ▪ Ig λ chain C region ▪ Azurocidin ▪ Heat shock protein β-1 ▪ High mobility group protein B1 ▪ Trypsin	▪ Haemoglobin subunits α and β ▪ Serum albumin ▪ Apolipoprotein A-I ▪ Peroxiredoxin-6 ▪ Carbonic anhydrase 3 ▪ 60S ribosomal protein L10a (fragment)
14 (16 kDa)	▪ Keratin type I cytoskeletal (9, 10, 14) ▪ Keratin type II cytoskeletal (1, 2, 5, 6A) ▪ 40S ribosomal protein S16 ▪ Trypsin	▪ Haemoglobin subunits α and β ▪ Peptidyl-prolyl cis-trans isomerase A ▪ Histone H4
15 (15 kDa)	▪ Keratin type I cytoskeletal (10) ▪ Keratin type II cytoskeletal (1) ▪ Histone H4	▪ Haemoglobin subunits α and β ▪ Fatty acid-binding protein (adipocyte) ▪ Trypsin

Following mass spectrometric analysis of the SDS PAGE acute wound protein mass fingerprint, between 5 and 19 proteins were identified per band (Table 5.4). This finding aids in the explanation of the relatively simple protein mass fingerprint of the wound tissue samples seen in Figures 5.3 and 5.4. In-gel trypsinisation and protein band identification were done to assess whether separating complex biological tissue using SDS PAGE into simplified fractions of the proteome would allow for the identification of less prominent wound-associated proteins and improve overall proteome coverage. This is based on the fact that fractionation on the protein level allows for isolation of highly abundant proteins for improved detection and coverage of low abundant proteins (Dzieciatkowska *et al.*, 2014).

It was found that the same protein identities were present in multiple, if not all of the 15 selected protein bands. Cytoskeletal type I and II keratins and haemoglobin were present in all protein bands including the sample well (Band #1), with collagen type III α 1 chain, serum albumin and cytoplasmic actin present in 60% of all sample bands. Vimentin was found in approximately 50% of the bands with decorin, complement C3, serotransferrin, annexin A1, prelamin-A/C, histone H4 and Ig λ chain C region all present in at least two bands on the gel. Trypsin was present in all protein bands, which is to be expected as this enzyme was added to all samples for proteolytic digestion in preparation for LC-MS/MS analysis (Section 5.2.2.2). While the same proteins were identified at different locations on the gel, the peptides providing the identification of these proteins during bioinformatics analysis were not the same. This suggests proteins of the same identity found in multiple bands may have been due to early protein cleavage in the frozen sections of the sample and that these cleavage products represent different regions of the same original protein. This is supported in literature where the prevention of rapid protease activity and degradation in biological tissue samples is known to be a challenge in proteomic analysis. Substantial disturbances in tissue homeostasis occur upon the removal of the sample from its natural environment, triggering the almost immediate release of proteolytic enzymes. Fresh tissue samples collected and snap frozen within one minute have been shown to already exhibit changes in proteome composition compared to the preserved, *in vivo* state of the tissue (Stingl *et al.*, 2014, Svensson *et al.*, 2009). Although wound tissue was almost immediately snap frozen upon excision in this study, instances such cutting the wound tissue in half using the

hydraulic cutter (Section 2.2.5) and tissue thawing during histological sectioning with the cryotome (Section 3.2.1) may have resulted in proteolytic activity within the tissue.

In addition, keratin has a high cysteine content that could cross-link to other proteins easily and although the samples are reduced and denatured prior to separation, on starting the electrophoretic process the reducing agent is diluted away and at high pH the thiol groups could again cross-link with available thiol groups of other small proteins. This offers an explanation as to why such proteins migrated to points other than those of their molecular weight on the gel and suggests that proteolytic cleavage occurred during sample preparation prior to SDS PAGE.

In-gel digestion of protein extracts from snap frozen wound biopsy tissue sections combined with LC-MS/MS analysis complicated the identification of the proteins within acute wound porcine tissue. In addition, multiple proteins were found in the sample well (Band #1, Table 5.4) that did not enter the gel, thus further complicating the ability to quantitate different proteins reliably and providing further reason to not use gel-based protein mass fingerprinting for the assessment of the acute wound proteome over time. It was decided that this technique did not provide a robust option for non-labelled quantitation of wound tissue and was not pursued for the analysis of the remaining wound kinetics.

5.3.3 In-solution vs on-tissue analysis

Due to the solubilisation procedure and protein concentration determination prior to analysis and the post-digestion clean-up step, in-solution analysis using MagReSyn HILIC beads (Section 5.2.1) is more time consuming and labour intensive than on-tissue analysis (Section 5.2.3), where minimal sample preparation, optimisation and processing is required. As on-tissue digestion loses spatial information, the data obtained should be comparable to the in-solution digestion and this was evaluated by comparing the numbers of proteins identified by both techniques for each of the six wound excision time points as well as in healthy tissue (Table 5.5).

Table 5.5: Comparison of in-solution and on-tissue analysis based on number of proteins identified

Tissue sample	In-solution analysis	On-tissue analysis
Wound Day 3	296	88
Wound Day 5	335	74
Wound Day 8	297	63
Wound Day 10	327	44
Wound Day 12	317	72
Wound Day 16	309	76
Healthy	274	39

The results revealed that in-solution analysis identified on average five times more proteins across the healthy tissue and six wound excision time points when compared to on-tissue analysis. While the exact extent was unknown, it was expected that in-solution analysis would be the superior method for protein identification. One of the reasons being that mass spectrometry for protein identification is based on the detection of peptide fragments rather than of the intact proteins, which is directly related to trypsin digestion efficiency and the yield of tryptic peptides (Kuzyk *et al.*, 2010). During on-tissue analysis, trypsin is sprayed across the surface of the tissue section creating a bias where dominant surface proteins are far more likely to be digested than those proteins embedded deeper within the tissue or within lipid membranes. Based on surface exposure, localised pH, liquid contact and cellular location, certain proteins are less likely to be effectively trypsinised to form peptides of suitable size to provide efficient mass spectrometer fragmentation and protein identification. During in-solution digestion, proteins are efficiently extracted from tissue sections and are in solution, therefore completely exposed to trypsin under optimal digestion conditions. The on-tissue digestion samples were analysed on a Thermo Scientific Orbitrap Fusion Mass Spectrometer as opposed to the SCIEX 6600 TripleTOF instrument used for the in-solution analysis. The Orbitrap is known for its superior high-resolution and sensitivity capabilities in high-end proteomic applications (Eliuk and Makarov, 2015). The results show that despite using an advanced capability mass spectrometer, in-solution analysis of wound tissue proteins is far superior to that of on-tissue analysis in protein identification.

Due to the different sample preparation approaches, it was of interest to determine the extent of overlap between protein identity from the in-solution and on-tissue

analyses. It was found that across the pool of seven samples representing the kinetic changes in acute wound healing phases and healthy tissue, more than 99% of the proteins identified using on-tissue analysis were present in the in-solution analysis. Only two proteins were found to be uniquely identified using on-tissue analysis as shown in Table 5.6.

Table 5.6: Proteins identified through on-tissue analysis only in both healthy and wound tissue (TheUniProtConsortium., 2014)

Accession no.	Protein description	Molecular function/Biological process	Tissue sample origin
P80272	Non-histone chromosomal protein HMG-17	Nucleosomal DNA binding	Healthy, Days 3, 8 & 16
Q95281	60S ribosomal protein L29	Ribonucleoprotein; cytoplasmic translation	Days 3, 5, 8, 10, 12 & 16

Only two proteins were found to be exclusively identified using the on-tissue analysis (proteins found to be in the contaminants sequence database were excluded), which is a considerably low number relative to the hundreds of proteins identified using in-solution analysis (Table 5.5). The significance of these proteins as potential markers in kinetic acute wound healing were assessed based on molecular/biological function.

Non-histone chromosomal protein HMG-17 is a DNA-binding protein that may aid in the unique chromatin conformation of transcribing genes, while 60S ribosomal protein is a component of the larger ribosomal subunit and plays a role in cytoplasmic protein synthesis (TheUniProtConsortium., 2014). Both proteins play a role in general cellular function with no evidence supporting any specific biological pathway related to wound healing. This is further supported by the presence of non-histone chromosomal protein in the healthy tissue sample as well as the presence of 60S ribosomal protein in all wound excision time points (Table 5.6).

While on-tissue analysis may be a faster and simpler sample preparation method for mass spectrometric proteomic analysis of tissue, based on these results its use in tissue protein identification for potential markers of the kinetic acute wound proteome is not supported. In-solution analysis proved to be the far superior sample preparation technique and was thus the method of choice for proteomic analysis of wound tissue for the remainder of this study.

5.3.4 In-solution analysis of the kinetic acute wound

In-solution analysis was used to assess the kinetic changes in the acute wound proteome at six selected time points over 16 days of wound repair. Proteins were extracted in duplicate (two sequential tissue sections per sample) from a section of non-wounded healthy tissue and from Day 3, 5, 8, 10, 12 and 16 wounded tissue samples using Extraction Method 1 and subjected to the semi-automated HILIC in-solution digestion and peptide clean-up protocol as described in Section 5.2.1. Following LC-MS/MS and bioinformatics analyses, protein identities were compared between the healthy tissue and the six different wound excision time points (only proteins identified with 100% confidence and at least 2 unique peptides were analysed, as described in Section 5.2.4). Proteins present in two or more of the seven sample groups (healthy tissue and six wound time points) were filtered out. This resulted in the isolation of protein identities unique to healthy tissue or one time point only, to reveal potential healing phase-specific kinetic changes in the acute wound proteome (Table 5.7). An unfiltered combined list of all the proteins identified across the six wound time points is provided in Appendix VIII.

Table 5.7: Protein identities with biological function for proteins unique to specific healing time points (TheUniProtConsortium., 2014)

	Accession no.	Protein description*	Basic molecular function/biological process
Healthy	P61958	Small ubiquitin-related modifier 2	Covalently attached to proteins for nuclear transport and DNA replication/repair
	Q5ISC6	Heme-binding protein 1	May bind free porphyrinogens and facilitate removal of toxic compounds
	P79280	Myosin light chain kinase (smooth muscle)	Regulates actin-myosin interaction
	Q29236	T-complex protein 1 subunit zeta	Molecular chaperone involved in protein folding
	P28839	Cytosol aminopeptidase	Involved in processing and regular turnover of intracellular proteins
	Q2QLE2	Caveolin-2	May act as a scaffolding protein within caveolar membranes
	Q07717	Beta-2-microglobulin	Component of the class I major histocompatibility complex (MHC)
	A1XQR7	MICOS complex subunit MIC13	Component of the MICOS complex
	P80895	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	Plays a role in repair/degradation of damaged proteins
	P09623	Dihydrolipoyl dehydrogenase (mitochondrial)	Component of the glycine cleavage system
	P12675	Calpastatin	Inhibitor of calpain (calcium-dependent cysteine protease)
Day 3	Q9GK37	Corticosteroid-binding globulin	A transport protein for glucocorticoids and progestins in the blood
	O46560	Pyridoxal kinase	Required for synthesis of pyridoxal-5-phosphate from vitamin B6
	P12309	Glutaredoxin-1	Reduces low molecular weight disulphides and proteins
	O02858	Acyl-CoA desaturase	Plays an important role in lipid biosynthesis.
	Q28833	von Willebrand Factor	Promotes adhesion of platelets at sites of vascular injury Acts as a chaperone for coagulation factor VIII
	Q767L8	Mediator of DNA damage checkpoint protein 1	Required for checkpoint mediated cell cycle arrest in response to DNA damage
Day 5	P14287	Osteopontin	Appears to form an integral part of the mineralised matrix in bone Acts as a cytokine involved in enhancing production of interferon- γ and IL-12
	P79324	60S ribosomal protein L15	Structural constituent of ribosome
	P21999	Nuclear factor 1 C-type	Capable of activating transcription and replication
	P51525	Prophenin-2	Exerts antimicrobial activity
	A7Y521	COP9 signalosome complex subunit 4	Essential regulator of the ubiquitin conjugation pathway
	Q2MJV8	Paralemmin-1	Involved in plasma membrane dynamics and cell process formation
	Q6PQD5	Serine-protein kinase ATM	Activates checkpoint signalling upon apoptosis and genotoxic stresses
* All proteins identified with 100% confidence and ≥ 2 unique peptides			

	Accession no.	Protein description*	Basic molecular function/biological process
Day 8	A5GFN6	mRNA export factor	Plays a role in mitotic bipolar spindle formation
	P12067	Lysozyme C-1	Has a bacteriolytic function associated with the monocyte-macrophage system
	P54613	Serine/threonine-protein phosphatase 2A subunit A β isoform	Involved in protein phosphatase regulator activity
	Q5PYH3	GTP-binding protein SAR1b	Involved in transport from the endoplasmic reticulum to the Golgi apparatus.
	P05027	Sodium/potassium-transporting ATPase subunit beta-1	Catalyses the hydrolysis of ATP coupled with Na ⁺ /K ⁺ exchange
	Q29073	Prostaglandin reductase 1	Involved in the initial and key step of metabolic inactivation of leukotriene B4
	Q95283	Cytochrome c oxidase subunit 4 isoform 1 (mitochondrial)	Polypeptide chain of the terminal oxidase in mitochondrial electron transport
	B2MVP8	Fat storage-inducing transmembrane protein 1	Plays a role in lipid droplet accumulation
	P47787	Thromboxane-A synthase	Involved in the prostaglandin biosynthetic process
	Q29090	Serine/threonine-protein phosphatase 2A subunit B α isoform	Involved in protein phosphatase regulator activity
	P46410	Glutamine synthetase	Essential for proliferation of fibroblasts and endothelial cell migration
Day 10	Q7M329	Ribonuclease T2	May play a role in cellular RNA catabolism
	P51524	Prophenin and tritrypticin precursor	Exert antimicrobial and haemolytic activities
	P49932	Antibacterial peptide PMAP-37	Exerts antimicrobial activity
	Q29548	Beta-hexosaminidase subunit beta	Responsible for degradation of GM2 gangliosides
	P42639	Tropomyosin alpha-1 chain	Binds to actin filaments and plays a role in the regulation of muscle contraction Involved in stabilising cytoskeletal actin filaments
	P26044	Radixin	Plays a role in the binding of actin filaments to the plasma membrane
	Q9GLV6	Probable ATP-dependent RNA helicase DDX58	Innate immune receptor which acts as a cytoplasmic sensor of viral nucleic acids Plays a role in the induction of type I interferons and proinflammatory cytokines
	O46374	DNA topoisomerase 2-alpha	Essential during mitosis and meiosis for segregation of daughter chromosomes
* All proteins identified with 100% confidence and ≥ 2 unique peptides			

	Accession no.	Protein description*	Basic molecular function/biological process
Day 12	P30356	Arachidonate 5-lipoxygenase-activating protein	Required for leukotriene biosynthesis
	A7TX81	COP9 signalosome complex subunit 6	Essential regulator of the ubiquitin conjugation pathway
	Q09136	5'-AMP-activated protein kinase catalytic subunit alpha-1	Plays a role in regulating cellular metabolism
	P12068	Lysozyme C-2	Has a bacteriolytic function associated with the monocyte-macrophage system
	P36887	cAMP-dependent protein kinase catalytic subunit alpha	Phosphorylates a large number of substrates in the cytoplasm and the nucleus
	Q5BLZ2	Perilipin-3	Required for transport of mannose 6-phosphate receptors
	I3LUP1	Mannose-1-phosphate guanyltransferase alpha	Regulatory subunit that allows allosteric feedback inhibition by GDP-mannose
	Q5D144	Transcription factor A (mitochondrial)	Functions in mitochondrial transcription regulation
	I3L5V6	Sterile alpha and TIR motif-containing protein 1	Promotes injury-induced axonal death
Day 16	Q866Y3	N-acetylmuramoyl-L-alanine amidase	Detection/defence against bacterium
	A4Z6H0	Adenylosuccinate synthetase isozyme 1	Component of the purine nucleotide cycle
	Q28942	Low affinity immunoglobulin gamma Fc receptor III	Receptor for the Fc region of complexed immunoglobulin gamma
	A1Y2K1	Tyrosine-protein kinase Fyn	Plays a role in cytoskeletal remodelling, immune response and axon guidance
	Q3BDI7	Desmoglein-1	Involved in mediating cell-cell adhesion
	Q9TTK7	Oxidized low-density lipoprotein receptor 1	Mediates degradation of oxidatively modified low-density lipoprotein
* All proteins identified with 100% confidence and ≥ 2 unique peptides			

Proteins uniquely identified per sample between the healthy tissue and six wound excision time points after in-solution analysis are shown in Table 5.7. Using mass spectrometry for protein identification of small tissue samples can be challenging for numerous reasons (Szabo and Janaky, 2015). One major challenge is that easily ionising peptides of abundant proteins tend to dominate mass spectrometric acquisition time thereby obscuring the identification of low abundant peptides (Dzieciatkowska *et al.*, 2014). The proteins at each healing time point were uniquely identified because these were the most abundant and easily ionised at this time point. Due to the dominance of specific peptide ions it can be assumed that the parent proteins were relatively more abundant than other proteins at the specific time point, providing some relative quantitative changes occurring during the different phases of the acute wound healing process. While the in-solution analysis of the kinetic changes in the acute wound tissue focussed on protein identities, the effect of quantitative variations on mass spectrometric protein identification does need be considered.

The healthy tissue and the Day 8 wound tissue samples yielded the highest number of unique proteins. All wound tissue was surrounded by varying amounts of healthy tissue when excised, so the presence of these “healthy tissue” proteins in samples from different time points is not precluded, but rather implies that identified proteins have increased quantitatively during the healing process and these high abundance proteins are obscuring the now relatively less abundant “healthy tissue” proteins. These filtered, unique “healthy tissue” proteins varied in function and included cellular related proteins such as small ubiquitin-related modifier 2, T-complex protein 1 subunit zeta and cytosol aminopeptidase; haeme-related proteins such as haeme-binding protein 1; and immunity-related proteins such as beta-2-microglobulin (Table 5.7).

The nature of the proteins highlighted per wound healing time point in Table 5.7 were then assessed from a wound healing context to determine their potential role in healing phase features for the kinetic acute wound. This was done based on protein molecular function and/or biological process involvement using Gene Ontology (GO) analysis. The results were as follows:

Day 3 wound tissue revealed six unique proteins with only a single protein showing relation to the wound healing process. The von Willebrand Factor is a blood glycoprotein involved in the maintenance of haemostasis, where it promotes adhesion of platelets to sites of vascular injury by forming a molecular bridge between the collagen matrix and platelet-surface receptor complex consisting of glycoprotein Ib-IX-V. The von Willebrand Factor also acts as a chaperone for coagulation Factor VIII, delivering it to the site of injury, stabilising its heterodimeric structure and protecting it from premature clearance from plasma (TheUniProtConsortium., 2014). Histological observations in this study show Day 3 wound tissue to be in the cusp of the haemostasis and inflammatory phases of wound healing (see Section 2.3.4) (Broughton *et al.*, 2006, Velnar *et al.*, 2009). The prominence of The von Willebrand Factor on Day 3 is therefore not an unexpected observation.

The seven unique proteins of Day 5 revealed osteopontin and prophenin-2 as potential features of wound healing. Osteopontin is often observed in wound healing tissue, being involved in neutrophil and monocyte recruitment through its chemotactic effects while also enhancing production of interferon-gamma (IFN- γ) and interleukin (IL)-12 and reducing production of IL-10. IFN- γ plays an important role in both innate and adaptive immunity through immuno-regulation and by activating macrophages and increasing the expression of Class II major histocompatibility complex (MHC-II) (Artis and Spits, 2015, Rothwell and Hopkins, 1995, TheUniProtConsortium., 2014). IL-12 stimulates the differentiation of naïve T cells, acts as a growth factor for activated T cells and natural killer cells and stimulates the production of IFN- γ from resting peripheral blood mononuclear cells (PBMC) (Oppmann *et al.*, 2000, TheUniProtConsortium., 2014). IL-10 is an anti-inflammatory cytokine, which inhibits the synthesis of various other cytokines, including IFN- γ (Grimbaldeston *et al.*, 2007, TheUniProtConsortium., 2014). The inflammatory phase was confirmed in Day 5 tissue using histology (Section 2.3.4) where the presence of osteopontin as a potentially dominant protein at this time, increasing and activating interleukins, interferons, T cells, natural killer cells and macrophages, is indicative of the inflammatory phase. This is further supported by the fact that osteopontin attenuates the anti-inflammatory activities of IL-10. The second unique protein, prophenin-2, exerts antimicrobial activity in a humoral

immune response mediated by antimicrobial peptides (TheUniProtConsortium., 2014). These two proteins suggest the occurrence of an immune response on Day 5. The inflammatory phase of acute wound healing is marked by immune infiltration where chemotactic agents and inflammatory mediators, including cytokines, attract neutrophils, lymphocytes and macrophages migrate into the wound area to clear invading microbes and debris from the wound site (see Section 1.1.3.1) (Broughton *et al.*, 2006). The increased production of IFN- γ , which has antiviral activity, may work in combination with prophenin-2 in exerting antimicrobial activity which would be expected in the wound tissue during this inflammatory healing phase.

Day 8 revealed both the highest number of unique and wound-related proteins across the six healing time points assessed in this study, where eleven unique proteins were identified, five of which were wound-related. This is of interest as Day 8 was consistently highlighted as the time point representing the peak of wound healing activity based on unidentified features during the MALDI-IMS analysis (Chapter 3). Lysozyme C-1, prostaglandin reductase 1, thromboxane-A synthase, arachidonate 15-lipoxygenase and glutamine synthetase were uniquely identified in Day 8 tissue that all show wound healing functions. Inflammation was subsiding, but still a dominant process in the wound on Day 8, (see Section 2.3.4), a fact further supported by some of the unique proteins found.

Lysozymes primarily have a lytic function, with those in tissues and body fluids being associated with the monocyte-macrophage system where they enhance the activity of numerous immunoagents (TheUniProtConsortium., 2014). This type of activity is not unexpected in tissue where inflammation is evident. Within support of a subsiding inflammatory phase, prostaglandin reductase 1 catalyses the conversion of leukotriene B₄, a pro-inflammatory mediator, into its biologically less active metabolite, 12-oxo-leukotriene B₄. This is a key initial step of metabolic inactivation of leukotriene B₄ (TheUniProtConsortium., 2014). Thromboxane-A synthase is involved in the chemical reactions and pathways of prostaglandin biosynthesis (TheUniProtConsortium., 2014), where different prostaglandins play a role in both the promotion or resolution of inflammation (Ricciotti and FitzGerald, 2011). Arachidonate 15-lipoxygenase most likely plays an important role in the immune and inflammatory responses. Arachidonate 15-lipoxygenase is involved in the cellular response to IL-13, an anti-inflammatory cytokine produced by a number of immune

cells. Interestingly, this protein was postulated to play a role in epithelial wound healing in the porcine cornea, highlighting this protein as a feature in the healing process (Minty *et al.*, 1993, Rael and Lockey, 2011, Suzuki *et al.*, 1994, TheUniProtConsortium., 2014). Glutamine synthetase is an enzyme that fixes nitrogen, including for nucleotide synthesis, and is a controlling enzyme in protein turn-over. It is reported to be essential for proliferation of skin fibroblasts and is required for endothelial cell migration during vascular development (TheUniProtConsortium., 2014). Histological analysis of Day 8 wound tissue visualised proliferative phase characteristics of acute wound healing (see Section 2.3.4). Some of the processes known to occur during the proliferative phase include fibroblast proliferation and angiogenesis (Broughton *et al.*, 2006) and glutamine synthetase levels may therefore be a useful feature of acute wound proliferation.

By Day 10 the number of unique proteins decreased to eight, three of which showed functionality related to wound healing. Antimicrobial activity seemed to occur in Day 10 wound tissue through the actions of the prophenin and tritrypticin precursor protein, antibacterial peptide PMAP-37 and probable ATP-dependent RNA helicase DDX58. Both prophenin-1 and antibacterial peptide PMAP-37 exert antimicrobial activity against Gram-positive and negative bacteria, while tritrypticin, a cathelicidin-like peptide has both antimicrobial and haemolytic activities. ATP-dependent RNA helicase DDX58 is an innate immune receptor, which acts as a cytoplasmic sensor of viral nucleic acids and plays a major role in the activation of a cascade of antiviral responses including the induction of type I interferons and proinflammatory cytokines. The remaining wound-associated protein, tropomyosin alpha-1 chain, regulates smooth muscle contraction and is implicated in stabilising cytoskeleton actin filaments in non-muscle cells (TheUniProtConsortium., 2014). Day 10 wound tissue falls within the proliferative and remodelling phases of acute wound healing where new granulation tissue deposition, angiogenesis and myofibroblast activity dominate the wound environment and offer an explanation for the detected presence of tropomyosin alpha-1 chain (Broughton *et al.*, 2006, Darby *et al.*, 2014).

On Day 12 nine unique proteins were found with three proteins showing potential as features of wound healing: Arachidonate 5-lipoxygenase-activating protein, lysozyme C-2 and sterile alpha and TIR motif-containing protein 1. Arachidonate 5-lipoxygenase-activating protein is required for leukotriene biosynthesis by 5-

lipoxygenase (TheUniProtConsortium., 2014). Leukotrienes are pro-inflammatory mediators and indicate a presence of inflammation in the wound (Salmon and Higgs, 1987). This is supported histologically, where inflammatory cells were visualised in the wound tissue on Day 12 (see Section 2.3.4). Lysozyme C-2 has a primary bacteriolytic function associated with the monocyte-macrophage system and enhances the activity of immune-mediators when in tissues and body fluids (TheUniProtConsortium., 2014), indicative of a still constant immune presence in the wound. Sterile alpha and TIR motif-containing protein 1 is a toll-like receptor transcription inhibitor and a negative regulator of the MYD88- and TRIF-dependent toll-like receptor signalling pathway that plays a pivotal role in activating axonal degeneration following injury. It also promotes Wallerian degeneration, an injury-induced axonal death pathway which involves degeneration of an axon distal to the injury site and thus implies the occurrence of nerve damage during wounding (TheUniProtConsortium., 2014).

On the last day of monitored healing, Day 16, a total of six unique proteins were revealed with three showing some relation to wound healing. N-acetylmuramoyl-L-alanine amidase is an enzyme that detects and provides defence against bacteria by digesting biologically active peptidoglycan into biologically inactive fragments, while low affinity immunoglobulin gamma Fc region receptor III functions as an immunoglobulin G (IgG) binding protein (TheUniProtConsortium., 2014). The third protein, tyrosine-protein kinase Fyn, is an enzyme involved in many biological processes where in addition to playing a role in adaptive immunity, is involved in regulation of cell growth, cell adhesion, cytoskeletal remodelling, cell motility and axon guidance. These functions may be associated with the remodelling phase of acute wound healing (Broughton *et al.*, 2006), which is the phase that is distinctly evident from the histological assessment of the Day 16 wound tissue

Tracking the unique proteins found at each of the six time points over the 16 days of wound healing revealed 6, 7, 11, 8, 9 and 6 unique proteins on Days 3, 5, 8, 10, 12 and 16 respectively. Interestingly, this distribution seems to mimic the average protein intensities of the MALDI-IMS average mass profiles of the kinetic changes in healing wound tissue (see Section 3.3.1 and Figure 3.7). In the MALDI-IMS data, increases in relative intensity and peak number are found from Day 3 to 5, reaching

a maximum on Day 8 then decreasing on Days 10 and 12, where Day 16 shows a decrease to more closely represent the proteomic activity of Day 3.

Proteins were revealed that show potential as future quantitative features for identifying the phases of acute wound healing. During haemostasis The von Willebrand Factor was found to be dominant; at the height of inflammation osteopontin and prophenin-2 presented as features with strong immune-related functions; during subsiding inflammation and initial proliferation lysozyme C-1, prostaglandin reductase 1, thromboxane-A synthase, arachidonate 15-lipoxygenase and glutamine synthetase were identified, playing roles in the immune and inflammatory responses as well as in fibroblast and endothelial cell proliferation.

Moving to the proliferative phase, proteins associated with antimicrobial activity were still found to be dominant through proteins prophenin and tritrypticin precursor protein, antibacterial peptide PMAP-37 and probable ATP-dependent RNA helicase DDX58. During proliferation and the start of remodelling, arachidonate 5-lipoxygenase-activating protein, lysozyme C-2 and sterile alpha and TIR motif-containing protein 1 were detected, relating to inflammatory, immune and neural functions. In the last measured time point of healing, representing the remodelling phase, N-acetylmuramoyl-L-alanine amidase, low affinity immunoglobulin gamma Fc region receptor III and tyrosine-protein kinase Fyn were identified, highlighting a continuous immune presence along with cellular growth, motility and remodelling functions.

Quantitative measurement of proteins that could define phases of healing could in turn be applied in a clinical model setting to non-healing chronic wound samples for diagnostic or treatment-based purposes.

5.4 Summary

Various sample preparation and processing techniques using nano LC-ESI-MS/MS analysis for small samples of wound tissue were assessed. The technique providing the highest number of identified proteins was then used to analyse samples of acute wounds over selected days during a 16-day healing period to reveal potential kinetic changes. In this way potential time-dependent healing phase protein features were revealed.

Tissue sample clean-up and protein extraction techniques were selected based on number of proteins identified following LC-MS/MS and bioinformatics analysis. Protein extracts were assessed by SDS PAGE followed by in-gel digestion and protein band identification to track the continuously changing wound proteome. The gel-based proteomic techniques did not provide useful proteomic results and showed surprising mass distribution of proteins of known mass across the entire mass range, which limits the useful data available for analysis of complex biological tissues.

The results obtained from the relatively simple on-tissue digestion of wound sections were compared to the more labour-intensive, but better controlled in-solution analysis. An average of five times the number of proteins were identified using the latter technique across several tissue samples. There are several potential reasons for the low protein identification rates from on-tissue digestion, but reproducibility will be completely dependent on parameters not under the researcher's control. On-tissue digestion showed poor reproducibility, low efficiency and very low protein identification ability and was not pursued any further in the study.

In-solution analysis was preceded by assessment of different sample preparation techniques from histological sections of fresh snap frozen tissue where protein extraction followed by HILIC in-solution digestion with additional post-digestion peptide clean-up, LC-MS/MS and bioinformatic processing proved to be the superior protocol for analysis of porcine wound tissue in this study.

In-solution analysis and an optimised protocol for LC-MS/MS analysis of the proteome from small wound tissue samples collected on selected days during the 16-day acute wound healing period, revealed a small number of potential proteomic features of acute wound healing phases. A total of 18 proteins showing functional relation to the healing process and absence/presence through LC-MS/MS analysis were revealed. Biological functions of these proteins included involvement in adaptive and innate immunity, inflammation, cellular adhesion and various biosynthetic processes. Further targeted quantitative analysis of these proteins across repeat sampling of multiple, controlled, healing acute wounds over time will allow for their assessment as future acute wound markers that may be used as validating features of acute wound healing in clinical settings or as new drug targets for healing therapies.

Chapter 6: Final Conclusions and Recommendations

6.1 Global Discussion and Conclusions

6.1.1 Overview

The purpose of this study was to make use of current mass spectrometric techniques to better define the kinetic changes occurring during acute wound healing across proteomic, metabolomic and lipidomic bases. Several sample preparation techniques, analytical approaches, data conversion methodologies and statistical tools were assessed to best visualise, characterise and identify molecules or features in the complex and continuously changing environment of the acute wound at six pre-selected time points over 16 days of healing. Different mass spectrometric approaches to analyse snap frozen histological sections of wound tissue collected from a well-controlled porcine wound healing model, allowed ‘omic’ analyses to provide a better understanding of the acute wound healing process. Unidentified features of interest found at key time points were compared to provide a foundation for an acute wound healing model, which could be applied to non-healing chronic wounds in future studies with the aim of identifying possible key indicators of hindered healing processes or potential drug targets. Chronic wounds include lower limb ulcers and diabetic foot ulcers, where key time points and changing features (proteins, metabolites or lipids) are still undefined. Any identified targets could be used as the basis of much-required development of new treatment options for chronic wounds.

In this study full-skin thickness wounds were surgically created in the porcine model where well planned and strictly controlled wound formation and healing could be accurately investigated. Scaled photography and histological analysis were used to track healing progression as wounds moved through the sequential yet overlapping phases of the acute wound healing pathway (Demidova-Rice *et al.*, 2012). Using the spatial distribution capabilities of matrix-assisted laser desorption ionisation imaging mass spectrometry (MALDI-IMS) correlated to sequential section histological staining, optimised multivariate analysis and feature statistical analysis, wound tissue was successfully defined within the surrounding healthy tissue. Kinetic changes were statistically characterised to reveal features and key time points during acute wound healing. This used proteomic, metabolomic and lipidomic assays from sequential

sections cut from the same wound tissue. MALDI-IMS has the advantage of being able to perform spatiotemporal identification of molecules making this technique beneficial for wound tissue analysis.

Methods for total protein extraction from wound tissue sections were compared to identify the protocols that provided the greatest protein yield and highest number of identifications. Protein extracts were further analysed using hydrophilic interaction liquid chromatography (HILIC)-based capture with in-solution tryptic digestion followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Extracted wound proteins were also assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) where protein mass fingerprints were visualised and major protein bands excised, in-gel digested and identified by LC-MS/MS analysis. All mass spectral proteomic data was subject to bioinformatic analysis.

A comparative analysis of in-solution versus on-tissue sample preparation was performed to determine optimal methodology for tissue protein extraction and identification. The optimised protocol was used to identify proteins across six time points and 16 days showing potential as predictive acute wound healing phase features.

6.1.2 Animal study and histological analysis

Full-skin thickness acute wounds were successfully created, monitored and excised from the porcine model 3, 5, 8, 10, 12 and 16 days post-wound creation. Scaled photographs taken under identical lighting were used to measure the wound healing rate with both wound perimeter and surface area decreasing over time and showing the greatest rate of reduction between Days 5 and 8. Healing progression was confirmed with histological analysis of FFPE wound tissue sections using H&E and PR stains visualised under bright-field and polarised light microscopy. Progressive histological changes seen included the presence and degree of inflammatory cells at the wound site, cellular oedema, structural markers (such as scabbing) and varying collagen deposition within the wound bed.

Histological analysis typically relies on biopsy tissue samples being chemically fixed, dehydrated, embedded in paraffin wax, sectioned into thin slices on a microtome and

visualised after staining with selected histological stains (Greenhalgh *et al.*, 1990, Lynch *et al.*, 1987, Nissen *et al.*, 1998, Tian *et al.*, 2007). Although cost-effective, this is a time-consuming and labour-intensive process, with visualisation being limited to the specific histological stains used. MALDI-IMS however, requires minimal sample preparation and has the ability to visualise thousands of molecules from a defined array of closely packed single spots across the entire surface of a histological section. Analysis of the ions generated from these molecules allows multiple compounds from tissue regions, intact cells and biological pathways to be detected simultaneously within very small areas, which is advantageous when analysing a biological system (Norris and Caprioli, 2013). MALDI-IMS and classic microscopy techniques should be used in conjunction with each other, where microscopy is used to identify morphological regions or features of interest and correlated to the MALDI-IMS-generated images (Deininger *et al.*, 2008, Spraggins and Caprioli, 2011).

6.1.3 MALDI-IMS

In order to advance in-depth understanding of the subtle molecular changes occurring during acute wound healing, analysis of the kinetic and spatial changes taking place in the wound and surrounding healthy tissue region throughout the healing process was performed. MALDI-IMS and H&E staining were done on sequential, snap frozen wound sections collected at six pre-selected time points spanning 16 days of the acute healing process. Spatial and composition changes in the proteins, metabolites and lipids in defined regions of interest (ROIs) incorporating the wound bed or wound edges were assessed. Proteome, metabolome and lipidome wound profiles of accurate mass data were generated for Days 3, 5, 8, 10, 12 and 16 where ROIs consisting of the wound bed, the left and right wound edges and a region of surrounding healthy tissue, were independently and comparatively tracked using 2D stack plots. Relative quantitative differences could be seen between the different ROIs within and between time points when comparing all three 'omic' data. Mass spectra and average mass profiles revealed peptides/protein features on Day 8 and lipid features on Day 5 as the major high intensity peaks relative to remaining time points. The wound metabolome did not show substantial intensity differences and displayed less prominent quantitative changes over the 16-day healing period.

Principal component analysis (PCA) was successfully utilised for comparative analyses of multiple datasets. Proteomic, metabolomic or lipidomic data derived from the ROIs representing the wound bed, wound edges and healthy tissue from all six wound time points were analysed using PCA. When assessing wound tissue variance relative to non-wounded healthy tissue it was found that the most significant changes occurred on Day 5 in the metabolomic and lipidomic analyses and on Day 8 in the proteomic analysis. Pseudo-gel visualisation supported these observations where the largest relative intensity increases of features were seen on Days 5 and 8. The time points of maximal healing activity variance in the wound metabolome and lipidome coincide, supporting evidence of this time point representing the height of wound healing associated processes. Wound-related proteomic activity started to increase on Day 5, reaching its peak variance at the next measured time point on Day 8. This delay may be due to changes in the expression of proteins lagging behind those of metabolites and lipids because of the additional time required for transcription and translation of new proteins. Due to the well-being of the animals based on ethical guidelines, it was not possible to obtain Day 6 or 7 wound tissue for analysis and therefore a three-day range for maximal wound proteome activity exists. If a single time point were to be the most representative of the height of acute wound healing across all three 'omic' bases, Day 5 showed the most substantial differences relative to healthy tissue. Day 5 is at the cusp of the inflammatory and the proliferation phase of acute wound healing, which is supported by the histological analysis of sequential histology sections from the same wounds. The timing of this switch from inflammatory tissue degradation to renewed growth is important as non-healing chronic wounds are often defined as being trapped in the degrading inflammatory phase. This implies that Day 5 would be a suitable time point for future comparative studies to assess the potential for chronic wound development. Transition from inflammation to tissue regeneration has been highlighted as possibly the most critical step for successful wound healing (Landén *et al.*, 2016).

The application of PCA and unsupervised ranking using the Kruskal-Wallis test to reveal potential features of acute wound healing was assessed. Peptides/proteins, metabolites and lipids were ranked using these statistical techniques and plotted as 2D peak distribution plots. The proteomic and metabolomic plots showed that ranked Kruskal-Wallis features better discerned wound from healthy tissue compared to

those revealed through PCA variance ranking. In the wound proteome and metabolome, ranked Kruskal-Wallis features distinguished healthy tissue from all wound time points except for that of Day 3 but had limited ability in classifying lipidomic wounds, where only Day 5 could be distinguished from healthy tissue. Using ranked PCA values, lipidomic Day 5 wound tissue was the only wound sample that could be discerned from healthy tissue across all three 'omic' bases. Overall, none of the statistical techniques revealed distinct features capable of completely distinguishing the six wound healing time points from one another. Proteomic data provided the best classification of early and late healing phases using Kruskal-Wallis-based techniques. Interestingly, features identified from Day 8 closely followed by features from Day 5 were ranked independently by Kruskal-Wallis, as time points of high relative variation in the proteomic healing process. The Kruskal-Wallis-based metabolomic distribution plot and all lipidomic distribution plots confirmed Day 5 as being the time point of maximal wound variation. Combining the results of PCA, peak statistics and spectral analysis, revealed 12 proteomic features (m/z 1531, 1723, 1756/8, 1815, 1977, 3330, 3849, 4772, 4794, 6719, 10724 and 15193), 9 metabolomic features (m/z 582, 772, 791, 884, 910, 956, 1803, 1940 and 1968) and 8 lipidomic features (m/z 611, 614, 619, 647, 671, 677, 687 and 886) as potential markers for further investigation of acute wound healing in the porcine model.

PCA was demonstrated to be a useful initial screening tool for the detection of significantly different physiological effects in differently treated wounds across proteomic, metabolomic and lipidomic bases. Using 3D PCA plots, various healing treatments were quickly and efficiently screened to determine whether any significantly different effects occurred relative to non-treated control wounds. Due to the relatively straightforward and time efficient tissue sample preparation required for MALDI-IMS, and easy analysis by PCA a simple, cost-effective solution to screen for potentially useful healing agents prior to any in-depth analysis can be implemented. If significant grouping or outliers are visually identified, complementary loading plots can be generated to reveal the masses of these variant features to better understand the molecular changes related to the altered healing observed. Unidentified variant features in specific wound treatments may also be highlighted through peak statistics analysis and visualised through 2D peak distribution plots where the features most

likely responsible for the differences can be observed. The application of these statistical and visualisation techniques provides insight into the features responsible for observed improved healing, potentially revealing new future drug targets.

MALDI-IMS has increasingly been used to differentiate tissue for disease classification (Deininger *et al.*, 2008, Kriegsmann *et al.*, 2015, Rauser *et al.*, 2010) making it a useful approach for classification of differences during acute wound healing phases or progression on which to generate potential models. MALDI-IMS is a costly process, and for complete acute wound healing model generation, multiple sets of wounds are needed for analysis to make allowance for intra- and inter-biological variance. This would need to take the natural biological variance observed within a single animal (different wound locations along the dorsum) as well as variance between animals into account, including the different rates of healing. This highlights the importance and relevance of using the data reducing abilities of multivariate statistical analysis to provide filtered and manageable datasets for reliable in-depth analysis and identification of features linked to complex changes occurring in healing tissue.

This study demonstrated the capability of MALDI-IMS coupled with histology, optimised multivariate analysis and unidentified feature statistical analysis as a powerful tool that possesses the unique ability to characterise and interpret large biological datasets without prior feature identification. This is useful across multiple 'omic' bases within single experiments, even when using limited size samples. The best time range to most accurately represent the essential molecular changes in the progression of healing of acute wounds across proteomic, metabolomic and lipidomic bases was found to coincide with a slight lag in the proteomic changes. Identification of a critical time range within a kinetic process allows a more comprehensive analysis, while creating manageable and representative datasets. The ranked Kruskal-Wallis significance values and PCA-based loading plots could identify potential features of interest in acute wound healing without the need to identify the individual features before analysis. These approaches can aid in comparative studies, such as those seeking to find unique differences that can be linked to chronic wounds. Using a filtered but well-defined dataset from acute wounds to identify potential markers of successful healing could establish a model to

simplify sample comparisons for identification of potential treatment targets in chronic wounds.

While MALDI-IMS provides a unique spatial advantage for mass spectrometric data that is especially applicable to the analysis of wound tissue samples, the absence of analyte enrichment or purification and lack of simultaneous MS/MS data collection means that IMS lacks the depth of coverage that LC-MS/MS can provide (Dilillo *et al.*, 2017). For this reason, LC-ESI-MS/MS is sometimes used in conjunction with MALDI MS as an additional complimentary technique (Banoub *et al.*, 2003).

6.1.4 LC-MS/MS analysis

The assessment and optimisation of various sample protocols for protein extraction from tissue sections was performed to achieve the highest number of protein identifications after using an optimised LC-MS/MS and bioinformatics pipeline.

The most efficient combination of sample preparation, digestion and analysis was then used to analyse the kinetic changes in the proteome of acute wound tissues of Days 3, 5, 8 10, 12 and 16 after wounding. The homogenisation of wound tissue proteins in an aqueous buffer containing SDS, Tris-HCl and protease inhibitor cocktail resulted in the highest number of proteins being identified after mass spectrometric analysis. Using this same sample extraction protocol followed by SDS PAGE produced unexpected results as protein mass fingerprints indicated numerous proteins at several unexpected mass locations on the gel. This raised the question of protein fragmentation and possible recombination of other smaller or larger peptides generated within the complex biological wound tissue. This highlighted the requirement of strict control over many aspects of the sample collection and workup that can limit the use of standard gel-based proteomics to monitor the kinetics of acute wound tissue.

In-solution digestion is a proteomic technique involving initial tissue protein extraction followed by enzymatic digestion of the whole extract. The specific protocol used in this study involved, reduction and acetamidation of the whole protein extract followed by a semi-automated HILIC bead-supported, in-solution digestion where the contaminating detergent and salts were washed from the bound protein to allow efficient tryptic digestion. This digestion was followed by off-line peptide clean-up

before injection onto the LC-MS/MS system. Results from this technique were compared to results obtained from on-tissue digestion, where proteins were trypsinised directly from the wound tissue sample section still mounted on the ITO slide. This involved minimal sample preparation but did show poor reproducibility and limited peptide recovery. Following mass spectrometric analysis and bioinformatics processing it was found that an average of five times more proteins were identified when using the in-solution analysis pipeline as opposed to the on-tissue analysis pipeline. This large discrepancy was observed despite the utilisation of a high-end proteomics type Orbitrap mass spectrometer for the on-tissue analysis, known for its overall superior sensitivity and high-resolution capabilities (Eliuk and Makarov, 2015). The substantial improvement in proteome coverage when using in-solution analysis justified the additional time, labour and optimisation required for the in-solution digestion technique.

By applying the in-solution digestion-based protocol to the analysis of the acute wound proteome from the various time points, 18 unique proteomic features were collected. Proteins were filtered based on unique identification per time point (*i.e.* being found exclusively at one wound time point only). These unique proteins were further filtered based on molecular function and/or biological process involvement, where only proteins showing potential relation to wound healing were retained. The final proteins revealed were The von Willebrand Factor during haemostasis; osteopontin and prophenin-2 during the inflammation phase, lysozyme C-1, prostaglandin reductase 1, thromboxane-A synthase, arachidonate 15-lipoxygenase and glutamine synthetase during subsiding inflammation and the initial proliferation phases; prophenin and tritrypticin precursor protein, antibacterial peptide PMAP-37 and probable ATP-dependent RNA helicase DDX58 at progression into the proliferation phase; arachidonate 5-lipoxygenase-activating protein, lysozyme C-2 and sterile alpha and TIR motif-containing protein 1 during proliferation and the start of the remodelling phase; and N-acetylmuramoyl-L-alanine amidase, low affinity immunoglobulin gamma Fc region receptor III and tyrosine-protein kinase Fyn during the remodelling phase.

Overall these proteins showed immune and inflammatory related functions as well as neural and cellular adhesion, motility and remodelling involvement. Revealing specific proteins as potential features of the acute wound healing process from the

thousands of molecules within the complex biological wound tissues requires robust sample enrichment and data reduction to enable manageable downstream validation.

Multiple repeats of targeted quantitative analysis of these 18 proteins in different wound samples would be required to compensate for inter- and intra-individual variability and could reveal features and intensity ranges as markers of progressive and predictive acute wound healing. These could be used as clinical diagnostic features or potential therapeutic drug targets for non-healing, chronic wounds.

6.1.5 Conclusion

This study highlighted the power and potential of combining MALDI-IMS and LC-ESI-MS/MS coupled with histology, optimised multivariate analysis and feature statistical analysis to characterise the complex kinetic process of acute wound healing over multiple time points across proteomic, metabolomic and lipidomic bases. These modern mass spectrometric techniques each offered a unique approach to reveal multiple analyte features, time points and identified proteins as potential features of predictive and successful acute wound healing.

Identification of such features within a kinetic process allows for a more comprehensive analysis, while providing smaller manageable and representative datasets. These approaches may aid in comparative healing studies, such as those seeking to better define differences that can be linked to chronic wounds. Using a well-defined but smaller sample set of acute wound-related data as a fixed model for comparison and to highlight potential markers of successful progression of healing, may simplify identification of potential drug targets for chronic wounds.

6.2 Study Limitations and Recommendations

Scientific weaknesses and constraints exist within all experimentation, and cognisance should be made of these when assessing a large study. Some limitations are contained within the foundations of the techniques utilised and may be optimised and corrected for during the study design or through adaptations that need to be made during the study.

While the aims and objectives of this study were achieved, certain limitations existed, some of which could be improved upon in future research.

6.2.1 Animal study

In the animal study, wounds were excised from three pigs according to a rotating schedule. This schedule strictly adhered to animal ethical guidelines whereby the exposure of each pig to anaesthesia over time had to be limited. This made inter-pig biological replicates at each of the six wound excision time points impossible. In future, increasing the number of animals used would aid in overcoming this challenge, however the costs and ethical boundaries incurred may be problematic. To overcome variation in the results due to inter-pig and subtle changes in environmental parameters inherent to this type of study, future studies could use time points closely clustered around the time points of healing phase switching (*i.e.* Days 5 and 8) to confirm histological and molecular features of healing progression, rather than using a fixed time measurement in days, as this can compensate for variability in healing rates.

Intra-pig wound variability may have occurred in the animal study where wound location along the dorsum has the ability to affect healing. Wounds in the upper dorsum near the scapula were exposed to more movement than those in the mid to lower regions, potentially causing healing disturbance. In future, reducing the number of wounds along each psoas muscle ridge would allow for comparable distribution in the mid-dorsal region.

While a biopsy punch was used to create all wounds, ensuring comparable overall size and shape, wound depth did vary subtly as this action was performed by hand. Placing a physical stop around the biopsy punch in future would minimise inter-wound depth variation. Another aspect of the study performed by hand was the excision of progressively healed wound tissue using a scalpel blade. This resulted in wound tissue samples being surrounded by varying amounts of healthy tissue and an inconsistent wound:healthy tissue ratio. This apparently small difference between samples complicated downstream analysis, making quantitative results of specific groups of proteins difficult to interpret. This could be overcome in future by utilising a punch biopsy measured a set distance from the wound edge, ensuring a set volume

of healthy and wound tissue within each individual sample. Consistent sample tissue sizes between the six excision time points would also allow for comparative spectral counts in the MALDI-IMS analysis as sections of tissue areas between different samples would then be the same.

6.2.2 MALDI-IMS

While MALDI-IMS provided feature rich data with the advantage of spatial specificity that is critical for the selective analysis of excised wound tissue in this type of study, the imaging technique presented with a number of noteworthy limitations. Unfortunately, MALDI-IMS exhibits a substantial decrease in sensitivity beyond a mass of 30 kDa when imaging proteins directly from tissue sections. This is mainly due to inefficient ionisation of larger molecules (Francese *et al.*, 2009, Goodwin *et al.*, 2008, Schober *et al.*, 2012). A progressive detection inadequacy exists in the micro-channel plate detectors with respect to increasing molecular mass (Wenzel *et al.*, 2005), where a relatively high ion velocity is essential for secondary electron production and therefore sensitivity decreases as ion mass increases for a fixed acceleration voltage (Brunelle *et al.*, 1997). In this study, proteins larger than 17 kDa were not efficiently detected, significantly limiting the proteomic data that could be collected. This mass limit corresponds to detecting proteins of only about 160 amino acids as the technique generally produces single charged ions. This suggests that the wound metabolome and lipidome would offer a more complete analysis of spatially obtained 'omic' data for potential marker assessment.

One of the complexities in analysing biological tissue is the presence of multiple molecular types, such as proteins, lipids, small organic molecules, matrix ions and salts, which have all previously been shown to cause matrix-dependent ion suppression of one another preventing optimal detection (MacAleese *et al.*, 2009, Stoeckli *et al.*, 2007). When one type of molecule ionises more easily than another or is in a significantly higher relative abundance, ion suppression occurs. An example of this occurrence is with haemoglobin chains, which are easily ionised and often dominate mass spectra, potentially compromising other protein signals in the mass spectrum (Schwartz *et al.*, 2003). This phenomenon is usually avoided by ensuring tissue does not make contact with blood upon sample collection, washing haemoglobin from the tissue surface or by performing tissue profiling where only

selected tissue regions are analysed, avoiding highly vascularised regions. Due to the fact that the blood is endogenous to the wound sample itself, haemoglobin contained within the tissue sections analysed in this study complicated analysis as removing haemoglobin results in the loss of other soluble proteins or peptides that are potential healing markers.

Another potentially limiting factor of MALDI imaging analysis is spatial resolution, which is dependent on the diameter of the laser beam of the instrument in question. To image tissue structures and single cells within mammalian tissue sections, one would require a laser beam with a maximal diameter of approximately 20 μm (Luxembourg *et al.*, 2004). The spatial resolution available for use in this study was 200 μm , limiting the analysis of individual features and complicating correlation with the LC-MS/MS protein identification results. The utilisation of more advanced mass spectrometric applications with progressions in technology such as laser beam filtration (Zavalin *et al.*, 2013), integration of a transmission geometry ion source (Zavalin *et al.*, 2015) and utilisation of 3D printers for matrix application (Tucker *et al.*, 2018) for example, make IMS analysis with high spatial resolution possible. It must however be kept in mind that with increased spatial resolution new challenges arise, including increased data acquisition times as well as increased data file sizes requiring increased processing times and storage capacity. Unfortunately, an improvement in resolution often results in a decrease in sensitivity as the tissue areas ablated by the laser are smaller and less sample is ionised with higher background noise that needs to be filtered out during analysis (Gessel *et al.*, 2014). Additionally it has been found that generating ionised molecules from matrix crystals within a smaller laser spot requires a higher laser fluence, ultimately resulting in extensive and more complex analyte fragmentation (Dreisewerd *et al.*, 1995, Spengler and Hubert, 2002).

Identification of complex molecules, especially peptides and proteins, directly from a mass spectrometry image is challenging (Mascini and Heeren, 2012, McDonnell *et al.*, 2010). Several approaches have been used in an attempt to identify proteins including high resolution on-tissue tryptic digestion (Schober *et al.*, 2012), enzyme spotting using array printers (Groseclose *et al.*, 2007), high resolution MALDI-IMS based on 15T Fourier transform ion cyclotron resonance (FTICR) mass spectrometry (Piga *et al.*, 2017) and utilising spatial restricting hydrogel methods (Harris *et al.*,

2013), which are all labour intensive and require sample specific optimisation. When imaging intact proteins, MALDI-TOF mass spectrometers cannot resolve co-registered peptides (Gessel *et al.*, 2014).

Lipid biochemistry within tissues may present with high complexity as a number of distinct families of lipids are synthesised by cells, all with diverse biological properties and functions. Identification of lipids following MALDI-IMS may be challenging due to ambiguity of identity due to isobaric masses for different lipids, and in some cases may not be possible. Complexities arise based on the molecular weight of the lipid, which following MALDI-IMS may be the theoretical weight $[M + H]^+$ or present as ammonium, sodium or potassium adducts. Additionally, the ion may be a fragment of a larger lipid, typical examples being cholesterol or ceramide with the loss of a water molecule, or phospholipids which easily form a dehydrated diacylglycerol fragment ion due to the loss of a polar head group. It would therefore be critical to assess all possibilities during the identification process. The accumulation of lipids in defined tissue regions are suggestive of certain biochemical processes occurring. With future developments in MALDI-IMS lipidomic analyses it is hoped that specific biochemical events can be directly connected to changes in lipid accumulation with biological relevance (Zemski Berry *et al.*, 2011).

While the absence of molecular identification lacks biological relevance for diagnostic MALDI-IMS applications, the identity of molecules is not always required (Gessel *et al.*, 2014, Schwamborn and Caprioli, 2010a). Multivariate analysis comparing mass profiles from experimental (e.g. wound) versus control (e.g. healthy) tissue can identify condition- or zone- related characteristic mass spectral features. Data from a large sample pool may be used to create prediction models to differentiate acute wound tissue from healthy tissue (and potentially can also be used to identify features unique to chronic wounds) based on distinctive protein, metabolite or lipid features that could be used to identify new drug targets. Trained and optimised multivariate analysis can be used to develop prediction models to perform marker recognition studies.

Processing and analysis of large numbers of well-defined IMS data is crucial for marker discovery and clinical research. Well-established multivariate analyses are used to reveal patterns in the localisation of molecules where each pixel is typically

treated as an independent sample. While such approaches are successfully applied, it must be noted that the complex nature of IMS data has resulted in multiple pre-processing and analytical methods where correct application and understanding of statistical techniques is essential for accurate results. Previous studies have recognised and addressed various methods to aid in statistical and computational imaging mass spectrometry challenges (Alexandrov, 2012, Bemis *et al.*, 2019, Jones *et al.*, 2012, Kooijman *et al.*, 2016). This allows for numerous options in alternative data analysis and data mining however, commercial software platforms often tend to be vendor-specific where external bioinformatics expertise may be required for customising software programs to best meet the statistical needs of the specific research study. Further development in statistical approaches to imaging data and possibly standardised methods in data classification would aid in resolving the current complexities faced in clinical applications of MALDI-IMS (Gessel *et al.*, 2014).

6.2.3 LC-MS/MS analysis

In this study LC-MS/MS analysis was successful in revealing and identifying proteins most likely to be unique features of the different acute wound healing phases. One of the main limitations in this analysis reverts back to the methodology utilised for sample collection in the animal study. Due to the variance in the wound:healthy tissue ratio between the six excision time point samples (see Section 6.2.1), comparative quantitative analysis could not be performed. While relatively abundant proteins per time point were revealed as potential healing phase features in this study, quantitative changes occurring as time progressed in the representative 16-day kinetic wound proteome could not be accurately measured. Wounds encompass endogenous tissue where increases or decreases in abundance of molecules are expected over time, rather than the sudden absence or appearance of molecules compared to non-wounded, healthy tissue. Restricting the ability to continuously measure all identified proteins over 16 days of healing significantly hinders opportunities to reveal potential healing phase features as markers for a model of the successful acute wound healing process. This limitation may be overcome in future studies by excising wounds a fixed distance from the wound edge using a biopsy punch, rather than by manual excision with a scalpel blade (as described in Section

6.2.1). This new approach however may introduce an alternate type of variance into the samples where, as samples heal and decrease in size, excised tissue will be taken closer to the wound centre. Analyses may need to be performed comparing sample tissue taken from the wound edge region versus the wound centre region, to be able to detect any potentially significant differences.

In this study the focus was on extraction of soluble proteins in wound tissue as potential proteomic features for the progression of acute wound healing. Large insoluble extracellular matrix (ECM) proteins are dominant in the skin where proteins such as collagen, elastin and fibronectin are expected to be present in high abundance in both wound and surrounding healthy tissue, complicating acute wound feature analysis. Protein extraction techniques in this study were aimed at excluding the low solubility ECM proteins, ensuring that they did not dominate label-free relative quantitation and obscure the detection and identification of less abundant, soluble proteins. In future studies where the ever-changing amount of wound versus healthy tissue can be controlled for at the different wound excision time points, ECM proteins can be investigated for potential break down or new products as wound healing progresses, to possibly identify additional insoluble proteins associated with acute wound healing as proteomic features.

6.3 Concluding Remarks

This study laid a foundation for the mass spectrometric analysis of potentially overwhelming and complex kinetic, time-based wound tissue datasets. The abilities of modern MALDI-IMS and semi-automated proteomic protocols using unique MagReSyn HILIC Beads with LC-MS/MS analysis were evaluated. Several protocols from sample collection through pre-analysis preparation were optimised to provide identities of soluble proteins involved in wound healing of controlled, uninfected wounds. Multiple analytical approaches and statistical techniques were assessed in their abilities to best characterise the acute wound healing process in a concise and interpretable manner.

Optimal statistical and visualisation tools, time points of most variable healing activity, proteomic, metabolomic and lipidomic wound-related features and protein identities of potential healing phase features, were identified. This knowledge

provides a working platform from which further in-depth analysis can be performed. Independent protocols have been created for future study where isolated tissues representing the height of healing, filtered wound feature masses and a number of identified wound-associated proteins can be further analysed for validation as acute wound healing markers. Future studies may include mass spectrometric analysis of time points clustered around Day 5 and/or 8 wound tissues using higher spatial resolutions inclusive of protein, metabolite and lipid identification as well as quantitative analysis of the continuously changing molecular healing environment to reveal progressive acute wound markers as potential diagnostic tools for monitoring impaired healing.

With the above recommendations and full data validation applied to this study, the analytical and statistical techniques demonstrated have the potential to create a model of predictive acute wound healing for clinical application in the discovery of new key targets in drug development therapy, particularly for non-healing chronic wounds.

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Appendix I: Ethical Approvals



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	An investigation of Carbohydrate Derived Fulvic Acid (CHD FA) as well as Absorbatox®/CHD FA wound dressings in wound dynamics in porcine model
PROJECT NUMBER	H013-15
RESEARCHER/PRINCIPAL INVESTIGATOR	K Sheva

STUDENT NUMBER (where applicable)	UP_02324873
DISSERTATION/THESIS SUBMITTED FOR	PhD

ANIMAL SPECIES	Pigs	
NUMBER OF ANIMALS	6	
Approval period to use animals for research/testing purposes	November 2015-November 2016	
SUPERVISOR	Prof. AD Cromarty	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 30 November 2015
CHAIRMAN: UP Animal Ethics Committee	Signature 

S4285-15



STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/07/30/C

APPLICANT: Professor A Cromarty

SCHOOL: Pharmacology

DEPARTMENT:

LOCATION:

PROJECT TITLE: An investigation of wound dynamics using Carbohydrate Derived Fulvic Acid gel (CHD FA) alone and Absorbatox®/CHD FA combination wound dressings

Number and Species

6 large white Porcine

Approval was given for the use of animals for the project described above at an AESC meeting held on 2015/07/28. This approval remains valid until 2017/08/13.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None

Signed: _____

(Chairperson, AESC)

Date: _____

21st August 2015

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____

(Registered Veterinarian)

Date: _____

21 August 2015

cc: Supervisor: N/A

Director: CAS

Works 2000/Ina0015/AESCCert.wps

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

13/07/2016

Approval Certificate
New Application

Ethics Reference No.: 397/2015

Title: Mass spectrometric proteomic profiling of acute wound healing processes in the porcine model

Dear Kim Sheva

The **New Application** as supported by documents specified in your cover letter dated 5/07/2016 for your research received on the 7/07/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 13/07/2016.

Please note the following about your ethics approval:

- Ethics Approval is valid for 3 years
- Please remember to use your protocol number (**397/2015**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed, PhD

Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

☎ 012 356 3085

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🌐 <http://www.up.ac.za/healthethics>

✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4-59, Gezina, Pretoria

Permission certificate

28 February 2017

Dear: Dr Stoyan Stoychev

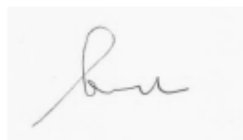
Title: **An investigation of wound dynamics using Carbohydrate Derived Fulvic Acid gel (CHD FA) alone and Absorbatox/CHD FA combination wound dressings (Ref: 205/2017).**

Thank you for submitting your application to the CSIR Research Ethics Committee (REC). The submission was reviewed by the REC on the 23rd of February 2017. The CSIR REC notes the University of Pretoria Animal Ethics Committee and the University of Pretoria Faculty of Health Sciences Research Ethics Committee clearance certificates. Furthermore, that ethics related work was performed at the University of Pretoria as per approved and that the CSIR's responsibility is the processing and analysis according to validated protocols of the wound biopsies samples collected during the animal study. The CSIR REC therefore grants permission for the study to proceed.

Kindly note that you are required to submit **bi-annual progress reports** to the CSIR REC and a **final report** on completion of the research in which you indicate (i) that the research has been completed; (ii) if any new or unexpected ethical issues emerged during the course of the study; and if so, (iii) how these ethical issues were addressed.

We wish you all of the best with your research project.

Kind regards



Dr Shenuka Singh
(CSIR REC Chair)

Appendix II: Animal Monitoring Sheet and Weight Chart

DATE	TIME	WEIGHT	PIA SWELLING	TEMP. °C	NOTES / OBSERVATIONS / INVESTIGATIONS / TREATMENTS	SIGN
18/01/16	9:00				ANIMALS (Pigs) ALLOCATED AS REQUESTED 3.4g (Limping)	NO
19/01/16	10:15	36.1 kg		39.2	Looking well, DENMARK and ART in distress. One pig - slight limp.	
		36 kg				
		36.65 kg				
20/01/16	10:10				Sleeping. Seem content. Started eating & drinking. One still limping.	
21/01/16	10:00				1 Pig (Limping) INJECTED PENICILLIN 36 kg x FANADONE 1.6g T 39.7	NO
21/01/16	10:20				Eating & drinking - content. 2 Pig limping.	
22/01/16					INJECTED FANADONE (Pig limping)	NO
22/01/16	10:10				Sleeping. Content. Ewors up to eat and drink.	
23/01/16					INJECT Limping Pig, FANADONE NO	X10
25/01/16	14:05				ALL PIGS UP & WALKING. Pig A1 seems to have recovered somewhat. ^{ALL EATING RAISINS.}	
27/01/16	08:10				PIG 3 DOESN'T WANT TO STAND UP.	
	10:45				PIGS AWAKE. PIG 3 STILL DOESN'T WANT TO STAND ON HIND LEGS	
	1:55pm				ALL PIGS UP & ABOUT. ALL EATING RAISINS.	
28/01/16					Pig # 3 T. 39.5.	
29/01/16	2:00pm				PIGS ALL UP. ALL EATING RAISINS	
1/02	8:20am				PIGS CONTENT WALKING AROUND	
3/02	13:50				PIGS SEEM FINE. ALL EATING RAISINS	
5/02	8:40				PIGS LOOKING CONTENT WOUNDS HEALING WELL IT SEEMS.	
5/02	13:00				PIGS ALL CONTENT WOUNDS ON AG B LOOK GOOD. ALL EATING RAISINS	
9/02	9:00				PIGS ALL WELL. A+B HEALED WELL.	

ANNUAL WEIGHT CHART

INVESTIGATOR: _____

AISC No. _____

DEPARTMENT:

- Lumping $\rightarrow$ Black Box

[illegible]

Appendix III: Wound Bed Proteome Peak Statistics

(ranked by feature mass)

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
1	1512,69	8,98	< 0.000001	14,9 ± 18,9	13,9 ± 22	10,7 ± 40,9	6,35 ± 35	8,18 ± 39,1	5,97 ± 33,5	7,68 ± 31,2
2	1517,99	10,36	< 0.000001	16,9 ± 20,7	14,6 ± 19,5	8,42 ± 29,8	6,54 ± 37,8	7,05 ± 46,5	6,86 ± 47,2	8,1 ± 43,7
3	1531,24	19,73	< 0.000001	25,8 ± 30	27,3 ± 21,7	13,1 ± 36,5	9,54 ± 39	8,21 ± 35,8	7,58 ± 44,2	9,94 ± 43,7
4	1554,38	10,87	< 0.000001	17,1 ± 19	15,5 ± 20,8	10,2 ± 25,8	7,48 ± 38	6,21 ± 29,7	6,56 ± 54,6	7,83 ± 34,5
5	1588,88	8,18	< 0.000001	13,9 ± 16,8	13,2 ± 16,4	10,3 ± 26,8	6,89 ± 41,2	5,78 ± 28,6	5,74 ± 57,4	6,2 ± 32,2
6	1722,92	7,17	< 0.000001	11,8 ± 18,7	10,6 ± 21,5	5,99 ± 34,3	4,6 ± 33,1	7,53 ± 25,4	5,63 ± 27,7	6,85 ± 27,6
7	1742,15	6,14	< 0.000001	10,8 ± 21,1	9,82 ± 21,4	5,71 ± 23,1	4,7 ± 33,4	7 ± 29,6	5,49 ± 40,4	6,42 ± 11,6
8	1745,25	5,41	< 0.000001	9,44 ± 27	8,39 ± 21,6	5,4 ± 29,5	4,03 ± 35,4	5,96 ± 39,9	4,7 ± 30,3	6,69 ± 16,9
9	1756,52	7,89	< 0.000001	13,5 ± 24,4	13,6 ± 18,5	5,97 ± 17,3	5,82 ± 29,9	7,34 ± 27,5	5,7 ± 40,2	7,53 ± 27,1
10	1780,8	7,67	< 0.000001	12,4 ± 18,1	11,2 ± 20,7	5,54 ± 23,7	5,64 ± 33,4	5,8 ± 25	4,74 ± 37,4	7,27 ± 31,7
11	1799,67	5,71	< 0.000001	11 ± 20,8	9,75 ± 16,7	5,65 ± 16,6	5,36 ± 27,8	6,33 ± 30,8	5,3 ± 42,3	6,42 ± 34,9
12	1815,57	9,88	< 0.000001	14,9 ± 21	14,5 ± 17,6	5,61 ± 22,4	8,46 ± 38,1	5,84 ± 26,9	4,98 ± 21,9	6,92 ± 20,2
13	1977,5	15,3	< 0.000001	13,5 ± 69,1	9,86 ± 23,5	5,88 ± 22,5	21,1 ± 130	13,6 ± 64,8	6,28 ± 24,9	5,81 ± 35,5
14	1985,61	3,89	< 0.000001	8,36 ± 28,3	8,06 ± 27,8	4,56 ± 26,6	4,67 ± 36	6,04 ± 35,7	6,03 ± 30,5	4,47 ± 36,8
15	1989,25	3,76	< 0.000001	7,69 ± 24,4	7,16 ± 27,6	4,67 ± 28,6	3,93 ± 29,1	6,57 ± 40,7	5,98 ± 31,4	5,43 ± 35,5
16	1993,3	3,82	< 0.000001	8,18 ± 22,3	7,7 ± 21,6	5,31 ± 23,8	4,36 ± 23,4	6,84 ± 28,4	5,74 ± 29,9	6,1 ± 32,9
17	2005,41	5,21	< 0.000001	9,68 ± 15,9	8,15 ± 21,2	5,06 ± 21,4	4,48 ± 22,7	6,83 ± 35,7	5,1 ± 31,3	5,26 ± 25,4
18	2008,5	4,78	< 0.000001	8,81 ± 29,2	8,28 ± 24,8	4,89 ± 25,4	4,02 ± 28,1	6,56 ± 43,1	4,3 ± 28,3	6,19 ± 33,2

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
19	2022,68	4,9	< 0.000001	9,66 ± 26,2	8,6 ± 21,4	5,83 ± 24,5	4,77 ± 22,9	6,95 ± 19,2	5,93 ± 33,9	6 ± 17,9
20	2029,2	4,79	< 0.000001	8,91 ± 25,1	7,8 ± 21,8	5,72 ± 21,2	4,12 ± 28,2	5,93 ± 21,9	4,35 ± 57,9	5,3 ± 19,4
21	2041,89	6,16	< 0.000001	11,7 ± 18,1	11,2 ± 19,3	5,79 ± 19,7	5,68 ± 26,5	5,59 ± 30,4	5,58 ± 37,6	6,56 ± 21,1
22	2065,55	5,8	< 0.000001	11,1 ± 18,4	9,54 ± 18,7	6,16 ± 20,5	5,82 ± 32,7	6,96 ± 28,5	5,29 ± 41,9	6,52 ± 14,1
23	2079,66	4,38	< 0.000001	8,98 ± 23	7,33 ± 21,3	6,08 ± 25,9	9,3 ± 102	5,74 ± 33	4,92 ± 29,2	5,09 ± 24,3
24	2179,02	12,2	9,03E-06	8,02 ± 29,7	6,52 ± 21,1	5,17 ± 23,8	16,9 ± 138	5,9 ± 25,1	4,69 ± 33	7,07 ± 40,1
25	3038,17	3,02	0,000215	4,47 ± 33,6	4,39 ± 29,8	4,77 ± 24,6	4,17 ± 41,9	6,59 ± 37,6	4,39 ± 34	3,58 ± 16,5
26	3199,52	8,77	< 0.000001	4,58 ± 23,1	4,57 ± 27,6	6,81 ± 45,7	7,55 ± 68	12,8 ± 57	4,08 ± 29,3	4,01 ± 24,5
27	3206,45	6,26	0,0196	4,72 ± 34,8	4,58 ± 25,2	4,55 ± 30,8	6,97 ± 85,9	10,3 ± 77,5	4,05 ± 38,6	3,99 ± 17,4
28	3221,23	2,95	< 0.000001	4,6 ± 25,7	4,61 ± 21,6	5,1 ± 22,8	3,93 ± 21,4	6,24 ± 27,1	6,87 ± 46,4	4,46 ± 10,1
29	3577,95	5,65	< 0.000001	4,22 ± 22,3	4,4 ± 22,3	4,81 ± 29,2	9,58 ± 101	4,3 ± 28,5	5,28 ± 30,1	3,93 ± 27,2
30	3847,14	2,32	0,000608	3,87 ± 32,4	3,76 ± 24,8	4,08 ± 20,4	5,61 ± 63,2	4,88 ± 28,8	3,62 ± 16,7	3,29 ± 20,7
31	4168,2	0,75	0,258	3,81 ± 25,3	3,67 ± 23,5	3,7 ± 22,1	4,29 ± 35,6	4,41 ± 27,7	3,8 ± 13,2	3,74 ± 23,4
32	4368,04	1,01	0,0549	3,36 ± 23,5	3,24 ± 26,4	3,82 ± 30	4,26 ± 62,5	3,72 ± 32,1	3,83 ± 20,5	3,4 ± 33,6
33	4525,45	2,01	0,000011	3,17 ± 30,2	3,18 ± 29,8	5,17 ± 52	4,25 ± 76,5	3,36 ± 30,2	3,95 ± 21,6	3,47 ± 19,2
34	4655,44	1,84	0,0000293	2,72 ± 31,5	3,11 ± 34,6	4,56 ± 44,5	3,36 ± 37,2	3,7 ± 26,3	3,46 ± 19,6	3,2 ± 24,6
35	4753,54	1,61	< 0.000001	2,97 ± 25,9	3,14 ± 22	3,49 ± 24,3	4,58 ± 45,2	3,3 ± 31,2	4,23 ± 18,6	2,97 ± 15
36	4771,45	29,8	< 0.000001	3,3 ± 40,3	3,37 ± 25,9	4,67 ± 39,2	33 ± 181	4,48 ± 49,6	4,65 ± 35,2	3,24 ± 17,2
37	4793,55	24,3	0,000173	3,21 ± 22,2	3,23 ± 24,9	3,71 ± 20	4,1 ± 56,9	3,75 ± 25,4	27,5 ± 107	3,34 ± 15,4
38	5017,25	5,95	< 0.000001	3 ± 21,3	3,97 ± 77,1	6,6 ± 55,7	8,95 ± 74	3,89 ± 43,2	3,62 ± 23,3	3,24 ± 19,9
39	5033,33	2,07	< 0.000001	2,95 ± 25,4	3,32 ± 29,7	4,42 ± 34,2	5,01 ± 58,5	3,64 ± 19,6	4,3 ± 18,5	3,59 ± 18,5

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
40	5146,99	1,29	< 0.000001	3,06 ± 31,7	2,87 ± 27,9	3,32 ± 29,7	4,16 ± 41,3	3,95 ± 24,8	3,67 ± 28,5	3,08 ± 15,8
41	5523,57	2,03	< 0.000001	2,62 ± 40,5	2,45 ± 28,2	2,96 ± 21,9	2,45 ± 24,8	3,52 ± 32,1	4,29 ± 29,3	4,48 ± 42
42	5536,16	2,9	0,0000061	2,59 ± 26,2	2,48 ± 27,7	2,7 ± 21,7	2,4 ± 26,8	3,3 ± 26,6	3,83 ± 32,8	5,3 ± 55,1
43	6287,43	1,08	< 0.000001	2,27 ± 24,8	2,3 ± 28,9	2,71 ± 24,3	3,29 ± 54,5	3,22 ± 29,5	3,35 ± 19,4	3,21 ± 27,2
44	6303,16	1,32	< 0.000001	2,08 ± 25	2,11 ± 34,4	2,44 ± 22,4	2,92 ± 38,9	2,71 ± 30,2	3,4 ± 32,5	3,39 ± 31,5
45	6317,69	1,91	< 0.000001	1,77 ± 28,7	2,09 ± 33,9	2,38 ± 21,9	2,28 ± 26,3	2,64 ± 31,8	3,68 ± 40,4	2,74 ± 20,3
46	6330,59	1,7	< 0.000001	1,82 ± 24,3	1,96 ± 30,7	2,38 ± 25,2	2,52 ± 35,9	2,74 ± 22,6	3,52 ± 34,2	2,48 ± 18,2
47	6343,26	5,08	< 0.000001	1,99 ± 28,4	2,09 ± 31	2,99 ± 27,3	7,07 ± 106	3,59 ± 47,1	3,44 ± 19,7	3,93 ± 48,6
48	6373,07	5,43	< 0.000001	2,09 ± 26,7	2,23 ± 24,5	2,74 ± 22,2	3,26 ± 50,8	2,84 ± 24,6	7,52 ± 63,2	3,17 ± 26,2
49	6514,44	1,14	< 0.000001	2 ± 27,3	2,1 ± 22,4	2,29 ± 18,3	2,3 ± 22,9	2,77 ± 36,2	2,94 ± 15,5	3,14 ± 17,5
50	6719,24	5,46	< 0.000001	2,07 ± 34,9	1,95 ± 27,7	2,86 ± 23	7,41 ± 107	3,67 ± 54,5	2,91 ± 33,9	4,64 ± 47,5
51	6730,29	6,81	< 0.000001	1,9 ± 30,1	1,95 ± 27,5	2,59 ± 22,9	3,56 ± 69,3	3,45 ± 50,8	3,54 ± 29,3	8,71 ± 59,5
52	6749,97	5,28	< 0.000001	2,13 ± 43,4	2,09 ± 25,4	2,74 ± 22,6	2,44 ± 24	2,84 ± 33,3	7,36 ± 56,7	3,42 ± 30,7
53	7547,39	1,23	< 0.000001	1,5 ± 30,2	1,61 ± 32,6	1,92 ± 23	2,08 ± 32,7	2,3 ± 26,6	2,73 ± 30,4	2,45 ± 40,9
54	7582,19	0,68	< 0.000001	1,85 ± 38,3	1,63 ± 34,3	1,97 ± 23,3	2,31 ± 27,4	2,28 ± 30,5	2,27 ± 33	2,28 ± 25,7
55	7599,87	2,2	< 0.000001	2,04 ± 67,8	1,83 ± 41,2	2,24 ± 21,6	4,03 ± 59,5	2,57 ± 22	2,2 ± 25,9	2,1 ± 36,1
56	7611,14	0,86	< 0.000001	1,82 ± 28,5	1,84 ± 34,4	2,23 ± 25,9	2,59 ± 35,5	2,66 ± 33,1	2,59 ± 30,9	2,68 ± 28,8
57	7635,25	1,4	< 0.000001	1,61 ± 43,8	1,58 ± 27	1,85 ± 21,8	2 ± 24,2	2,12 ± 20,6	2,98 ± 37,4	2,27 ± 18,9
58	7656,78	0,75	0,000116	1,66 ± 65,5	1,62 ± 37,6	1,7 ± 31,1	1,75 ± 28,1	2,14 ± 30,3	2,27 ± 22,4	2,37 ± 22
59	7666,09	1,06	1,73E-06	1,63 ± 39,4	1,67 ± 32,2	1,84 ± 18,5	1,91 ± 23,2	1,94 ± 17,3	2,69 ± 30	2,31 ± 12,2
60	7703,16	0,83	< 0.000001	1,91 ± 40,2	1,61 ± 31,9	1,84 ± 26,2	2,22 ± 29,9	2,33 ± 22,4	2,38 ± 17,1	2,44 ± 38,8

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
61	7725,93	1,23	< 0.000001	1,65 ± 40,6	1,58 ± 33,9	1,78 ± 31,4	1,9 ± 23,1	1,92 ± 22,1	2,8 ± 38,3	2,29 ± 15,7
62	8283,46	0,86	< 0.000001	1,48 ± 44,4	1,69 ± 32,5	2,12 ± 40,5	2,34 ± 53,6	1,84 ± 19,2	2,27 ± 21,3	2,07 ± 18,1
63	8380,96	1,09	< 0.000001	1,46 ± 31,7	1,56 ± 26,6	1,98 ± 26,9	2,56 ± 38,6	2,41 ± 38,1	1,9 ± 18	1,93 ± 23,9
64	8537,85	0,81	< 0.000001	1,32 ± 36,7	1,52 ± 33,5	2,13 ± 31,6	2,09 ± 31,4	1,57 ± 21,9	2,01 ± 28,9	1,64 ± 31,9
65	8586,83	0,86	< 0.000001	1,28 ± 30,1	1,34 ± 32,3	1,71 ± 24,8	1,62 ± 21,7	1,64 ± 12,8	2,14 ± 47,3	1,7 ± 27,2
66	8654,01	0,51	0,0000204	1,49 ± 36,5	1,59 ± 35,4	1,78 ± 29	1,92 ± 28,5	1,64 ± 26,2	2 ± 29	1,84 ± 27,3
67	8880,3	1,48	< 0.000001	1,1 ± 35,3	1,34 ± 33,7	1,51 ± 24,7	1,85 ± 68,3	1,64 ± 25,7	2,58 ± 45,6	1,67 ± 26,4
68	9041,73	1,35	< 0.000001	1,14 ± 30,6	1,2 ± 29,7	1,5 ± 27,1	1,45 ± 28,4	1,76 ± 37,6	2,49 ± 42,4	1,66 ± 24
69	9213,76	1,14	< 0.000001	1,48 ± 35,3	1,27 ± 28,8	1,54 ± 24,5	2,41 ± 61,5	1,83 ± 14,7	1,7 ± 37,9	1,57 ± 27,9
70	10724,05	5,51	< 0.000001	1,15 ± 28,7	1,06 ± 29,3	1,42 ± 29,3	6,57 ± 105	2,02 ± 59,7	1,58 ± 32,3	1,84 ± 31,4
71	10767,17	1,34	< 0.000001	0,86 ± 32,7	0,9 ± 36,8	1,22 ± 35,3	2,2 ± 69,7	1,47 ± 26,9	2,13 ± 41,3	1,19 ± 18,6
72	10933,17	0,74	< 0.000001	0,9 ± 55,3	0,71 ± 41,7	1,07 ± 36,8	1,45 ± 39,3	1,05 ± 29,6	1,24 ± 20,9	1,18 ± 36,7
73	10982,58	0,68	< 0.000001	0,82 ± 43,6	0,82 ± 37	1,07 ± 32,2	1,49 ± 35,2	1,23 ± 42,4	1,26 ± 25	1,09 ± 14,9
74	11374,41	0,68	< 0.000001	0,95 ± 46,8	0,82 ± 43,3	1,13 ± 23,3	1,41 ± 29,7	1,22 ± 35,8	1,24 ± 32,6	1,5 ± 41,1
75	11405,33	1,47	< 0.000001	0,81 ± 31,4	0,92 ± 54,7	1,16 ± 27,2	2,28 ± 48,1	1,9 ± 60,4	1,4 ± 26,1	1,42 ± 30,1
76	11424,22	1,48	< 0.000001	0,81 ± 44,1	0,75 ± 50,3	1,1 ± 34,9	2,24 ± 64,2	1,46 ± 48,5	1,29 ± 35,5	1,33 ± 58,2
77	11440,55	1,28	< 0.000001	0,93 ± 46,6	0,84 ± 38,8	1,19 ± 26,9	2,12 ± 53,2	1,5 ± 32,1	1,54 ± 21,2	2,07 ± 46,6
78	11466,95	1,3	< 0.000001	0,8 ± 41,4	0,76 ± 42,6	1,06 ± 29,9	2,06 ± 60,6	1,21 ± 36,7	1,75 ± 38,1	1,4 ± 27,3
79	11477,92	1,37	< 0.000001	0,83 ± 30,1	0,83 ± 44,3	1,09 ± 25,8	1,78 ± 51	1,19 ± 23,8	2,2 ± 34,3	1,67 ± 19,8
80	11520,79	1,31	< 0.000001	0,74 ± 49,1	0,75 ± 35	1,03 ± 37,4	1,39 ± 31	1,09 ± 28,9	2,05 ± 30,7	1 ± 26,5
81	11559,55	0,86	< 0.000001	0,65 ± 46,2	0,69 ± 43,3	0,96 ± 32,5	1,09 ± 26,9	0,99 ± 40,8	1,5 ± 31,7	1,11 ± 27,2

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
82	12118,57	0,52	< 0.000001	0,61 ± 55	0,56 ± 38,2	0,91 ± 32,8	0,87 ± 27,9	0,88 ± 24,1	1,07 ± 27,9	1,02 ± 24,9
83	12245,13	0,59	< 0.000001	0,64 ± 43,6	0,6 ± 35,2	0,91 ± 26,2	0,94 ± 25,3	0,81 ± 29,1	1,16 ± 26,6	1,19 ± 34
84	12270,73	0,64	< 0.000001	0,74 ± 43,7	0,65 ± 40,1	0,96 ± 32,5	0,95 ± 21,1	0,97 ± 15	0,99 ± 25,8	1,29 ± 35,7
85	13081,18	0,25	< 0.000001	0,63 ± 61,7	0,59 ± 40,1	0,81 ± 29,9	0,76 ± 27,8	0,75 ± 36,6	0,84 ± 42,4	0,72 ± 26
86	13440,71	0,49	< 0.000001	0,52 ± 32,1	0,58 ± 44	0,74 ± 32,3	0,77 ± 24,4	0,64 ± 22,9	1,01 ± 23,6	0,89 ± 36,5
87	13549,14	0,47	< 0.000001	0,52 ± 43	0,47 ± 56,7	0,68 ± 35,1	0,76 ± 30,9	0,58 ± 31,2	0,93 ± 36	0,94 ± 37,9
88	13654,68	0,66	< 0.000001	0,53 ± 48,5	0,49 ± 41,9	0,73 ± 28,7	0,74 ± 22	0,65 ± 24,8	1 ± 27,8	1,15 ± 31,9
89	13759,68	0,5	< 0.000001	0,6 ± 68,2	0,51 ± 49,9	0,73 ± 31,4	1,01 ± 64,7	0,69 ± 28,9	0,95 ± 40,3	0,8 ± 31,2
90	13782,87	0,72	< 0.000001	0,54 ± 55,1	0,51 ± 37,9	0,76 ± 30,9	0,98 ± 42,4	0,66 ± 39	1,02 ± 37,7	1,22 ± 38,6
91	13806,27	0,63	< 0.000001	0,43 ± 44,7	0,44 ± 54,1	0,63 ± 33,6	0,88 ± 40,1	0,64 ± 30,3	0,89 ± 40,4	1,06 ± 22,8
92	13827,27	0,58	< 0.000001	0,45 ± 48,2	0,48 ± 54,6	0,66 ± 30,7	1,03 ± 49,2	0,73 ± 54,3	0,98 ± 28,2	0,84 ± 47,1
93	13855,03	0,77	< 0.000001	0,51 ± 89,2	0,39 ± 43,6	0,6 ± 32,5	0,86 ± 37,2	0,73 ± 37,2	0,82 ± 38,2	1,15 ± 17,6
94	13920,99	1,13	< 0.000001	0,71 ± 51,4	0,6 ± 36,8	0,79 ± 20	1,74 ± 72,8	1,03 ± 45	1,17 ± 33,1	1,31 ± 43,3
95	13944,66	1,03	< 0.000001	0,57 ± 46	0,5 ± 41,7	0,74 ± 39,2	1,52 ± 70,4	0,82 ± 45	1,24 ± 41	1,46 ± 40,4
96	13965,78	1,02	< 0.000001	0,46 ± 43,1	0,49 ± 46,8	0,69 ± 38,2	1,34 ± 60,8	0,75 ± 35,2	1,48 ± 50,2	1,35 ± 44,7
97	14020,68	0,99	< 0.000001	0,47 ± 39,6	0,38 ± 49,2	0,67 ± 29,1	1,23 ± 44	0,93 ± 59,5	1,37 ± 36,5	1,17 ± 51,3
98	14041,67	1,45	< 0.000001	0,67 ± 63,4	0,51 ± 37,7	0,73 ± 27,2	1,25 ± 43,8	0,82 ± 48,6	1,34 ± 35,4	1,95 ± 54,4
99	14083,59	0,89	< 0.000001	0,49 ± 49,1	0,41 ± 44,8	0,67 ± 27,8	1,13 ± 46,2	0,72 ± 43,5	1,27 ± 41,4	1,31 ± 45,5
100	14108,53	0,93	< 0.000001	0,46 ± 51,1	0,37 ± 44,5	0,67 ± 32,4	1,21 ± 51,9	0,65 ± 39,4	1,1 ± 30,1	1,3 ± 40,6
101	14146,8	2	< 0.000001	0,68 ± 41,7	0,54 ± 38,4	0,94 ± 30	2,54 ± 78,4	0,86 ± 32,5	1,5 ± 37,9	1,2 ± 27,1
102	14212,19	1,04	< 0.000001	0,5 ± 47,2	0,43 ± 39,2	0,71 ± 31,8	1,19 ± 49,2	0,65 ± 19,9	1,46 ± 43	0,81 ± 23,9

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
103	14256,05	0,84	< 0.000001	0,53 ± 50,8	0,42 ± 43	0,65 ± 32,8	1 ± 33,8	0,71 ± 33,7	1,26 ± 35,6	0,84 ± 21,3
104	14350,59	0,54	< 0.000001	0,52 ± 66,9	0,42 ± 44,9	0,67 ± 45,3	0,91 ± 35,7	0,61 ± 20,1	0,96 ± 57,7	0,72 ± 31,1
105	14676,76	0,5	< 0.000001	0,45 ± 45,1	0,42 ± 52,2	0,65 ± 34,4	0,65 ± 29,9	0,6 ± 34,5	0,92 ± 32,6	0,65 ± 25,6
106	14737,52	0,47	< 0.000001	0,47 ± 44,5	0,42 ± 39,6	0,69 ± 30,3	0,69 ± 30,2	0,64 ± 42,6	0,87 ± 25,6	0,89 ± 43,9
107	14831,82	0,59	< 0.000001	0,58 ± 53,9	0,45 ± 36,1	0,72 ± 31,7	0,71 ± 29,2	0,52 ± 18,3	1,04 ± 35,2	0,92 ± 29
108	15033,88	1,12	< 0.000001	0,57 ± 53,8	0,51 ± 36,5	0,73 ± 23,2	1,31 ± 70,9	1,63 ± 86,6	0,95 ± 29,7	1,03 ± 18,3
109	15058,65	1,07	< 0.000001	0,55 ± 36,5	0,48 ± 35,1	0,7 ± 25,5	0,98 ± 43,2	0,98 ± 40,5	0,89 ± 29,6	1,55 ± 42,5
110	15193,17	4,39	< 0.000001	1,23 ± 126	0,75 ± 73,4	1,49 ± 50,6	5,13 ± 98,5	2,17 ± 80,9	1,06 ± 32,7	2,03 ± 57
111	15342,68	0,74	< 0.000001	0,46 ± 58,8	0,42 ± 53,7	0,73 ± 39,4	1,16 ± 57	0,93 ± 46,2	0,77 ± 34,3	0,77 ± 23,2
112	15364,44	0,85	< 0.000001	0,57 ± 61,2	0,51 ± 39,5	0,91 ± 34,2	1,36 ± 55,5	0,86 ± 28,4	0,74 ± 25,5	0,92 ± 25,7
113	15399,61	1,09	< 0.000001	0,58 ± 63,2	0,47 ± 51,5	0,86 ± 41,7	1,56 ± 66	0,88 ± 39,7	0,87 ± 22,1	0,9 ± 33,6
114	15479,08	0,74	< 0.000001	0,56 ± 40,6	0,53 ± 37,8	0,8 ± 28,3	1,27 ± 41	0,78 ± 33,1	0,87 ± 34,4	0,99 ± 21,8
115	15518,38	0,6	< 0.000001	0,56 ± 48,2	0,52 ± 35,2	0,73 ± 31	1,12 ± 38,4	0,69 ± 26	1,07 ± 38,5	0,94 ± 39,6
116	15631,17	0,47	< 0.000001	0,54 ± 61,8	0,43 ± 41	0,68 ± 38,2	0,91 ± 30,3	0,6 ± 31,7	0,75 ± 47	0,74 ± 31,9
117	16199,27	0,79	< 0.000001	0,56 ± 36,5	0,47 ± 40,3	0,72 ± 29	1,26 ± 70,7	0,55 ± 25,2	0,72 ± 27,2	0,71 ± 26,6
118	16394,5	0,48	< 0.000001	0,38 ± 55,3	0,41 ± 42,7	0,6 ± 32,3	0,86 ± 46,1	0,45 ± 12,5	0,55 ± 45,8	0,62 ± 23,1
119	18996,04	0,2	< 0.000001	0,25 ± 54,9	0,28 ± 84,6	0,45 ± 32,4	0,43 ± 34,8	0,25 ± 39,1	0,34 ± 33,1	0,34 ± 41,2
120	19531,03	0,23	< 0.000001	0,22 ± 60,1	0,23 ± 48,8	0,45 ± 42,9	0,42 ± 39,4	0,24 ± 28,3	0,34 ± 42,9	0,32 ± 26,2
121	21139,75	0,21	< 0.000001	0,22 ± 50,8	0,22 ± 106	0,42 ± 32,5	0,41 ± 43,4	0,21 ± 40,4	0,36 ± 34	0,34 ± 25,2
122	23079,08	0,25	< 0.000001	0,16 ± 41,2	0,18 ± 161	0,37 ± 34	0,41 ± 38,6	0,22 ± 61,5	0,32 ± 59,2	0,3 ± 37,8
123	24553,68	0,23	< 0.000001	0,26 ± 116	0,15 ± 54	0,34 ± 36,6	0,37 ± 43,2	0,13 ± 35,5	0,26 ± 29,9	0,28 ± 25,5

Index	Mass	D Ave	PKW	Ave H $\pm$ % CV	Ave D3 $\pm$ % CV	Ave D5 $\pm$ % CV	Ave D8 $\pm$ % CV	Ave D10 $\pm$ % CV	Ave D12 $\pm$ % CV	Ave D16 $\pm$ % CV
124	25027,53	0,24	< 0.000001	0,2 $\pm$ 159	0,14 $\pm$ 44,3	0,35 $\pm$ 38,1	0,38 $\pm$ 43,5	0,19 $\pm$ 31,7	0,32 $\pm$ 38,2	0,31 $\pm$ 38,7
125	25433,16	0,21	< 0.000001	0,3 $\pm$ 140	0,21 $\pm$ 58,3	0,42 $\pm$ 39,2	0,39 $\pm$ 41,2	0,21 $\pm$ 47,2	0,31 $\pm$ 39,9	0,39 $\pm$ 28,4
126	27281,01	0,23	< 0.000001	0,24 $\pm$ 127	0,14 $\pm$ 70,4	0,38 $\pm$ 38,6	0,35 $\pm$ 45,9	0,19 $\pm$ 78	0,23 $\pm$ 31,1	0,27 $\pm$ 33,9

(**D Ave**: Difference between the maximal and minimal average feature intensity for healthy tissue and all six time points (arb. u.); **PKW**: P-value calculated by the Kruskal-Wallis test; **Ave *N* $\pm$ % CV**: Feature intensity average of class *N* where 'H'= healthy tissue and 'D'= Day post-wounding; $\pm$ coefficient of variation in % of class *N*)

Appendix IV: Wound Edge Proteome Peak Statistics

(ranked by feature mass)

Index	Mass	D Ave	PKW	Ave H $\pm$ % CV	Ave D3 $\pm$ % CV	Ave D5 $\pm$ % CV	Ave D8 $\pm$ % CV	Ave D10 $\pm$ % CV	Ave D12 $\pm$ % CV	Ave D16 $\pm$ % CV
1	1513,38	7,87	< 0.000001	14,9 $\pm$ 18,9	13 $\pm$ 21,2	10,2 $\pm$ 25,8	7,08 $\pm$ 27,7	8,24 $\pm$ 36,8	9,71 $\pm$ 39,4	10,8 $\pm$ 34,1
2	1517,43	9,41	< 0.000001	16,9 $\pm$ 20,7	13,5 $\pm$ 19,8	9,05 $\pm$ 33,1	7,49 $\pm$ 27,62	8,09 $\pm$ 31,1	10,3 $\pm$ 34,7	12,8 $\pm$ 42,4
3	1531,65	16,15	< 0.000001	25,8 $\pm$ 30	23,8 $\pm$ 29	13,1 $\pm$ 27,2	10,84 $\pm$ 37,27	9,67 $\pm$ 35,9	15,3 $\pm$ 56,6	22,1 $\pm$ 41,7
4	1553,63	9,59	< 0.000001	17,1 $\pm$ 19	14,1 $\pm$ 19,5	11,3 $\pm$ 28,6	8,8 $\pm$ 34,44	7,48 $\pm$ 26	10,5 $\pm$ 44,2	14 $\pm$ 33,1
5	1588,38	7,17	< 0.000001	14 $\pm$ 16,7	12,4 $\pm$ 18,9	10,8 $\pm$ 28,6	7,61 $\pm$ 28,34	6,85 $\pm$ 29,3	11,9 $\pm$ 57,1	12,2 $\pm$ 46,2
6	1723,43	6,01	< 0.000001	11 $\pm$ 21,3	9,59 $\pm$ 25,4	6,25 $\pm$ 28,4	4,95 $\pm$ 22,8	6,27 $\pm$ 30,2	6,25 $\pm$ 41,4	8,65 $\pm$ 36
7	1741,47	5,47	< 0.000001	10,9 $\pm$ 20,4	10,2 $\pm$ 22,7	6,75 $\pm$ 29,6	5,43 $\pm$ 19,34	7,01 $\pm$ 29,4	6,52 $\pm$ 37	8,21 $\pm$ 30,8
8	1758,56	7,13	< 0.000001	13,5 $\pm$ 24,4	13,1 $\pm$ 23,4	7,32 $\pm$ 22,9	6,36 $\pm$ 26,47	7,31 $\pm$ 30,5	7,96 $\pm$ 42	12,2 $\pm$ 31,9
9	1779,86	6,81	< 0.000001	13,2 $\pm$ 20,4	11 $\pm$ 18,6	7,11 $\pm$ 23,3	6,67 $\pm$ 27,14	6,42 $\pm$ 24,1	7,99 $\pm$ 46,2	9,63 $\pm$ 30,2
10	1800,35	5,02	< 0.000001	11 $\pm$ 21	9,73 $\pm$ 19,8	6,42 $\pm$ 22,7	6 $\pm$ 22,52	5,97 $\pm$ 22,7	6,86 $\pm$ 36,5	8,82 $\pm$ 27,9
11	1815,44	9,27	< 0.000001	14,9 $\pm$ 21	13,4 $\pm$ 19,9	6,78 $\pm$ 26,1	9,3 $\pm$ 35,99	6,2 $\pm$ 21,7	8,01 $\pm$ 59,9	15,5 $\pm$ 71
12	1977,15	31,72	< 0.000001	13,6 $\pm$ 68,9	9,33 $\pm$ 18,4	6,27 $\pm$ 20,8	4,98 $\pm$ 23,02	36,7 $\pm$ 103	7,17 $\pm$ 25,9	6,45 $\pm$ 31,7
13	1993,9	3,7	< 0.000001	8,18 $\pm$ 22,3	7,27 $\pm$ 27,6	6,86 $\pm$ 23,9	4,48 $\pm$ 21,55	7,5 $\pm$ 24,4	6,4 $\pm$ 42,8	6,83 $\pm$ 25,3
14	1998,12	3,95	< 0.000001	7,88 $\pm$ 27,3	6,59 $\pm$ 27,4	5,87 $\pm$ 24,8	3,92 $\pm$ 22,04	7,42 $\pm$ 45,4	6,16 $\pm$ 31,3	5,6 $\pm$ 27,8
15	2005,77	4,98	< 0.000001	9,42 $\pm$ 18	7,51 $\pm$ 24,2	5,65 $\pm$ 24,2	4,44 $\pm$ 21,99	7,04 $\pm$ 35,9	5,92 $\pm$ 32,9	6 $\pm$ 27,6

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
16	2008,61	4,19	< 0.000001	8,85 ± 28,5	7,99 ± 20,6	6,13 ± 26,3	4,66 ± 28,3	5,99 ± 32,6	5,54 ± 36,6	7,65 ± 31,9
17	2028,68	4,92	< 0.000001	9,61 ± 23,1	7,92 ± 21,9	6,13 ± 27,1	4,69 ± 20,43	6,47 ± 24,5	6,14 ± 35,6	7,15 ± 18,8
18	2043,41	5,36	< 0.000001	11,3 ± 17,8	10,1 ± 20,3	6,83 ± 23,7	5,91 ± 25,72	6,07 ± 24,1	7,63 ± 28,1	9,45 ± 34,2
19	2065,94	4,89	< 0.000001	10,9 ± 19,3	8,83 ± 18,1	8,86 ± 45,8	5,98 ± 21,63	6,36 ± 19,4	7,5 ± 30	8,6 ± 31,3
20	2178,77	6,88	< 0.000001	7,71 ± 30,3	6,67 ± 27,8	5,39 ± 23,7	4,2 ± 21,99	11,1 ± 76,9	4,92 ± 28,7	6,07 ± 30,9
21	2256,56	5,43	< 0.000001	7,9 ± 15,4	6,96 ± 24,3	5,66 ± 22,5	4,18 ± 19,39	9,62 ± 91,7	5,44 ± 37,9	6,15 ± 25,9
22	2552,73	2,74	< 0.000001	6,78 ± 26,3	6,61 ± 20,2	5 ± 20,2	4,04 ± 20,7	4,95 ± 27,8	5,9 ± 30,1	5,57 ± 29,8
23	3039,23	2,89	< 0.000001	5,39 ± 24,3	5,29 ± 23,3	5,38 ± 20,8	4,13 ± 19,36	7,02 ± 58,4	5,2 ± 27,6	5,23 ± 23,7
24	3199,74	10,94	< 0.000001	5,17 ± 24,8	4,93 ± 20,6	6,24 ± 35	5,74 ± 29,32	15,3 ± 76,9	4,38 ± 19,1	4,4 ± 16,9
25	3566,31	1,31	< 0.000001	4,39 ± 34,5	4,36 ± 22,9	4,31 ± 25,1	3,5 ± 17,44	4,81 ± 47,4	4,23 ± 27	3,6 ± 19,3
26	3577,45	2,39	< 0.000001	4,1 ± 24,3	4,47 ± 24,3	4,2 ± 20,2	5,38 ± 40,06	6,25 ± 56	4 ± 22,6	3,86 ± 25,3
27	3846,6	5,71	< 0.000001	4,12 ± 28,2	4,1 ± 26,2	4,77 ± 33	9,79 ± 46,18	4,24 ± 21,7	4,12 ± 20,2	4,08 ± 25,3
28	3904,88	0,75	0,0892	3,61 ± 30,2	3,98 ± 25,5	3,66 ± 22,2	3,67 ± 23	4,1 ± 31,9	3,78 ± 19,9	3,35 ± 29,8
29	4166,49	2,74	0,000861	3,9 ± 27,5	3,75 ± 28,4	3,72 ± 26,2	3,56 ± 24,02	5,86 ± 58,2	3,41 ± 19,1	3,13 ± 32,7
30	4655,6	1,61	< 0.000001	2,98 ± 24,6	3,83 ± 32,6	4,6 ± 40,1	3,79 ± 29,66	3,18 ± 24,2	3,55 ± 25,1	3,76 ± 28,3
31	4672,42	1,48	< 0.000001	3,23 ± 22,3	3,78 ± 25,4	4,71 ± 29,8	3,36 ± 20,63	3,29 ± 20,3	3,87 ± 29,9	3,51 ± 32,5
32	4770,5	4,41	0,000162	3,25 ± 41,4	3,49 ± 27,4	3,74 ± 43,2	3,41 ± 26,28	7,34 ± 105	3,36 ± 14,3	2,93 ± 20,2
33	5017,47	7,47	< 0.000001	2,92 ± 23,6	4,98 ± 98,9	6,55 ± 52,8	10,4 ± 48,03	3,8 ± 23,4	3,4 ± 18,6	3,84 ± 38,2

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
34	5034,35	3,36	< 0.000001	2,87 ± 27,5	3,59 ± 36,5	5,54 ± 45,1	6,23 ± 55,8	3,56 ± 31,2	4,4 ± 25,7	3,91 ± 28,5
35	5055,8	1,16	< 0.000001	3,1 ± 50,7	3,13 ± 30,5	3,84 ± 33,1	3,08 ± 22,39	2,68 ± 22,3	3,01 ± 27,1	2,98 ± 26,6
36	5130,59	0,7	0,00114	2,71 ± 31,9	3,08 ± 29,8	3,02 ± 22,1	3,41 ± 22,16	2,97 ± 24,5	3,1 ± 24	2,93 ± 28
37	5146,8	1,63	< 0.000001	3,21 ± 30	3,51 ± 28,6	3,15 ± 23,5	4,78 ± 37,36	3,6 ± 24,9	3,4 ± 22,7	3,26 ± 26,1
38	5539,7	0,89	0,0255	2,2 ± 38	2,42 ± 26,7	2,39 ± 29,4	2,32 ± 23,98	2,75 ± 24,2	2,99 ± 35,3	3,08 ± 58,8
39	5600,68	0,93	0,00108	2,26 ± 27,1	2,74 ± 31,4	3 ± 33,4	3,03 ± 24,02	2,85 ± 35,5	2,85 ± 26,1	3,19 ± 51,6
40	6304,46	1,41	< 0.000001	2,24 ± 26,4	2,59 ± 26,7	2,36 ± 25,4	2,6 ± 21,61	2,85 ± 14,7	3,65 ± 36,3	2,69 ± 28,8
41	6323,44	2,05	6,28E-06	1,63 ± 32,9	2,02 ± 35,8	2,01 ± 26,1	2,04 ± 26,68	2,45 ± 29,3	3,68 ± 48,6	2,23 ± 41,9
42	6341,1	1,14	0,0000631	1,95 ± 26,8	2,4 ± 44,8	2,26 ± 32,8	3,09 ± 80,09	2,68 ± 34,9	3,04 ± 35,8	2,36 ± 27
43	6366,06	3,28	< 0.000001	2,3 ± 32,1	2,68 ± 32,9	2,37 ± 22,4	2,98 ± 28,76	2,91 ± 22	5,58 ± 69,6	3,31 ± 60,4
44	6719,04	1,38	< 0.000001	2,08 ± 34,7	2,42 ± 63,1	2,37 ± 30,2	3,46 ± 69,74	3,32 ± 56,7	2,74 ± 35,1	2,54 ± 28,8
45	6735,8	1,57	< 0.000001	1,68 ± 32,1	2,01 ± 32,3	2,09 ± 29,7	2,39 ± 29,7	2,29 ± 37,1	3,17 ± 39,1	3,24 ± 95,1
46	6743,43	2,85	0,0384	1,88 ± 29,8	2,11 ± 31,3	2,07 ± 24,4	2,17 ± 21,41	2,16 ± 31,7	4,72 ± 81,7	2,51 ± 75
47	7363,84	0,83	< 0.000001	1,58 ± 45,6	1,82 ± 33,5	1,86 ± 28,4	2,22 ± 34,79	2,04 ± 32,5	2,42 ± 20,1	2,06 ± 36,7
48	7546,66	1,44	< 0.000001	1,5 ± 30,9	1,79 ± 25,6	1,92 ± 26,8	2,94 ± 40,66	2,17 ± 28,7	1,8 ± 28,5	1,84 ± 50,9
49	7570	0,51	< 0.000001	1,74 ± 47,1	1,68 ± 38	1,85 ± 24,8	2,18 ± 23,58	2,16 ± 33,6	1,86 ± 30,1	1,88 ± 37,5
50	7599,7	1,46	< 0.000001	2,25 ± 56,8	2,12 ± 31,7	2,45 ± 32,5	3,58 ± 49,19	2,86 ± 30,1	2,4 ± 32,2	2,14 ± 40,6
51	7626,57	0,86	1,22E-06	1,75 ± 41,1	1,74 ± 31,4	1,96 ± 27,9	2,2 ± 30,55	2,22 ± 16,5	2,6 ± 40	1,82 ± 44

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
52	7641,97	0,77	2,43E-06	1,47 ± 44,2	1,59 ± 30,6	1,7 ± 27,4	1,96 ± 29,4	2,02 ± 33,2	2,24 ± 29,9	1,78 ± 31,5
53	7701,92	0,46	0,00237	2 ± 36,8	2,11 ± 35,5	2,04 ± 24,3	2,3 ± 24,76	2,3 ± 21,8	2,18 ± 21,8	1,84 ± 28,4
54	7719,59	0,36	0,000038	1,68 ± 40,9	1,74 ± 40,1	1,75 ± 28,4	2,03 ± 24,08	2,04 ± 19,4	1,94 ± 43,2	1,76 ± 38,7
55	7922,37	1,58	< 0.000001	1,57 ± 36,5	1,92 ± 30,8	1,95 ± 35,4	3,16 ± 44,1	2,04 ± 32	2,31 ± 26,4	1,99 ± 32,4
56	8111,47	1,04	< 0.000001	1,51 ± 35,8	1,68 ± 29,4	1,84 ± 23,2	2,55 ± 35,29	1,84 ± 27,4	1,97 ± 23,8	1,59 ± 24,2
57	8282,95	2,83	< 0.000001	1,58 ± 38,9	1,75 ± 27,4	2,1 ± 30,5	4,41 ± 61,81	1,95 ± 21,6	2,16 ± 21,2	1,87 ± 35,3
58	8303,52	0,8	< 0.000001	1,15 ± 37,6	1,51 ± 36,7	1,66 ± 32,6	1,95 ± 35,1	1,38 ± 28,2	1,53 ± 41,7	1,8 ± 41,9
59	8380,83	1,06	< 0.000001	1,47 ± 31,5	1,84 ± 32,7	1,88 ± 26,4	2,53 ± 25,86	2,03 ± 24,1	1,69 ± 47,7	1,59 ± 27,5
60	8539,75	0,68	< 0.000001	1,56 ± 24,5	1,82 ± 32,5	2,24 ± 31,7	2,23 ± 24,63	1,69 ± 24,8	2,1 ± 28,3	1,82 ± 24,6
61	8654,98	0,34	0,00149	1,53 ± 34	1,67 ± 36,5	1,65 ± 28,7	1,87 ± 19	1,74 ± 36,4	1,83 ± 33,4	1,8 ± 24
62	9212,54	0,51	5,81E-06	1,49 ± 31,6	1,4 ± 26,4	1,63 ± 32,1	1,75 ± 26,6	1,91 ± 33,5	1,79 ± 52,9	1,59 ± 26,7
63	9823,09	0,41	< 0.000001	1,15 ± 36,2	1,18 ± 31,5	1,24 ± 25,1	1,55 ± 24,65	1,5 ± 38,2	1,39 ± 27,1	1,32 ± 36,6
64	9906,92	0,53	1,71E-06	0,89 ± 53,4	1,14 ± 40,4	1,18 ± 33,8	1,36 ± 25,73	1,19 ± 30,5	1,42 ± 32,1	1,2 ± 30,6
65	10722,1	0,9	< 0.000001	1,01 ± 30,8	1,09 ± 42	1,19 ± 30,8	1,69 ± 39,88	1,91 ± 26,9	1,4 ± 32,4	1,04 ± 26,1
66	10741,9	0,5	< 0.000001	0,95 ± 39,7	0,91 ± 42	1,05 ± 30,8	1,28 ± 25,23	1,41 ± 35	1,21 ± 26,5	0,95 ± 33,3
67	11205,3	0,38	< 0.000001	0,74 ± 39,7	0,78 ± 35,8	0,91 ± 30,4	1,12 ± 25,6	0,97 ± 39	1,09 ± 40,3	0,91 ± 23,8
68	11405,5	1,3	< 0.000001	0,98 ± 36,3	1,19 ± 50,1	1,18 ± 30	2,28 ± 44,01	1,6 ± 34,6	1,48 ± 32,7	1,3 ± 43,9
69	11433,8	0,93	< 0.000001	0,8 ± 52,9	0,87 ± 47,4	1,02 ± 31,8	1,73 ± 36	1,41 ± 55,6	1,23 ± 33,1	1,23 ± 66,5

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
70	11465,8	1,05	< 0.000001	0,8 ± 32,2	0,92 ± 47,3	1,04 ± 29,5	1,37 ± 30,58	1,48 ± 51,4	1,85 ± 64,5	1,24 ± 87,6
71	11539	0,7	< 0.000001	0,79 ± 54,2	0,9 ± 40	0,97 ± 31,7	1,22 ± 25,05	1,15 ± 36,3	1,49 ± 46,3	1,03 ± 56,1
72	11588,3	0,46	< 0.000001	0,8 ± 46,6	0,75 ± 41,2	0,87 ± 30,8	1,16 ± 23,56	1,06 ± 40,3	1,21 ± 30,6	0,92 ± 56,7
73	11611,5	0,99	< 0.000001	0,73 ± 43,2	0,87 ± 39,5	0,92 ± 32,1	1,72 ± 36,53	1,05 ± 49,1	1,09 ± 34,5	0,84 ± 42,6
74	11626,6	0,77	< 0.000001	0,73 ± 54,4	0,84 ± 39,3	0,92 ± 35,8	1,51 ± 30,45	1,08 ± 50,8	1,08 ± 28,3	0,92 ± 33,3
75	11830,7	0,37	< 0.000001	0,81 ± 37,4	0,86 ± 35,3	0,95 ± 27,6	1,13 ± 21,88	0,98 ± 35,2	1,18 ± 31,7	1,01 ± 32
76	12106,3	0,53	< 0.000001	0,67 ± 45,4	0,73 ± 35,6	0,89 ± 32,6	1,2 ± 30,25	0,81 ± 30,1	0,92 ± 36,3	0,83 ± 29,9
77	13038,3	0,29	7,83E-06	0,64 ± 52,2	0,62 ± 43,8	0,71 ± 28,9	0,79 ± 26,89	0,69 ± 36,5	0,9 ± 44,5	0,7 ± 33,5
78	13411,6	0,28	< 0.000001	0,47 ± 50,6	0,55 ± 42,4	0,71 ± 36,3	0,75 ± 27,16	0,57 ± 22,2	0,74 ± 36,8	0,65 ± 57,6
79	13823,3	0,32	< 0.000001	0,48 ± 43,8	0,54 ± 41,3	0,67 ± 35,6	0,76 ± 28,73	0,8 ± 47,4	0,66 ± 43,4	0,75 ± 67,7
80	13854	0,23	< 0.000001	0,51 ± 87,8	0,49 ± 46	0,59 ± 33,8	0,71 ± 29,02	0,67 ± 50,3	0,69 ± 43,4	0,7 ± 58
81	13908,9	0,44	< 0.000001	0,54 ± 36,5	0,59 ± 48,7	0,71 ± 32,8	0,9 ± 35,8	0,98 ± 57	0,97 ± 32,3	0,79 ± 68,6
82	13925,9	0,42	< 0.000001	0,53 ± 78,1	0,51 ± 65,8	0,61 ± 36,3	0,83 ± 37,75	0,93 ± 66,3	0,83 ± 37,4	0,76 ± 82,4
83	13946,5	0,5	< 0.000001	0,54 ± 50	0,54 ± 55,7	0,63 ± 35,5	0,81 ± 32,59	1,04 ± 63,5	0,81 ± 40,8	0,97 ± 117
84	13980,8	0,5	< 0.000001	0,55 ± 53,7	0,57 ± 58,5	0,64 ± 28,3	0,81 ± 27,54	1,05 ± 53,7	1,03 ± 73	0,86 ± 99,7
85	14020,1	0,7	< 0.000001	0,63 ± 58,5	0,57 ± 46,8	0,73 ± 36,6	0,87 ± 30,09	1,28 ± 61,4	1,02 ± 48,7	0,9 ± 99
86	14054	0,5	< 0.000001	0,62 ± 54,5	0,52 ± 39,8	0,69 ± 33,4	0,82 ± 28,31	1,02 ± 63	0,92 ± 49,2	1,02 ± 111
87	14120,2	0,39	< 0.000001	0,5 ± 40,9	0,47 ± 54,2	0,61 ± 38,9	0,74 ± 35,47	0,86 ± 65,2	0,77 ± 55,5	0,78 ± 76,6

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
88	14149,4	0,45	< 0.000001	0,67 ± 42,4	0,63 ± 56,9	0,74 ± 29,9	1,07 ± 56,54	1,08 ± 59,3	1,01 ± 34,9	0,8 ± 64,3
89	14190	0,45	< 0.000001	0,46 ± 47,8	0,47 ± 52,2	0,59 ± 37,5	0,81 ± 30,59	0,86 ± 63,3	0,9 ± 60	0,74 ± 67
90	14206,4	0,53	< 0.000001	0,5 ± 39,8	0,48 ± 41,3	0,63 ± 37,8	0,77 ± 27,24	1 ± 67,4	1,01 ± 65,3	0,76 ± 57,6
91	14255,9	0,46	< 0.000001	0,55 ± 54,7	0,49 ± 58,1	0,63 ± 33,7	0,75 ± 22,99	0,76 ± 56,1	0,95 ± 52,9	0,64 ± 60,5
92	14735,6	0,28	< 0.000001	0,45 ± 49,4	0,43 ± 43,2	0,61 ± 32,3	0,65 ± 26,33	0,66 ± 44,4	0,71 ± 44,6	0,66 ± 56,2
93	14931	0,26	< 0.000001	0,42 ± 39,8	0,36 ± 47,4	0,59 ± 32,5	0,62 ± 29,01	0,55 ± 47,6	0,58 ± 33,8	0,58 ± 31,9
94	14978,8	0,28	< 0.000001	0,43 ± 49,4	0,47 ± 47,8	0,6 ± 28,8	0,71 ± 28,23	0,62 ± 40,3	0,66 ± 46,7	0,61 ± 44,9
95	15032,7	0,67	< 0.000001	0,57 ± 53,9	0,58 ± 46	0,68 ± 34,5	1,14 ± 58,28	1,25 ± 52,6	0,68 ± 34,8	0,68 ± 42
96	15072,2	0,4	< 0.000001	0,5 ± 43,3	0,46 ± 38,2	0,65 ± 33,3	0,84 ± 37,77	0,85 ± 37,6	0,77 ± 47,6	0,69 ± 51,7
97	15193,4	3,18	< 0.000001	1,2 ± 130	0,65 ± 42,4	1,66 ± 96,2	3,83 ± 93,68	2,04 ± 66	0,9 ± 26,8	0,78 ± 46,5
98	15366,1	0,69	< 0.000001	0,54 ± 64,2	0,48 ± 50,3	0,95 ± 65,8	1,17 ± 48,19	0,85 ± 46,1	0,65 ± 49,7	0,56 ± 57,2
99	15406,9	0,84	< 0.000001	0,68 ± 53	0,57 ± 37,2	1,04 ± 59,2	1,41 ± 50,04	1,03 ± 35,7	0,81 ± 37,9	0,74 ± 31,1
100	15629,1	0,4	< 0.000001	0,55 ± 57,5	0,41 ± 45,1	0,67 ± 35,9	0,81 ± 32,73	0,61 ± 45,3	0,64 ± 52,4	0,5 ± 36,6
101	16199,4	0,55	< 0.000001	0,44 ± 38,2	0,4 ± 46,2	0,69 ± 43,1	0,94 ± 51,47	0,52 ± 31,1	0,39 ± 34,4	0,41 ± 26,4
102	16291,3	0,29	< 0.000001	0,45 ± 43	0,42 ± 46	0,72 ± 50,2	0,67 ± 35,74	0,54 ± 32,4	0,46 ± 32,4	0,45 ± 25,7
103	16323,4	0,37	< 0.000001	0,5 ± 45	0,42 ± 44	0,79 ± 61,3	0,71 ± 34,02	0,55 ± 23,6	0,53 ± 38,2	0,44 ± 39,2
104	16407,3	0,35	< 0.000001	0,4 ± 48,4	0,42 ± 40	0,7 ± 54,4	0,75 ± 32,9	0,56 ± 32,5	0,53 ± 35,6	0,48 ± 34,6
105	20100,6	0,19	< 0.000001	0,23 ± 47,1	0,26 ± 90,7	0,38 ± 40,9	0,42 ± 31,14	0,25 ± 46,6	0,33 ± 30,9	0,29 ± 31,7

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
106	24134	0,22	< 0.000001	0,21 ± 57,6	0,17 ± 54,4	0,36 ± 50,6	0,39 ± 40,86	0,19 ± 57,5	0,23 ± 42,1	0,29 ± 32,6
107	25359	0,21	< 0.000001	0,18 ± 42,4	0,17 ± 56,1	0,34 ± 49,8	0,38 ± 38,59	0,19 ± 41,8	0,2 ± 41,5	0,29 ± 39,1
108	26853,3	0,2	< 0.000001	0,19 ± 46,9	0,16 ± 60,7	0,36 ± 54,2	0,37 ± 39,4	0,17 ± 36,6	0,19 ± 27,7	0,3 ± 34
109	27270,5	0,21	< 0.000001	0,24 ± 127	0,16 ± 48,8	0,36 ± 56,1	0,37 ± 42,83	0,17 ± 37,3	0,21 ± 28,3	0,28 ± 31,5

(**D Ave**: Difference between the maximal and minimal average feature intensity for healthy tissue and all six time points (arb. u.); **PKW**: P-value calculated by the Kruskal-Wallis test; **Ave N ± % CV**: Feature intensity average of class N where 'H'= healthy tissue and 'D'= Day post-wounding; ± coefficient of variation in % of class N)

Appendix V: Whole Wound Proteome Peak Statistics

(ranked by feature mass)

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
1	1513,07	7,97	< 0.000001	14,9 ± 18,9	13,3 ± 19,4	10,3 ± 30,8	6,97 ± 27,6	7,79 ± 41	8,11 ± 44,9	9,84 ± 37,3
2	1517,37	9,58	< 0.000001	16,9 ± 20,7	14,1 ± 17,6	8,89 ± 32,4	7,31 ± 29,8	7,33 ± 38,9	8,8 ± 42,7	11,5 ± 46,7
3	1531,49	18,56	< 0.000001	25,8 ± 30	26,8 ± 27,9	13,1 ± 29,7	10,9 ± 33,6	8,26 ± 37,2	12 ± 64,9	18,7 ± 52,4
4	1553,76	10,32	< 0.000001	17,3 ± 17,5	15,5 ± 19,3	12,7 ± 30,6	8,82 ± 30,6	7 ± 31,9	9,68 ± 62,2	12,4 ± 39,2
5	1588,26	8,18	< 0.000001	13,9 ± 16,8	13 ± 19,2	10,6 ± 28,4	7,79 ± 29,7	5,75 ± 36,4	9,48 ± 66	10,5 ± 52,5
6	1723,05	6,77	< 0.000001	12 ± 19,3	10,6 ± 19,6	6,77 ± 25,8	5,19 ± 22,7	7,56 ± 37,4	7,29 ± 42,9	8,6 ± 35,2
7	1741,32	5,72	< 0.000001	10,8 ± 20,9	9,92 ± 17,5	6,35 ± 30,3	5,11 ± 23,4	6,78 ± 33,6	5,89 ± 40,8	7,59 ± 29,8
8	1758,51	7,24	< 0.000001	13,6 ± 23,5	13,4 ± 17,8	7,11 ± 22,8	6,35 ± 25,7	7,39 ± 29,5	7,49 ± 43,3	10,9 ± 37,2
9	1780,37	7,36	< 0.000001	13,2 ± 20,4	11,2 ± 19,6	6,66 ± 25,5	6,48 ± 25,3	5,87 ± 25,7	6,71 ± 43,7	8,87 ± 34,1
10	1797,67	5,37	< 0.000001	10,6 ± 22,5	9,29 ± 23,8	5,75 ± 24,2	5,22 ± 23,3	5,22 ± 31	5,65 ± 35,2	7,1 ± 32,9
11	1801,54	5,11	< 0.000001	10,1 ± 23,1	9,1 ± 18,9	5,92 ± 24,2	5,73 ± 22,4	5 ± 26,1	5,56 ± 49,9	8,01 ± 32,1
12	1815,45	9,45	< 0.000001	14,9 ± 21	15,1 ± 20,6	6,58 ± 25,9	9,56 ± 33,8	5,65 ± 23,2	6,74 ± 58,4	13,1 ± 76,9
13	1977,43	18,23	< 0.000001	13,5 ± 69,7	8,89 ± 18,5	6,14 ± 21,5	7,78 ± 119	24,4 ± 159	6,72 ± 26,8	6,23 ± 32
14	1985,71	4,38	< 0.000001	8,65 ± 25,5	8,19 ± 24	5,27 ± 28,8	4,27 ± 28,2	7,37 ± 44	6,59 ± 38,9	6,15 ± 36,4
15	1993,57	3,77	< 0.000001	8,18 ± 22,3	7,19 ± 25	6,46 ± 26,2	4,41 ± 21,4	6,57 ± 28,9	6,12 ± 38,1	6,62 ± 27,1

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
16	2005,53	5,13	< 0.000001	9,7 ± 16	7,9 ± 23,2	5,74 ± 24,1	4,57 ± 19,1	7,06 ± 45,3	5,7 ± 33	6,01 ± 27,4
17	2008,86	4,4	< 0.000001	8,92 ± 28,6	7,75 ± 20,6	5,88 ± 27,3	4,53 ± 26,9	5,67 ± 30,2	5,08 ± 34,7	7,31 ± 33,5
18	2022,15	4,39	< 0.000001	9,08 ± 27,4	7,76 ± 20,8	5,97 ± 22,6	4,69 ± 21,5	6,02 ± 30,5	6,15 ± 36,2	6,57 ± 22,8
19	2029,92	5,05	< 0.000001	9,85 ± 21,8	8,3 ± 22,5	6,39 ± 23,9	4,8 ± 19,4	6,2 ± 25,3	5,93 ± 42,7	7,28 ± 26,3
20	2039,87	5,43	< 0.000001	10,7 ± 20,4	9,59 ± 20,9	5,75 ± 24,9	5,24 ± 24	5,27 ± 33	5,86 ± 40,1	7,2 ± 37,5
21	2043,16	5,57	< 0.000001	11,1 ± 19,1	10,8 ± 18,5	6,6 ± 23,7	5,93 ± 24,7	5,57 ± 29,5	6,57 ± 35,3	8,7 ± 35,1
22	2066,14	5,26	< 0.000001	11,1 ± 18,6	8,76 ± 16,8	8,15 ± 45,7	5,82 ± 20	6,94 ± 28,1	6,47 ± 35,8	7,99 ± 31,5
23	2178,8	4,38	< 0.000001	7,87 ± 29,5	6,66 ± 22	5,43 ± 24,2	6,29 ± 106	9,09 ± 93,6	4,71 ± 32,8	6,6 ± 39,9
24	3037,32	6,27	< 0.000001	5,2 ± 25,3	5,13 ± 23,5	5,2 ± 21,8	4,05 ± 20,4	10,3 ± 63,9	4,78 ± 29,1	5,14 ± 24,7
25	3199,61	17,93	< 0.000001	5,18 ± 24,4	4,7 ± 22,1	6,42 ± 37,8	5,81 ± 37	22,3 ± 75,6	4,41 ± 22,2	4,38 ± 18,1
26	3220,27	5,22	< 0.000001	4,62 ± 25,3	4,73 ± 22,2	5,13 ± 22	3,85 ± 18,8	9,07 ± 46,4	5,83 ± 41,2	4,58 ± 21,6
27	3567,3	1,5	< 0.000001	4,35 ± 34,9	4,11 ± 23,2	4,21 ± 24,8	3,47 ± 21,7	4,97 ± 42	4,26 ± 26,3	3,76 ± 22,1
28	3577,7	2,97	< 0.000001	4,25 ± 22,1	4,55 ± 20,6	4,44 ± 22,9	6,63 ± 61,5	6,96 ± 31,1	4,76 ± 27,1	3,99 ± 24,7
29	3846,62	4,39	< 0.000001	3,98 ± 29,7	3,86 ± 21,4	4,56 ± 32,1	8,13 ± 53,9	4,38 ± 25,7	3,94 ± 18,4	3,74 ± 25,3
30	4167,56	2,12	0,058	3,95 ± 27,2	3,75 ± 22,3	3,73 ± 24,1	3,67 ± 23	5,44 ± 65,2	3,7 ± 14,9	3,32 ± 32,2
31	4368,5	0,47	0,236	3,41 ± 22,2	3,52 ± 23,5	3,53 ± 26,9	3,47 ± 26,9	3,87 ± 25,4	3,81 ± 19	3,5 ± 26,2
32	4525,64	1,1	< 0.000001	3,23 ± 30,2	3,2 ± 24,6	4,04 ± 48,2	2,94 ± 22,6	3,68 ± 36,5	3,54 ± 26	3,4 ± 21,9
33	4655,41	1,66	< 0.000001	2,94 ± 24,8	3,11 ± 24,7	4,6 ± 40,9	3,67 ± 31,7	3,54 ± 25,6	3,67 ± 24,3	3,63 ± 26,2

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
34	4672,47	1,36	< 0.000001	3,23 ± 22,3	3,31 ± 20,1	4,58 ± 28,5	3,24 ± 22,5	3,23 ± 23,9	4,1 ± 25,6	3,57 ± 29,1
35	4771,06	3,77	< 0.000001	3,22 ± 41,9	3,14 ± 26,3	3,92 ± 44,1	4,24 ± 83	6,74 ± 103	3,81 ± 35,2	2,97 ± 19
36	4793,57	10,73	< 0.000001	3,21 ± 22,2	3,15 ± 26	3,72 ± 23,5	3,29 ± 18,8	3,78 ± 23,4	13,9 ± 160	3,41 ± 27,4
37	5017,41	7,02	< 0.000001	2,92 ± 23,6	3,26 ± 25,1	6,55 ± 53,5	9,94 ± 64,1	4,04 ± 29,8	3,52 ± 19,9	3,64 ± 36,2
38	5034,2	2,82	< 0.000001	2,95 ± 25,4	3,38 ± 26,2	5,24 ± 43,9	5,77 ± 58	3,65 ± 25,6	4,36 ± 23,2	3,87 ± 25,7
39	5056,02	0,92	< 0.000001	3,21 ± 45,1	3,04 ± 21,2	3,96 ± 32,1	3,1 ± 22,3	3,21 ± 24,7	3,89 ± 33,6	3,29 ± 25,4
40	5146,7	1,57	< 0.000001	3,21 ± 30	2,99 ± 24,6	3,23 ± 25,1	4,56 ± 39,3	3,57 ± 24,3	3,47 ± 20,9	3,21 ± 23,7
41	5413,6	1,01	0,0000133	2,68 ± 25,6	2,74 ± 28,6	2,78 ± 24,2	2,58 ± 19,1	3,23 ± 25,1	3,12 ± 25,6	3,59 ± 41,4
42	5600,2	1,32	9,31E-06	2,44 ± 29,8	2,55 ± 25,3	3,12 ± 30,1	2,93 ± 26,6	3,09 ± 27,4	3,13 ± 26,3	3,75 ± 51,4
43	6286,87	0,93	< 0.000001	2,22 ± 26,4	2,11 ± 30,5	2,24 ± 26,6	2,7 ± 34,5	3,04 ± 24,9	2,91 ± 29,4	2,74 ± 27,7
44	6304,09	1,38	< 0.000001	2,12 ± 29,4	2,19 ± 26,5	2,29 ± 25,8	2,56 ± 22,1	3 ± 25,4	3,5 ± 35,8	2,88 ± 32,4
45	6318,11	1,64	< 0.000001	1,7 ± 33,2	2,04 ± 29,4	2,05 ± 26,6	2,16 ± 24,5	2,65 ± 36,4	3,34 ± 45,1	2,35 ± 35,1
46	6329,81	1,63	< 0.000001	1,78 ± 24,8	1,94 ± 33,9	2,02 ± 28,4	2,11 ± 27,7	2,67 ± 30,3	3,41 ± 33,9	2,12 ± 29,9
47	6342,5	1,79	< 0.000001	2,01 ± 28,1	2,15 ± 27,6	2,48 ± 33,1	3,8 ± 79,4	3,42 ± 37,3	3,21 ± 29,3	2,77 ± 39,2
48	6367,52	3,49	< 0.000001	2,12 ± 39,7	2,12 ± 24,6	2,46 ± 25,3	3,13 ± 32,6	3,25 ± 32,4	5,6 ± 65,8	3,59 ± 55,3
49	6469,82	1,02	0,00113	2 ± 47,7	1,85 ± 29,4	1,97 ± 28,5	1,92 ± 29,2	2,32 ± 36,3	2,87 ± 43	2,07 ± 40,2
50	6718,89	2,24	< 0.000001	2,08 ± 34,6	2,03 ± 30	2,49 ± 29,6	4,27 ± 89,2	4,13 ± 52,8	2,8 ± 31,8	3,45 ± 68,6
51	6732,94	3,03	< 0.000001	1,79 ± 30,9	1,94 ± 30,5	2,22 ± 27,1	2,64 ± 46	2,76 ± 29,5	3,41 ± 34,9	4,83 ± 91,3

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
52	6747,56	3,9	< 0.000001	2,24 ± 38,4	2,26 ± 23,7	2,45 ± 22,6	2,45 ± 19,6	3,26 ± 67,8	6,14 ± 62,7	2,78 ± 47
53	6962,48	1,02	< 0.000001	1,52 ± 34,7	1,75 ± 32,6	1,78 ± 32,2	1,94 ± 26,2	2,55 ± 30,5	2,39 ± 31,9	2,14 ± 33,2
54	7365,38	1,07	< 0.000001	1,69 ± 42,8	1,8 ± 32,1	1,92 ± 27,7	2,25 ± 33,5	2,16 ± 29,7	2,76 ± 31,9	2,21 ± 32,9
55	7546,09	1,07	< 0.000001	1,55 ± 26,9	1,66 ± 29,3	1,95 ± 25,7	2,62 ± 39,4	2,35 ± 28,5	2,16 ± 36,5	2,06 ± 47,3
56	7582,77	0,59	< 0.000001	1,8 ± 39,6	1,65 ± 30,5	1,87 ± 27,3	2,24 ± 27,3	2,18 ± 33	2,16 ± 38,4	1,76 ± 31,6
57	7599,58	2,23	< 0.000001	2,08 ± 66	2 ± 40,1	2,27 ± 32,2	4,03 ± 55,7	2,93 ± 35	2,21 ± 21,7	1,8 ± 35,3
58	7670,39	0,78	7,34E-06	1,48 ± 46,4	1,67 ± 36,1	1,74 ± 31,7	1,89 ± 23,8	1,82 ± 26,7	2,26 ± 31,4	1,76 ± 33,8
59	7704,2	0,65	< 0.000001	1,92 ± 38,5	1,73 ± 39,8	1,94 ± 27,5	2,37 ± 27,4	2,05 ± 22,2	2,34 ± 14	1,99 ± 35,5
60	7720,89	0,79	< 0.000001	1,87 ± 32,8	1,78 ± 22,6	1,94 ± 24,5	2,2 ± 22	2,21 ± 17,7	2,57 ± 35,1	2,17 ± 30,8
61	7922,81	1,14	< 0.000001	1,6 ± 35,5	1,66 ± 23,2	2,01 ± 32,7	2,74 ± 43,2	2,29 ± 22,3	2,46 ± 21,2	2,06 ± 27,5
62	8110,26	0,88	< 0.000001	1,42 ± 40	1,49 ± 34,6	1,74 ± 25,4	2,3 ± 34,5	1,65 ± 28,3	2,2 ± 35,6	1,56 ± 28,5
63	8178	0,5	< 0.000001	1,42 ± 35,8	1,41 ± 31,7	1,63 ± 28,7	1,87 ± 22,8	1,89 ± 21,5	1,91 ± 30	1,7 ± 32,9
64	8282,81	1,95	< 0.000001	1,57 ± 38,9	1,76 ± 29	2,13 ± 32,5	3,52 ± 66,3	2,06 ± 20,4	2,24 ± 20,8	1,95 ± 30,9
65	8381,73	1,36	< 0.000001	1,48 ± 30,8	1,58 ± 27,7	1,91 ± 26,4	2,84 ± 37,7	2,02 ± 24,7	1,75 ± 38,5	1,71 ± 29
66	8538,98	0,96	< 0.000001	1,33 ± 36	1,59 ± 37,6	2,16 ± 34	2,29 ± 27,4	1,7 ± 30,5	2,03 ± 29,7	1,7 ± 29,4
67	8653,91	0,51	< 0.000001	1,47 ± 38	1,56 ± 34	1,67 ± 29,3	1,98 ± 26,4	1,66 ± 33,2	1,94 ± 29,4	1,78 ± 23,2
68	9213,12	0,65	< 0.000001	1,5 ± 31,3	1,37 ± 30,8	1,65 ± 30,2	1,85 ± 46,6	2,02 ± 31,5	1,8 ± 44,3	1,57 ± 26,5
69	10237,92	0,47	< 0.000001	1,08 ± 30,6	0,98 ± 38,4	1,13 ± 25,8	1,45 ± 25,9	1,27 ± 34,6	1,31 ± 31,7	1,21 ± 29,3

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
70	10473,33	0,59	< 0.000001	0,91 ± 31,3	0,88 ± 33,7	1,11 ± 24,9	1,23 ± 24,4	1,18 ± 22,9	1,47 ± 29,1	1,05 ± 32,9
71	10723,54	3,15	< 0.000001	1 ± 34,3	0,94 ± 34	1,2 ± 30,7	4,1 ± 160	1,91 ± 37,5	1,45 ± 33,9	1,23 ± 43,5
72	10766,23	0,93	< 0.000001	0,73 ± 40,1	0,79 ± 39,2	1,03 ± 32,4	1,66 ± 69,3	1,29 ± 32,8	1,49 ± 56,3	0,92 ± 31,5
73	10950,96	0,39	< 0.000001	0,85 ± 40,7	0,85 ± 37,1	1 ± 27,7	1,24 ± 34,9	1,18 ± 30,6	1,23 ± 39,5	1,02 ± 34,9
74	11406,12	1,34	< 0.000001	0,96 ± 36,3	0,91 ± 33,2	1,16 ± 31,1	2,25 ± 43,6	1,39 ± 39,1	1,47 ± 29,3	1,39 ± 38,1
75	11424,49	1,09	< 0.000001	0,74 ± 50	0,71 ± 37,6	0,99 ± 36,8	1,8 ± 45,9	1,29 ± 45,4	1,17 ± 45,8	1,2 ± 63,8
76	11435,11	1,09	< 0.000001	0,79 ± 54,3	0,74 ± 33,1	1,02 ± 31,2	1,83 ± 39,6	1,12 ± 40	1,32 ± 28,2	1,39 ± 62,3
77	11463,64	1,06	< 0.000001	0,84 ± 41	0,81 ± 37,7	1,06 ± 31	1,57 ± 37,4	1,33 ± 32,3	1,87 ± 52,9	1,44 ± 82,2
78	11539,09	0,82	< 0.000001	0,84 ± 54	0,74 ± 30,9	1 ± 31,8	1,26 ± 24,6	1,03 ± 27,8	1,56 ± 43,3	1,12 ± 45,9
79	11582,25	0,67	< 0.000001	0,88 ± 38,6	0,7 ± 32,9	1 ± 28,9	1,24 ± 25,9	1,1 ± 28,9	1,37 ± 34,1	1,02 ± 44,4
80	11612,09	0,82	< 0.000001	0,74 ± 43,8	0,75 ± 37,2	0,91 ± 31	1,56 ± 37,7	0,95 ± 27,9	1,25 ± 34,7	0,9 ± 38,3
81	12082,56	0,35	< 0.000001	0,63 ± 55,8	0,6 ± 33,9	0,82 ± 30,8	0,92 ± 26,8	0,8 ± 29,1	0,96 ± 35	0,93 ± 32
82	12106,45	0,46	< 0.000001	0,68 ± 42,8	0,71 ± 34,2	0,95 ± 31,1	1,15 ± 30,4	0,88 ± 37,9	1,02 ± 35	0,95 ± 34,2
83	12349,97	0,48	< 0.000001	0,65 ± 34,6	0,74 ± 36,2	0,9 ± 31,3	0,98 ± 25,1	0,93 ± 29,1	1,13 ± 25,4	1,03 ± 35,9
84	12431,76	0,31	< 0.000001	0,58 ± 60	0,55 ± 39,8	0,75 ± 36,6	0,84 ± 29,1	0,81 ± 38,6	0,87 ± 33,5	0,78 ± 33,1
85	13557,4	0,35	< 0.000001	0,52 ± 42,7	0,46 ± 49,6	0,66 ± 25,9	0,8 ± 30,1	0,61 ± 40,2	0,81 ± 44,3	0,62 ± 36,5
86	13780,69	0,36	< 0.000001	0,54 ± 55,1	0,54 ± 45,6	0,71 ± 28,2	0,86 ± 27,8	0,68 ± 33,1	0,9 ± 40	0,9 ± 48,3
87	13826,24	0,38	< 0.000001	0,51 ± 43,2	0,51 ± 38,1	0,68 ± 34,7	0,88 ± 31,3	0,79 ± 36,6	0,84 ± 39,9	0,8 ± 58,2

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
88	13923,55	0,61	< 0.000001	0,54 ± 77,3	0,43 ± 33,9	0,62 ± 34,6	1,04 ± 49,8	0,85 ± 35,1	0,89 ± 36,4	0,9 ± 74,7
89	13947,1	0,62	< 0.000001	0,55 ± 49,1	0,5 ± 34,8	0,67 ± 36,6	0,99 ± 43	0,81 ± 30,4	1,01 ± 45,4	1,12 ± 90,6
90	13971,21	0,75	< 0.000001	0,57 ± 40,3	0,52 ± 40	0,71 ± 34,1	0,98 ± 34,5	0,82 ± 33,2	1,27 ± 60,3	1,12 ± 85
91	14017,99	0,8	< 0.000001	0,62 ± 59,1	0,45 ± 35,3	0,72 ± 35	1,02 ± 37,1	1,04 ± 32,7	1,25 ± 47,1	1,04 ± 82,9
92	14040,97	0,79	< 0.000001	0,65 ± 52,6	0,51 ± 45,6	0,73 ± 31,7	0,97 ± 32,8	0,89 ± 38,9	1,11 ± 48	1,3 ± 90,6
93	14147,57	0,95	< 0.000001	0,56 ± 42,8	0,47 ± 34,2	0,71 ± 30	1,43 ± 75,2	0,92 ± 51,7	1,11 ± 47,5	0,87 ± 57,9
94	14206,62	0,81	< 0.000001	0,57 ± 41,9	0,51 ± 35,1	0,7 ± 36,1	0,95 ± 36,7	0,81 ± 26,8	1,32 ± 54,3	0,82 ± 47,6
95	14255,22	0,59	< 0.000001	0,59 ± 48,7	0,51 ± 45,3	0,66 ± 31,5	0,86 ± 27	0,75 ± 33,2	1,1 ± 43,5	0,74 ± 45,4
96	14931,03	0,26	< 0.000001	0,42 ± 41,1	0,4 ± 44,1	0,6 ± 32,5	0,64 ± 27,3	0,59 ± 30	0,65 ± 46,2	0,61 ± 30,9
97	14951,26	0,36	< 0.000001	0,5 ± 45,6	0,38 ± 36,1	0,59 ± 30,8	0,69 ± 29,4	0,6 ± 31,7	0,74 ± 39,9	0,63 ± 31,2
98	14979,31	0,33	< 0.000001	0,38 ± 45	0,42 ± 49,7	0,59 ± 31,2	0,72 ± 29,5	0,59 ± 42,7	0,67 ± 37,1	0,68 ± 43,2
99	15032,14	0,87	< 0.000001	0,57 ± 54,3	0,48 ± 37,2	0,69 ± 31,6	1,3 ± 65	1,35 ± 61,3	0,76 ± 33,3	0,78 ± 39,6
100	15192,95	3,96	< 0.000001	1,23 ± 126	0,81 ± 74,1	1,64 ± 86,6	4,76 ± 96,2	2,11 ± 67	1,09 ± 43,8	1,17 ± 74,6
101	15363,86	0,84	< 0.000001	0,62 ± 54,6	0,53 ± 54,1	0,98 ± 54,1	1,37 ± 51,5	0,85 ± 35,3	0,76 ± 40,9	0,7 ± 46,9
102	15399,85	1,03	< 0.000001	0,71 ± 50,3	0,61 ± 43,6	1,04 ± 52	1,64 ± 56,4	1 ± 32	0,92 ± 32,8	0,83 ± 37,1
103	15475,8	0,65	< 0.000001	0,56 ± 40,2	0,48 ± 47,1	0,8 ± 41,3	1,13 ± 39,1	0,81 ± 38,2	0,85 ± 34,1	0,72 ± 46,1
104	15519,03	0,56	< 0.000001	0,53 ± 52,5	0,45 ± 43,4	0,75 ± 39,5	1,01 ± 37,2	0,67 ± 27,5	0,91 ± 43,6	0,65 ± 46,8
105	15593,57	0,51	< 0.000001	0,44 ± 53,3	0,43 ± 49,4	0,69 ± 36,9	0,93 ± 34,4	0,58 ± 37,2	0,7 ± 48,5	0,6 ± 40,5

Index	Mass	D Ave	PKW	Ave H $\pm$ % CV	Ave D3 $\pm$ % CV	Ave D5 $\pm$ % CV	Ave D8 $\pm$ % CV	Ave D10 $\pm$ % CV	Ave D12 $\pm$ % CV	Ave D16 $\pm$ % CV
106	15630,24	0,49	< 0.000001	0,52 $\pm$ 65,5	0,4 $\pm$ 42,5	0,65 $\pm$ 36,3	0,89 $\pm$ 32	0,53 $\pm$ 37,4	0,66 $\pm$ 52,5	0,57 $\pm$ 41,4
107	16101,75	0,36	< 0.000001	0,38 $\pm$ 51,5	0,33 $\pm$ 36,7	0,55 $\pm$ 33,7	0,68 $\pm$ 27,5	0,52 $\pm$ 47	0,56 $\pm$ 48,7	0,5 $\pm$ 32,3
108	16199,84	0,69	< 0.000001	0,6 $\pm$ 31,2	0,56 $\pm$ 42,2	0,78 $\pm$ 34,3	1,25 $\pm$ 63,1	0,64 $\pm$ 20,6	0,68 $\pm$ 29,9	0,6 $\pm$ 32
109	16320,32	0,42	< 0.000001	0,53 $\pm$ 39,6	0,44 $\pm$ 32,3	0,78 $\pm$ 55,9	0,86 $\pm$ 36,4	0,53 $\pm$ 21,9	0,58 $\pm$ 31,1	0,53 $\pm$ 36,9
110	16386,78	0,48	< 0.000001	0,39 $\pm$ 42,6	0,4 $\pm$ 42,5	0,67 $\pm$ 52,2	0,86 $\pm$ 43,2	0,49 $\pm$ 35,4	0,51 $\pm$ 42,3	0,49 $\pm$ 39,7
111	16406,79	0,48	< 0.000001	0,4 $\pm$ 49,4	0,39 $\pm$ 44,6	0,68 $\pm$ 51	0,87 $\pm$ 40,1	0,47 $\pm$ 34,5	0,52 $\pm$ 43,1	0,49 $\pm$ 34,2
112	16526,58	0,38	< 0.000001	0,48 $\pm$ 67,7	0,36 $\pm$ 37,9	0,67 $\pm$ 45,6	0,74 $\pm$ 33,7	0,45 $\pm$ 22,1	0,45 $\pm$ 23	0,45 $\pm$ 34,8

(**D Ave**: Difference between the maximal and minimal average feature intensity for healthy tissue and all six time points (arb. u.); **PKW**: P-value calculated by the Kruskal-Wallis test; **Ave N $\pm$ % CV**: Feature intensity average of class N where 'H'= healthy tissue and 'D'= Day post-wounding; $\pm$ coefficient of variation in % of class N)

Appendix VI: Whole Wound Metabolome Peak Statistics

(ranked by feature mass)

Index	Mass	D Ave	PKW	Ave H $\pm$ % CV	Ave D3 $\pm$ % CV	Ave D5 $\pm$ % CV	Ave D8 $\pm$ % CV	Ave D10 $\pm$ % CV	Ave D12 $\pm$ % CV	Ave D16 $\pm$ % CV
1	524,89	2,89	< 0.000001	1,72 $\pm$ 46,3	1,84 $\pm$ 35,5	4,61 $\pm$ 45,2	3,03 $\pm$ 183	3,37 $\pm$ 95,9	3,24 $\pm$ 69	2,93 $\pm$ 43
2	525,66	2,27	< 0.000001	2,02 $\pm$ 34,9	2,71 $\pm$ 90,2	2,11 $\pm$ 46,9	4,21 $\pm$ 75,6	2,65 $\pm$ 58,1	2,37 $\pm$ 53,2	4,29 $\pm$ 55,2
3	526,92	4,59	< 0.000001	2,63 $\pm$ 67,9	1,98 $\pm$ 40,8	6,57 $\pm$ 34,8	2,47 $\pm$ 56,6	2,92 $\pm$ 81,6	4,08 $\pm$ 72,9	3,28 $\pm$ 49,9
4	541,68	4,42	< 0.000001	1,56 $\pm$ 61,4	2,09 $\pm$ 74,1	1,93 $\pm$ 62,7	3,19 $\pm$ 67,6	5,99 $\pm$ 119	2,43 $\pm$ 70,6	3,61 $\pm$ 53,7
5	545,68	3,45	< 0.000001	2,55 $\pm$ 39,2	4,51 $\pm$ 83,6	1,07 $\pm$ 89,7	2,72 $\pm$ 47,4	1,49 $\pm$ 64,5	1,54 $\pm$ 56,4	3,25 $\pm$ 46,5
6	547,7	3,94	< 0.000001	4,02 $\pm$ 38,9	4,3 $\pm$ 68,4	6,03 $\pm$ 91,2	4,48 $\pm$ 66,7	2,09 $\pm$ 71,1	2,69 $\pm$ 56,6	5,57 $\pm$ 54,1
7	559,78	5,28	< 0.000001	3,5 $\pm$ 42	4,72 $\pm$ 44,2	1,27 $\pm$ 40,1	5,64 $\pm$ 42,6	3,9 $\pm$ 91,8	3,6 $\pm$ 72,8	6,55 $\pm$ 41,8
8	560,79	2,14	< 0.000001	2,85 $\pm$ 57,5	3,51 $\pm$ 46,2	1,83 $\pm$ 54,2	3,97 $\pm$ 32,8	2,78 $\pm$ 58,3	2,47 $\pm$ 52,9	3,63 $\pm$ 29,2
9	561,78	2,61	< 0.000001	2,59 $\pm$ 31,6	3,17 $\pm$ 29,5	1,37 $\pm$ 40,1	3,98 $\pm$ 38,6	2,46 $\pm$ 66,2	2,2 $\pm$ 60,5	3,92 $\pm$ 35,3
10	563,78	1,93	< 0.000001	1,87 $\pm$ 37,6	2,33 $\pm$ 53,5	3,46 $\pm$ 71,7	2,83 $\pm$ 66,9	1,79 $\pm$ 62,2	1,52 $\pm$ 67,1	2,92 $\pm$ 49,6
11	566,01	7,57	< 0.000001	8,69 $\pm$ 114	8,91 $\pm$ 108	11,6 $\pm$ 57,6	8,77 $\pm$ 81,4	10,7 $\pm$ 71,2	4,52 $\pm$ 73,6	4,05 $\pm$ 59,3
12	567,01	5,4	< 0.000001	4,55 $\pm$ 95,9	5,1 $\pm$ 103	8,45 $\pm$ 58,7	6,36 $\pm$ 80,8	7,89 $\pm$ 61,1	3,42 $\pm$ 52,9	3,06 $\pm$ 58,3

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
13	568,02	3,23	< 0.000001	5,6 ± 37,6	5,51 ± 52,1	4,97 ± 56,3	4,88 ± 57,2	5,06 ± 61,7	2,38 ± 50,8	2,93 ± 31,1
14	576,87	8,4	< 0.000001	3,01 ± 45,9	5,1 ± 33,8	1,08 ± 110	4,78 ± 53,2	1,57 ± 102	4,47 ± 137	9,48 ± 65,6
15	577,79	47,83	< 0.000001	33,1 ± 49,8	53 ± 38	5,12 ± 207	38,7 ± 58,5	8,55 ± 167	11,9 ± 114	41,2 ± 58,3
16	579,84	32,47	< 0.000001	33,6 ± 42,1	39,4 ± 28,9	6,88 ± 147	37,9 ± 63,6	7,72 ± 142	9,79 ± 99,9	36,4 ± 65,1
17	582,16	58,64	< 0.000001	63,6 ± 56,4	50,4 ± 51,5	4,99 ± 128	59,3 ± 58,3	9,03 ± 168	6,97 ± 95,3	22,6 ± 53,7
18	583,14	12,89	< 0.000001	13,4 ± 56,4	12,6 ± 56,2	1,74 ± 90,8	14,6 ± 58,7	3,45 ± 118	2,76 ± 73,4	5,92 ± 45,4
19	599,95	3,63	< 0.000001	5,09 ± 52,1	4,83 ± 45,3	1,7 ± 72	5,22 ± 67,2	1,64 ± 111	2,01 ± 81,8	5,27 ± 51,7
20	604,42	15,36	< 0.000001	16,8 ± 42,2	17 ± 44,9	3,97 ± 49	4,09 ± 65,7	1,61 ± 59,1	3,34 ± 66,8	7,78 ± 45
21	605,41	5,41	< 0.000001	6,98 ± 38,7	6,88 ± 42,3	1,95 ± 43,4	2,38 ± 44,1	1,57 ± 41,3	1,8 ± 60	3,62 ± 42
22	606,47	20,19	< 0.000001	22,5 ± 39,7	21,5 ± 44,3	5,74 ± 50,6	3,91 ± 87,6	2,26 ± 63,5	5,55 ± 93,2	14,6 ± 53,4
23	607,49	7,03	< 0.000001	8,82 ± 40,3	8,56 ± 46,4	2,57 ± 57,3	2,71 ± 57,8	1,79 ± 53,6	3,1 ± 68,2	6,23 ± 47,2
24	608,49	7,35	< 0.000001	9,33 ± 36,2	9,05 ± 51,4	3,55 ± 47,2	3,2 ± 57,6	1,98 ± 48,3	5,31 ± 57,8	7,07 ± 51
25	609,46	2,12	< 0.000001	3,58 ± 35,8	3,91 ± 39	1,79 ± 44,9	2,61 ± 42	1,84 ± 46,7	2,98 ± 78,9	3,22 ± 38,1
26	617,2	13,18	< 0.000001	13,1 ± 130	14,1 ± 125	18,4 ± 55,3	12,7 ± 93,6	16,9 ± 76,7	9,46 ± 74,6	5,25 ± 103

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
27	618,19	9,06	< 0.000001	9,39 ± 121	9,97 ± 115	13,7 ± 54,7	10,2 ± 81,9	13,5 ± 59,8	7,71 ± 74,7	4,61 ± 75,8
28	619,23	208,2	< 0.000001	144 ± 122	173 ± 111	270 ± 44	193 ± 91,2	285 ± 62,6	147 ± 93	76,9 ± 133
29	620,22	163,7	< 0.000001	103 ± 128	123 ± 120	205 ± 48,7	140 ± 95,7	215 ± 57,4	108 ± 87,8	50,9 ± 135
30	621,19	67,09	< 0.000001	42,9 ± 129	47,2 ± 130	77,3 ± 54,6	53,4 ± 96	84,7 ± 58	40,9 ± 82,1	17,6 ± 126
31	622,15	17,75	< 0.000001	14,5 ± 105	14,1 ± 118	21,6 ± 50,2	17,1 ± 79,9	24,8 ± 64,1	12,4 ± 96,8	7,09 ± 71,5
32	623,18	5,25	< 0.000001	5,79 ± 75	5,79 ± 87,5	6,68 ± 51,5	6,6 ± 62	8,21 ± 54,6	3,83 ± 101	2,96 ± 47,2
33	624,11	2,18	< 0.000001	3,93 ± 35,4	3,94 ± 42,5	3 ± 51,4	4,3 ± 40,5	4,18 ± 52,4	2,12 ± 72,2	3,43 ± 40,1
34	632,32	1,56	< 0.000001	2,43 ± 26	3,14 ± 20	1,97 ± 37,3	3,52 ± 30,3	1,98 ± 50,9	2,93 ± 57,5	3,24 ± 26,3
35	633,28	4,95	< 0.000001	3,79 ± 72,5	4,97 ± 87,7	7,73 ± 69,1	6,79 ± 75,7	5,02 ± 67,1	2,86 ± 57,3	2,79 ± 36,4
36	634,26	3,68	< 0.000001	4,03 ± 52,1	5,02 ± 65,4	6,79 ± 55,6	6,73 ± 62,4	4,49 ± 54,5	3,11 ± 45,4	3,47 ± 25,6
37	635,26	2,89	< 0.000001	4,27 ± 66,8	3,85 ± 55,5	4,93 ± 44,8	5,78 ± 67,8	3,75 ± 47,5	2,89 ± 44	3,86 ± 58,2
38	636,23	2,35	< 0.000001	4,13 ± 60,8	3,94 ± 36,7	3,34 ± 35,2	5,2 ± 36,8	3,02 ± 48,3	2,85 ± 34,7	4,23 ± 44,2
39	637,45	3,53	< 0.000001	5,28 ± 27,5	5,79 ± 43,4	2,63 ± 28	5,88 ± 42,2	2,62 ± 74,3	2,35 ± 50,7	3,03 ± 34,9
40	642,27	3,67	< 0.000001	3,39 ± 58,9	3,03 ± 47,1	6,05 ± 49,3	3,8 ± 48,1	2,73 ± 50,7	2,69 ± 59,2	2,38 ± 32

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
41	646,15	6,33	< 0.000001	2,75 ± 30,3	3 ± 40,7	1,23 ± 30,9	3,57 ± 49,5	1,84 ± 56,5	7,56 ± 186	3,36 ± 33,6
42	649,4	1,27	< 0.000001	2,44 ± 56,4	2,63 ± 50,8	3,27 ± 39,3	3,47 ± 52,6	2,53 ± 45	2,2 ± 44,8	2,27 ± 28,4
43	650,37	1,76	< 0.000001	2,69 ± 48,5	2,9 ± 45,4	3,66 ± 36,3	4,01 ± 50,7	2,86 ± 41,8	2,25 ± 34	2,57 ± 30,2
44	656,32	2,98	< 0.000001	2,17 ± 31,7	2,39 ± 33,6	1,52 ± 44,2	4,51 ± 73,3	1,96 ± 57,5	2,5 ± 48,5	2,72 ± 31,8
45	658,35	2,17	< 0.000001	2,16 ± 26,9	2,36 ± 30	4,17 ± 36,7	4,33 ± 44,6	3,42 ± 52,6	3,52 ± 39,7	2,9 ± 30
46	664,37	2,66	< 0.000001	2,87 ± 34	3,29 ± 34,2	1,71 ± 40,3	4,23 ± 60,5	1,57 ± 72,1	1,67 ± 52,6	3,76 ± 44,4
47	666,36	3,4	< 0.000001	2,98 ± 35,9	3,63 ± 36,5	1,3 ± 61	4,7 ± 62,7	1,64 ± 60,7	1,73 ± 56,7	3,77 ± 52,9
48	668,36	2,63	< 0.000001	2,9 ± 32,2	3,49 ± 43,8	1,16 ± 42,5	3,79 ± 51,6	1,43 ± 57,9	1,59 ± 50,7	3,42 ± 37,6
49	671,63	9,04	< 0.000001	4,63 ± 80,7	3,25 ± 58,7	11,3 ± 53,5	4,45 ± 59,2	3,88 ± 50,3	2,3 ± 47,2	2,36 ± 38,6
50	687,66	6,63	< 0.000001	2,32 ± 22,7	2,18 ± 24	8,69 ± 49,7	2,8 ± 38,7	3,99 ± 53,8	2,06 ± 62,8	2,1 ± 27,2
51	688,47	1,91	< 0.000001	2,19 ± 21,7	2,44 ± 31,3	3,87 ± 43	2,85 ± 39,4	2,74 ± 39,6	1,96 ± 60,1	2,61 ± 35
52	707,06	6,23	< 0.000001	3,97 ± 81,9	3 ± 56,3	9,24 ± 38,8	3,1 ± 64,6	6,43 ± 91,6	8,67 ± 70	3,49 ± 47,5
53	708,09	2,64	< 0.000001	2,2 ± 50,8	2,31 ± 32,8	4,28 ± 35,8	2,18 ± 40,9	4,36 ± 67,7	4,82 ± 59	2,19 ± 29,1
54	718,83	30,45	< 0.000001	33,7 ± 46,8	27,7 ± 52,4	3,23 ± 89,2	30,4 ± 56,7	7,07 ± 139	4,96 ± 70,9	9,57 ± 48,3

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
55	719,82	8,96	< 0.000001	8,23 ± 41,9	9,35 ± 56,8	1,92 ± 63,5	10,9 ± 55,7	3,57 ± 99,6	2,47 ± 67,8	3,57 ± 37,8
56	720,71	1,85	< 0.000001	3,04 ± 27,8	3,16 ± 30,7	1,32 ± 31,2	2,83 ± 35,4	2,02 ± 46	1,87 ± 60,9	2,32 ± 29,9
57	736,31	7,01	< 0.000001	2,47 ± 33,6	2,79 ± 27,3	5,46 ± 37	3,26 ± 62,7	9,48 ± 72,5	8,28 ± 61,8	4,66 ± 41,6
58	738,25	15,26	< 0.000001	5,28 ± 73	6,81 ± 84,6	19,5 ± 37	5,14 ± 104	15,1 ± 60,9	20,4 ± 53,3	9,58 ± 47,2
59	760,31	7,29	< 0.000001	2,07 ± 25,8	2,51 ± 34,6	9,36 ± 30	2,33 ± 48,6	3,59 ± 48,1	3,55 ± 49,9	2,45 ± 24,6
60	762,33	25,97	< 0.000001	6,98 ± 81,1	5,69 ± 86,8	29,6 ± 35,9	3,58 ± 101	13 ± 79,8	11,3 ± 61	5,53 ± 48,5
61	763,3	12,93	< 0.000001	3,44 ± 74,7	2,95 ± 61,6	15,2 ± 34,8	2,31 ± 64,1	7,85 ± 70,7	6,45 ± 58,7	3,03 ± 44
62	764,37	44,56	< 0.000001	9,77 ± 82	10,6 ± 89,6	53,6 ± 34,7	9,02 ± 127	24,8 ± 65	26,4 ± 56,9	12,8 ± 50
63	765,3	18,66	< 0.000001	4,91 ± 80,2	5,09 ± 87,7	22,9 ± 34,2	4,22 ± 127	12,7 ± 57,1	12,4 ± 57,7	5,7 ± 48,4
64	766,32	7,67	< 0.000001	3,07 ± 72,7	3,27 ± 65,2	10,5 ± 33,1	2,87 ± 77,2	6,4 ± 52,5	8,72 ± 75	3,13 ± 40,2
65	770,75	3,06	< 0.000001	3,56 ± 47	3,81 ± 26,7	1,67 ± 35,4	4,25 ± 39,6	2,41 ± 52	2,44 ± 52,7	4,73 ± 42,9
66	771,91	23,69	< 0.000001	26,9 ± 134	18,7 ± 112	8,87 ± 74,4	8,46 ± 110	12,8 ± 84,5	3,21 ± 93,5	4,67 ± 83,2
67	772,86	17,36	< 0.000001	20,8 ± 131	15,2 ± 107	6,79 ± 73,8	8,23 ± 78	10,7 ± 72	3,48 ± 72,4	5,58 ± 46
68	773,9	7,77	< 0.000001	10,3 ± 123	7,77 ± 95,8	4,32 ± 42,3	4,56 ± 71,1	6,65 ± 63,3	2,49 ± 59,4	3,07 ± 33,8

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
69	774,78	2,91	< 0.000001	5,41 ± 88,9	4,7 ± 65,1	3,51 ± 24	3,71 ± 36,5	4,35 ± 55,5	2,51 ± 43,5	3,67 ± 29,6
70	776,39	8,78	< 0.000001	3,11 ± 44	2,94 ± 27,3	11,5 ± 37,4	2,7 ± 22,2	3,09 ± 44,5	3,17 ± 42	2,85 ± 29,5
71	778,46	4,34	< 0.000001	1,89 ± 23	2,43 ± 28,5	6,23 ± 39,2	2,03 ± 37,5	2,45 ± 44	2,38 ± 40,9	2,73 ± 56,9
72	784,42	7,67	< 0.000001	1,85 ± 23,3	1,88 ± 26,4	9,37 ± 26,8	2,03 ± 40,5	2,49 ± 59,5	2,19 ± 73,4	1,69 ± 26
73	786,45	22,04	< 0.000001	3,1 ± 47,1	3,29 ± 54,9	25,1 ± 28	3,43 ± 110	5,93 ± 54,4	7,02 ± 50,1	4,14 ± 43,5
74	787,53	8,92	< 0.000001	2,76 ± 38,4	2,91 ± 27	11,7 ± 28,8	3,44 ± 56,5	3,82 ± 47,4	4,08 ± 54,4	2,95 ± 37,7
75	788,57	15,7	< 0.000001	7,48 ± 58,9	8,82 ± 33,9	22,1 ± 32,6	8,15 ± 77,9	6,36 ± 62	7,11 ± 43,6	7,25 ± 71,6
76	789,6	6,26	< 0.000001	4,78 ± 45,7	5,61 ± 30,6	10,2 ± 33,4	4,96 ± 70	3,93 ± 49,2	4,25 ± 50,2	4,76 ± 58,3
77	790,52	28,17	< 0.000001	26 ± 92,7	16,9 ± 54,7	37,9 ± 46,7	9,87 ± 69,1	11,3 ± 69,4	12,7 ± 53,1	9,73 ± 60,4
78	791,44	13,39	< 0.000001	13,5 ± 90,8	8,63 ± 52,3	19 ± 44,5	5,59 ± 60	7,45 ± 65,7	8,3 ± 61,3	7,24 ± 61,8
79	792,56	12,47	< 0.000001	13,1 ± 84,7	9,57 ± 40,1	20 ± 47,4	7,53 ± 61,2	7,5 ± 59,9	8,97 ± 43,5	7,54 ± 48,5
80	794,65	3,22	< 0.000001	3,32 ± 33,1	3,91 ± 35,7	5,73 ± 35,8	3,79 ± 52,5	2,51 ± 52,2	3,32 ± 69,4	4,11 ± 43,2
81	796,05	15,46	< 0.000001	7,22 ± 110	5,18 ± 85,5	18,6 ± 49,5	5,62 ± 76,2	5,98 ± 55,2	3,96 ± 58	3,18 ± 43,8
82	796,97	6,43	< 0.000001	4,48 ± 78,7	3,31 ± 63,4	9,16 ± 43,5	3,72 ± 51,5	3,96 ± 49	2,72 ± 47,4	2,77 ± 30,3

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
83	800,55	6,84	< 0.000001	1,65 ± 29,8	1,89 ± 30,6	8,49 ± 25,3	2,07 ± 38	2,28 ± 54,9	2,63 ± 46,6	1,88 ± 26,5
84	802,52	9,75	< 0.000001	1,9 ± 21,4	1,98 ± 26,3	11,5 ± 29,2	1,98 ± 30,3	2,74 ± 58	2,16 ± 37,8	1,72 ± 27,6
85	804,48	5,89	< 0.000001	2,58 ± 59,3	2,4 ± 40,7	7,6 ± 35,4	1,97 ± 25,4	1,98 ± 49	2,09 ± 85,4	1,72 ± 32,8
86	810,71	5,79	< 0.000001	2,59 ± 38,9	3,34 ± 38,7	8,04 ± 26	3,8 ± 45,7	2,48 ± 43,4	2,26 ± 42,1	3,37 ± 41,5
87	812,51	19,96	< 0.000001	4,83 ± 37,4	4,28 ± 31,6	24,2 ± 37,1	6,41 ± 46,2	10,2 ± 63,8	5,05 ± 40,8	4,71 ± 35,5
88	814,64	17,99	< 0.000001	4,48 ± 35,4	3,83 ± 29,4	21,8 ± 38,6	5,06 ± 70,2	5,8 ± 55,3	7,85 ± 49,3	4,99 ± 30,5
89	816,66	6,74	< 0.000001	2,83 ± 32	2,69 ± 23,1	9,43 ± 36,1	2,76 ± 43	3,46 ± 45,8	5,56 ± 51,7	3,23 ± 22,1
90	817,75	4,1	< 0.000001	2,45 ± 38,6	2,26 ± 33,3	6,19 ± 33,2	2,1 ± 33,8	2,85 ± 44,7	4,05 ± 50,7	2,32 ± 33,5
91	818,64	3,6	< 0.000001	2,37 ± 41,2	2,18 ± 34,2	5,74 ± 25,3	2,23 ± 44,8	2,89 ± 46,3	3,19 ± 40,5	2,14 ± 29,2
92	820,66	3,15	< 0.000001	2,41 ± 41	2,28 ± 33,9	5,03 ± 31	2 ± 34	1,96 ± 42,9	2,22 ± 41,9	1,87 ± 30,2
93	828,68	6,56	< 0.000001	1,64 ± 23,2	1,81 ± 25,6	8,04 ± 34,7	1,79 ± 30,2	1,76 ± 41,7	1,48 ± 47,7	1,74 ± 26,8
94	834,9	1,62	< 0.000001	3,12 ± 44,3	2,79 ± 39,8	3,63 ± 26,9	2,48 ± 45,8	2,04 ± 45,7	2,01 ± 64,2	3,01 ± 44,4
95	839,22	4,39	< 0.000001	5,45 ± 126	6,6 ± 105	5,84 ± 25,6	4,03 ± 104	3,01 ± 77,4	2,22 ± 44,6	3,56 ± 93,4
96	840,26	2,77	< 0.000001	3,56 ± 99,6	4,34 ± 96,8	5,33 ± 29,6	3,4 ± 89,8	2,73 ± 52,5	2,77 ± 96,3	2,56 ± 73,8

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
97	855,85	4,29	< 0.000001	5,85 ± 42,5	4,16 ± 42,3	3,15 ± 31,6	2,02 ± 20,7	1,56 ± 56,3	1,7 ± 112	2,8 ± 36,2
98	856,85	2,24	< 0.000001	3,95 ± 45,3	3,16 ± 38	3,04 ± 21,4	2,44 ± 42,1	1,71 ± 48,1	1,8 ± 51,7	2,87 ± 35,1
99	857,85	8,18	< 0.000001	9,81 ± 43,4	8,56 ± 43,7	4,39 ± 43,6	2,41 ± 48,9	1,63 ± 52,8	2,02 ± 55,9	5,8 ± 40,6
100	858,84	5,31	< 0.000001	6,83 ± 42,1	4,6 ± 40,2	3,43 ± 29,6	2,08 ± 42,3	1,52 ± 55,1	1,95 ± 59	4,03 ± 43,2
101	859,87	9,15	< 0.000001	10,7 ± 40,2	7,13 ± 46,5	3,07 ± 49,2	1,89 ± 55,1	1,55 ± 66	1,99 ± 77,4	5,56 ± 52,8
102	860,85	3,84	< 0.000001	5,34 ± 40	4,33 ± 40,4	3,8 ± 30,1	1,89 ± 33,8	1,49 ± 58,7	1,85 ± 71,9	3,28 ± 43,6
103	881,95	10,88	< 0.000001	12,4 ± 43,4	9,45 ± 45	5,46 ± 51,9	2,18 ± 47,2	1,49 ± 50,5	2,29 ± 71,1	4,15 ± 37,5
104	884,01	41,97	< 0.000001	43,3 ± 43,3	32 ± 46,7	12,5 ± 60,9	3,36 ± 132	1,32 ± 52	3,18 ± 59	15,5 ± 48,9
105	884,91	19,37	< 0.000001	20,7 ± 45,1	15,4 ± 46,4	8,17 ± 59,7	2,52 ± 106	1,37 ± 51,7	2,77 ± 91,7	9,15 ± 47,1
106	886,03	56,26	< 0.000001	57,7 ± 41,6	36 ± 49,5	15,3 ± 63,8	4,57 ± 152	1,43 ± 48	4,29 ± 81,8	26,5 ± 52,5
107	887,96	18,62	< 0.000001	20 ± 39,5	14,8 ± 44,7	7,93 ± 70,2	2,23 ± 87,8	1,34 ± 51,7	2,94 ± 112	9,86 ± 56
108	900,09	3,43	< 0.000001	5,75 ± 54,9	3,5 ± 48,6	5,33 ± 33	2,31 ± 40,4	3,81 ± 71,7	3,11 ± 58,3	2,96 ± 31,9
109	902,07	2,84	< 0.000001	4,74 ± 65,8	2,84 ± 62,4	4,63 ± 46,5	1,9 ± 40,1	1,9 ± 49,8	1,99 ± 72	2,97 ± 37,4
110	906,04	3,5	< 0.000001	4,92 ± 45	3,02 ± 39,9	2,53 ± 39,8	1,78 ± 32	1,41 ± 46	1,54 ± 44,9	1,98 ± 147

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
111	909,02	7,26	< 0.000001	7,56 ± 42,8	5,4 ± 43,2	2,92 ± 39,5	1,63 ± 34,2	1,09 ± 40	2,6 ± 75,1	8,36 ± 52,1
112	910,11	13,93	< 0.000001	15,2 ± 42,3	12 ± 48,1	7,4 ± 60,2	1,83 ± 81,8	1,24 ± 48,5	2,44 ± 73,7	7,02 ± 37,4
113	912,16	15,05	< 0.000001	16,4 ± 43,7	13,2 ± 47,4	7,41 ± 68,1	2,53 ± 124	1,3 ± 46,6	2,47 ± 93,9	8,9 ± 43,8
114	914,14	7,44	< 0.000001	9,11 ± 41,6	7,56 ± 45,9	4,43 ± 60	2,28 ± 64	1,67 ± 48,9	2,51 ± 109	5,39 ± 46,3
115	949,19	5,18	< 0.000001	2,23 ± 39,7	1,93 ± 23,2	6,75 ± 107	1,84 ± 30,6	1,66 ± 42,9	1,93 ± 85,1	1,56 ± 24,5
116	957,28	2,93	< 0.000001	1,89 ± 33,5	1,88 ± 30,4	4,52 ± 38,8	1,94 ± 46,8	3,22 ± 61,1	2,43 ± 46,3	1,59 ± 21,2
117	977,38	5,35	< 0.000001	1,63 ± 39,3	1,66 ± 30,2	6,74 ± 87,2	1,63 ± 30,8	1,63 ± 43,9	1,8 ± 97,5	1,38 ± 33
118	983,67	1,58	< 0.000001	1,89 ± 29,6	2,28 ± 29,8	3,36 ± 41,4	2,51 ± 35,1	3,47 ± 55,9	3,11 ± 65,2	2,16 ± 32,7
119	999,66	1,56	< 0.000001	1,92 ± 34,7	2,53 ± 29,8	3,28 ± 59,4	2,76 ± 62,5	1,73 ± 57,1	1,77 ± 65,7	2,37 ± 72,4
120	1001,68	2,39	< 0.000001	2,19 ± 44,7	2,8 ± 38,2	3,59 ± 86,3	4,05 ± 95,5	1,67 ± 82	2,12 ± 111	2,9 ± 69,4
121	1003,67	2,02	< 0.000001	2,08 ± 41,4	3,03 ± 43,7	2,65 ± 82,4	3,79 ± 74,2	1,76 ± 85,8	2,64 ± 83,8	3,07 ± 74,6
122	1025,8	0,66	< 0.000001	2,35 ± 27,1	2,38 ± 27,8	2,03 ± 34,3	2,57 ± 42,6	1,92 ± 36,6	2,36 ± 57,8	2,54 ± 25
123	1043,88	1,59	< 0.000001	2,42 ± 38,7	2,12 ± 32,7	1,41 ± 32,5	2,35 ± 39,6	1,55 ± 40,3	1,64 ± 49	3 ± 69,2
124	1045,88	1,29	< 0.000001	2,59 ± 43,6	2,2 ± 31,8	1,39 ± 36,5	2,47 ± 46,3	1,49 ± 44,2	1,9 ± 59	2,68 ± 35,4

Index	Mass	D Ave	PKW	Ave H $\pm$ % CV	Ave D3 $\pm$ % CV	Ave D5 $\pm$ % CV	Ave D8 $\pm$ % CV	Ave D10 $\pm$ % CV	Ave D12 $\pm$ % CV	Ave D16 $\pm$ % CV
125	1047,87	1,08	< 0.000001	2,33 $\pm$ 38,3	2,17 $\pm$ 37	1,47 $\pm$ 42,3	2,33 $\pm$ 45,3	1,4 $\pm$ 45,5	1,62 $\pm$ 40,1	2,48 $\pm$ 35,5
126	1237,74	5,14	< 0.000001	6,89 $\pm$ 147	7,4 $\pm$ 176	7,9 $\pm$ 69,9	3,78 $\pm$ 87,8	5,8 $\pm$ 76,1	3,85 $\pm$ 74,5	2,77 $\pm$ 38,3
127	1266,86	3,58	< 0.000001	1,53 $\pm$ 31,1	1,75 $\pm$ 30,8	0,99 $\pm$ 37,8	1,83 $\pm$ 47,8	4,57 $\pm$ 109	1,97 $\pm$ 46,9	1,9 $\pm$ 27,7
128	1270,82	2,35	< 0.000001	2,11 $\pm$ 43,2	2,27 $\pm$ 35,6	1,07 $\pm$ 84,9	3,42 $\pm$ 73,5	1,97 $\pm$ 87,4	3,08 $\pm$ 83,6	2,96 $\pm$ 45,8
129	1272,87	2,25	< 0.000001	2,24 $\pm$ 48,8	2,17 $\pm$ 37,6	1,11 $\pm$ 78,9	3,36 $\pm$ 65,4	1,72 $\pm$ 37,9	2,46 $\pm$ 74,8	3,13 $\pm$ 34,6
130	1501,83	0,97	< 0.000001	2,26 $\pm$ 56,9	1,45 $\pm$ 32,4	1,58 $\pm$ 29,5	1,96 $\pm$ 38,6	1,95 $\pm$ 55,3	2,42 $\pm$ 38,6	2,2 $\pm$ 47,1
131	1939,72	3,54	< 0.000001	1,07 $\pm$ 27,7	1,03 $\pm$ 34,2	1,12 $\pm$ 36,9	1,46 $\pm$ 56,6	2,37 $\pm$ 121	4,57 $\pm$ 86,5	1,36 $\pm$ 39,4
132	1967,91	27,76	< 0.000001	1,04 $\pm$ 24,5	4,42 $\pm$ 151	1,27 $\pm$ 32	4,64 $\pm$ 215	14,2 $\pm$ 247	28,8 $\pm$ 161	1,3 $\pm$ 40,6

(**D Ave**: Difference between the maximal and minimal average feature intensity for healthy tissue and all six time points (arb. u.); **PKW**: P-value calculated by the Kruskal-Wallis test; **Ave N $\pm$ % CV**: Feature intensity average of class N where 'H'= healthy tissue and 'D'= Day post-wounding; $\pm$ coefficient of variation in % of class N)

Appendix VII: Whole Wound Lipidome Peak Statistics

(ranked by feature mass)

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
1	502,29	5,58	< 0.000001	1,11 ± 63,8	1,73 ± 74,9	6,67 ± 51,7	1,37 ± 45,3	1,08 ± 43,3	2,32 ± 88,7	1,76 ± 35,8
2	511,91	2,51	< 0.000001	3,89 ± 65,6	2,72 ± 80	2,22 ± 73,1	1,45 ± 41,9	3,95 ± 65,7	2,38 ± 168	2,66 ± 53,2
3	524,13	2,54	< 0.000001	2,56 ± 33	3,86 ± 54,8	1,67 ± 65,4	2,09 ± 57,8	1,32 ± 34,5	2,82 ± 89,7	1,98 ± 32,2
4	525,1	4,65	< 0.000001	5,96 ± 113	2,8 ± 126	1,78 ± 56,9	1,53 ± 30,5	4,02 ± 163	3,28 ± 87,2	6,18 ± 46,7
5	527,07	3,95	< 0.000001	1,57 ± 50,7	2,2 ± 47,1	2,48 ± 50,1	1,36 ± 34,5	4,16 ± 60,2	1,69 ± 60,1	5,31 ± 30,2
6	559,99	4,88	< 0.000001	4,64 ± 66,1	3,04 ± 93	5,13 ± 69,4	1,43 ± 46	6,31 ± 56,3	2,9 ± 191	3,83 ± 61
7	560,94	3,35	< 0.000001	2,74 ± 52,3	2,24 ± 75,4	4,59 ± 63,4	1,24 ± 33,7	3,81 ± 59	1,83 ± 131	2,19 ± 50
8	569,78	2,7	< 0.000001	3,77 ± 31,5	2,4 ± 30,7	1,88 ± 32,1	3,59 ± 37,9	4,58 ± 38,3	3,05 ± 61,9	3,12 ± 21,8
9	571,09	11,96	< 0.000001	15,4 ± 62,9	10,4 ± 98,3	11,5 ± 68,6	4,21 ± 70,3	16,2 ± 45,3	8,57 ± 163	11,3 ± 59,7
10	571,97	4,95	< 0.000001	6,68 ± 59,4	4,38 ± 82,6	4,98 ± 63,6	2,27 ± 57,6	7,1 ± 54,4	7,22 ± 126	4,85 ± 56,7
11	582,22	8,11	< 0.000001	7,98 ± 32,5	4,26 ± 53,2	2,28 ± 43,9	7,81 ± 47,3	10,4 ± 45,3	6,42 ± 66,2	7,77 ± 27,4
12	596,24	8,22	< 0.000001	3,1 ± 29,5	1,95 ± 36	2,39 ± 32,8	3,36 ± 43,7	4,96 ± 42,4	4,55 ± 65,4	10,2 ± 29,1
13	611,14	14,65	< 0.000001	2,35 ± 33,2	1,62 ± 31,2	16,3 ± 51,2	2,05 ± 22,6	2 ± 18	2,69 ± 32,3	3,72 ± 20,4
14	613,33	7,78	< 0.000001	2,66 ± 36,2	1,72 ± 41,1	2,66 ± 39,2	3,26 ± 41,2	4,45 ± 43,1	4,41 ± 55,5	9,49 ± 29,6
15	617,26	4,55	< 0.000001	3,55 ± 54,4	2,99 ± 79,8	6,34 ± 66,3	1,79 ± 43,8	4,25 ± 39,3	3,81 ± 89,8	3,33 ± 50,9
16	619,31	56,48	< 0.000001	45,9 ± 63,2	36,5 ± 103	65,5 ± 92,2	13,1 ± 96,3	69,6 ± 43,2	23,7 ± 182	44,5 ± 64,6

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
17	620,27	33,02	< 0.000001	23,1 ± 65,8	16,5 ± 104	39,3 ± 97,6	6,29 ± 83,3	36 ± 51,6	12,8 ± 164	21,9 ± 63,3
18	621,28	10,58	< 0.000001	8,58 ± 62,8	5,31 ± 87,3	13,9 ± 95,2	3,3 ± 50	12,2 ± 61,3	4,62 ± 138	7,72 ± 51,6
19	622,15	5,46	< 0.000001	4,62 ± 47	3,39 ± 49,8	4,87 ± 81	3,15 ± 43,9	5,88 ± 44,9	5,05 ± 116	8,61 ± 31,8
20	637,28	2,44	< 0.000001	2,74 ± 24,4	2,53 ± 45,2	4,56 ± 42,2	2,54 ± 27,2	2,12 ± 15,7	3,45 ± 52,1	3,32 ± 20,7
21	639,32	6,4	< 0.000001	3,58 ± 29,4	3,07 ± 40,3	4,19 ± 46,4	3,77 ± 42,3	4,06 ± 32,5	4,3 ± 33,5	9,47 ± 30,7
22	647,29	25,19	< 0.000001	1,78 ± 22,1	2,47 ± 41,4	26,5 ± 62,5	1,84 ± 32,2	1,35 ± 17,6	1,46 ± 76,6	1,73 ± 44,2
23	648,2	8,17	< 0.000001	1,85 ± 25,5	2,19 ± 45	9,52 ± 58,7	1,79 ± 31,8	1,35 ± 17,5	1,57 ± 44,8	2,16 ± 32,2
24	653,33	14,1	< 0.000001	2,99 ± 34,2	4,02 ± 56,3	16,7 ± 41,6	2,57 ± 32,9	3,94 ± 39,3	4,35 ± 75,4	3,23 ± 34,5
25	657,57	9,79	< 0.000001	1,37 ± 27,4	1,45 ± 45,5	10,8 ± 70,3	1,37 ± 34,7	2,66 ± 70,7	1,27 ± 34,4	1,02 ± 24
26	659,37	7,41	< 0.000001	4,72 ± 78,8	9,02 ± 103	4,42 ± 69,6	3,84 ± 71,3	1,6 ± 57,5	6,34 ± 121	4,04 ± 52,7
27	665,23	3,2	< 0.000001	3,29 ± 26,8	5,15 ± 53,7	3,54 ± 36,8	2,61 ± 40,1	1,95 ± 20,3	2,21 ± 32,4	3,72 ± 34
28	667,11	1,95	< 0.000001	2,61 ± 18,4	4 ± 45,8	2,4 ± 29,8	2,48 ± 28	2,05 ± 20,9	2,49 ± 36,7	3,08 ± 19,8
29	669,42	3,25	< 0.000001	2,47 ± 41,4	4,31 ± 52,5	5,01 ± 29,6	3,17 ± 61,9	2,58 ± 63	4,19 ± 73,6	1,76 ± 40,2
30	671,45	6,73	< 0.000001	1,77 ± 29,3	3,02 ± 147	8,1 ± 108	2,82 ± 107	1,38 ± 25,7	8,02 ± 148	3,4 ± 190
31	675,42	12,27	< 0.000001	9,27 ± 79,8	14 ± 73,8	2,22 ± 65,1	7,63 ± 89,6	1,75 ± 105	9,24 ± 114	6,45 ± 51,5
32	680,83	12,57	< 0.000001	10,5 ± 30,2	6,45 ± 47	1,73 ± 33,6	9,48 ± 45,6	14,3 ± 42,8	7,84 ± 70,4	8,64 ± 18,9
33	687,42	2,53	< 0.000001	2,04 ± 31,4	2,74 ± 59,9	2,46 ± 56,1	3,17 ± 89,8	1,53 ± 26,4	4,06 ± 120	3,72 ± 100
34	689,41	2,69	< 0.000001	3,02 ± 29,1	1,9 ± 39,9	2,01 ± 35,6	3,11 ± 56,8	2,21 ± 23,8	3,34 ± 75,6	4,58 ± 38,3
35	691,49	8,16	< 0.000001	7,39 ± 60,6	10,6 ± 63,7	3,98 ± 38,5	7,02 ± 66,5	2,44 ± 41,3	6,61 ± 92,9	4,93 ± 40

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
36	692,28	4,69	< 0.000001	5,46 ± 49,5	5,86 ± 53,1	2,25 ± 31,5	4,11 ± 26,9	5,29 ± 39,8	6,94 ± 86,4	5,31 ± 26,2
37	695,1	1,81	< 0.000001	3,54 ± 18,5	4,38 ± 47,9	2,58 ± 29,8	2,85 ± 22,6	3,81 ± 28,5	3,8 ± 42,8	3,66 ± 24,6
38	696,88	5,42	< 0.000001	6,12 ± 29,9	4,4 ± 44,8	1,78 ± 29,1	4,66 ± 34,5	7,2 ± 36,3	5,99 ± 50	4,37 ± 17,2
39	706,97	12,35	< 0.000001	2,63 ± 57,6	3,39 ± 43,9	13,6 ± 50,4	2,93 ± 50,6	1,42 ± 20	2,74 ± 58,9	1,28 ± 59
40	712,11	6,63	< 0.000001	3,28 ± 21,9	3,41 ± 42,9	2,44 ± 29,7	3,64 ± 29,5	5,66 ± 40,2	4,83 ± 44,8	9,07 ± 23,3
41	728,23	6,56	< 0.000001	3,15 ± 32,4	3,39 ± 64,5	1,82 ± 31,2	4,01 ± 39,6	6,75 ± 43,1	4,48 ± 54,8	8,37 ± 27,3
42	729,19	4,64	< 0.000001	5,23 ± 19,6	4,82 ± 54,8	2,84 ± 37,3	5,52 ± 33,9	7,33 ± 37,2	7,47 ± 46,7	5,95 ± 14,4
43	738,33	8,68	< 0.000001	3,98 ± 32,6	3,56 ± 41,8	1,68 ± 24,2	3,87 ± 39,5	10 ± 52,6	5,57 ± 57,1	10,4 ± 21,8
44	745,3	2,57	< 0.000001	3,84 ± 22,4	3,23 ± 45,9	2,18 ± 55	4,01 ± 42,1	4,56 ± 36,2	4,75 ± 45,4	2,8 ± 21,3
45	754,19	6,02	< 0.000001	3,47 ± 34,4	3,44 ± 49,9	1,18 ± 28,5	4,44 ± 53,9	4,81 ± 35,3	4,16 ± 54,3	7,21 ± 32,6
46	760,57	13,62	< 0.000001	3,94 ± 25,5	2,55 ± 42	1,15 ± 47,9	5,14 ± 56,4	8,57 ± 52,9	6,21 ± 70,3	14,8 ± 25
47	762,58	5,13	< 0.000001	2,9 ± 35,8	2,47 ± 32,4	2,03 ± 30,6	3,06 ± 37	4,12 ± 38,4	4,26 ± 51,2	7,16 ± 33,4
48	764,52	4,78	< 0.000001	3,6 ± 23	4,75 ± 42,5	1,92 ± 28,4	3,54 ± 37,7	6,24 ± 43,4	4,75 ± 27,5	6,7 ± 25,1
49	776,57	9,55	< 0.000001	3,81 ± 28,4	2,74 ± 40,8	0,96 ± 52,6	4,51 ± 50,3	7,78 ± 50,2	6,02 ± 68,5	10,5 ± 31,3
50	780,15	4,31	< 0.000001	2,99 ± 35,8	5,11 ± 62	0,8 ± 57,8	2,95 ± 47,2	2,49 ± 29,3	2,84 ± 37,9	3,36 ± 34,2
51	786,68	11,34	< 0.000001	4,35 ± 33,5	4,4 ± 51,6	1,14 ± 27,6	5,42 ± 84,6	6,27 ± 48,1	5,1 ± 56,3	12,5 ± 31,7
52	788,55	5,51	< 0.000001	3,38 ± 26,1	2,85 ± 56,8	1,3 ± 26,7	3,59 ± 48	4,56 ± 45,4	4,11 ± 37,5	6,8 ± 26,4
53	791,36	4	< 0.000001	3,96 ± 22,2	3,47 ± 43,5	1,34 ± 25,2	3,19 ± 29,3	5,34 ± 32,7	3,36 ± 43,1	5,09 ± 26,7
54	793,27	3,07	< 0.000001	3,57 ± 28,4	3,72 ± 46,2	1,2 ± 37,6	3,17 ± 33,9	4,27 ± 29,5	3,77 ± 45,5	4,16 ± 26,3

Index	Mass	D Ave	PKW	Ave H ± % CV	Ave D3 ± % CV	Ave D5 ± % CV	Ave D8 ± % CV	Ave D10 ± % CV	Ave D12 ± % CV	Ave D16 ± % CV
55	802,66	8,6	< 0.000001	3,9 ± 35,5	4,11 ± 57,2	0,82 ± 43,7	4,92 ± 68,2	5,25 ± 42,2	5,1 ± 55,9	9,42 ± 30,7
56	804,42	4,24	< 0.000001	2,63 ± 21,9	2,41 ± 59,1	1 ± 33,1	3,29 ± 57,4	3,38 ± 37,7	3,56 ± 46,3	5,24 ± 31,4
57	807,24	1,11	< 0.000001	2,34 ± 25,3	2,35 ± 47,6	2,72 ± 57,5	2,43 ± 30	3,26 ± 28,1	2,15 ± 42,6	2,22 ± 27,6
58	808,34	0,93	0,0428	2,51 ± 33,6	2,27 ± 34,6	3,19 ± 74	2,33 ± 46,3	2,71 ± 27,3	2,39 ± 48,3	2,56 ± 29,7
59	812,62	4,91	< 0.000001	3,57 ± 49,7	5,93 ± 59,7	1,02 ± 31	3,04 ± 47,7	2,66 ± 35,2	2,65 ± 38,6	4,17 ± 40,6
60	814,66	3,22	< 0.000001	2,9 ± 31,3	3,72 ± 54	0,87 ± 47,7	2,75 ± 41,6	3,19 ± 35,7	2,73 ± 41,1	4,1 ± 33,7
61	828,66	4,38	< 0.000001	3,08 ± 27,4	5,69 ± 67,2	1,3 ± 28,9	2,95 ± 58,2	2,21 ± 31	2,5 ± 49,2	3,54 ± 38,8
62	830,65	2,51	< 0.000001	2,33 ± 33,1	3,64 ± 63,1	1,14 ± 33,1	2,56 ± 54	2,08 ± 28,8	2,36 ± 41,6	3,01 ± 31,8
63	859,26	10,75	< 0.000001	1,5 ± 57,4	1,82 ± 47,6	12 ± 54	1,78 ± 33	1,21 ± 20,3	1,81 ± 31,9	1,67 ± 34,6
64	865,35	3,27	< 0.000001	2,11 ± 54	4,14 ± 72,2	4,47 ± 55,5	2,79 ± 54,9	1,44 ± 49,7	3,8 ± 109	1,19 ± 65,1
65	881,85	3,39	< 0.000001	2,2 ± 49,2	3,59 ± 59,9	4,88 ± 52,9	2,61 ± 54,1	1,64 ± 62,1	4,61 ± 118	1,5 ± 52,3
66	884,03	10,69	< 0.000001	2,84 ± 64,5	4,65 ± 116	11,9 ± 52,9	2,08 ± 47,2	1,17 ± 30,4	5,47 ± 115	3,6 ± 91
67	886,1	14,79	< 0.000001	2,68 ± 61,2	4,58 ± 123	15,8 ± 56,1	1,59 ± 31,1	1,03 ± 24,1	3,44 ± 104	4,5 ± 71
68	897,7	3,32	< 0.000001	2,67 ± 59	4,65 ± 68,5	3,43 ± 42,1	3,01 ± 61	1,96 ± 95,5	4,62 ± 93,6	1,34 ± 75,4
69	899,49	2,93	< 0.000001	1,95 ± 62	3,62 ± 69,3	2,8 ± 33,8	3,56 ± 81,6	1,34 ± 56,8	4,27 ± 104	2,7 ± 127
70	908,17	3,31	< 0.000001	1,44 ± 50,1	1,93 ± 54,9	4,19 ± 58,7	1,56 ± 31,1	0,96 ± 24,7	1,25 ± 44,1	0,88 ± 55,1
71	910,23	5,42	< 0.000001	1,8 ± 66	2,24 ± 78,9	6,44 ± 60,1	1,54 ± 39,3	1,02 ± 25,6	1,31 ± 31,9	1,35 ± 69,8
72	912,27	10,63	< 0.000001	1,46 ± 40,9	2,07 ± 88,8	11,6 ± 41,1	1,45 ± 32,1	0,97 ± 17,5	1,33 ± 27,5	1,83 ± 80,8
73	915,46	4,79	< 0.000001	1,4 ± 51,4	2,49 ± 50,1	5,91 ± 40,6	3,48 ± 66,5	1,12 ± 23,5	2,26 ± 74,7	1,62 ± 113

Index	Mass	D Ave	PKW	Ave H $\pm$ % CV	Ave D3 $\pm$ % CV	Ave D5 $\pm$ % CV	Ave D8 $\pm$ % CV	Ave D10 $\pm$ % CV	Ave D12 $\pm$ % CV	Ave D16 $\pm$ % CV
74	918,23	5,5	< 0.000001	1,21 $\pm$ 82,1	1,86 $\pm$ 62,2	6,58 $\pm$ 51,3	2,43 $\pm$ 64,1	1,08 $\pm$ 26,6	4,17 $\pm$ 144	1,49 $\pm$ 118
75	970,3	1,68	< 0.000001	1,79 $\pm$ 32,3	2,82 $\pm$ 37,6	1,18 $\pm$ 50,7	1,97 $\pm$ 39,7	1,14 $\pm$ 23,4	1,71 $\pm$ 51,9	1,48 $\pm$ 84,1
76	1014,47	2,66	< 0.000001	2,4 $\pm$ 60,4	3,66 $\pm$ 51,8	1 $\pm$ 27,2	2,25 $\pm$ 42,7	1,16 $\pm$ 28,4	1,13 $\pm$ 31,6	1,13 $\pm$ 50,9
77	1030,5	1,67	< 0.000001	1,8 $\pm$ 36,7	2,62 $\pm$ 37,6	1,17 $\pm$ 26,8	2,19 $\pm$ 42,5	1,2 $\pm$ 24,1	1,07 $\pm$ 33,7	0,95 $\pm$ 39
78	1058,73	5,16	< 0.000001	2,78 $\pm$ 48,3	5,95 $\pm$ 51,5	0,78 $\pm$ 34,2	2,89 $\pm$ 47,8	1,26 $\pm$ 29,3	1,18 $\pm$ 36,3	1,53 $\pm$ 90,4
79	1065,37	3,48	< 0.000001	1,13 $\pm$ 67	1,06 $\pm$ 72,8	4,18 $\pm$ 68,6	1,43 $\pm$ 30,8	1,05 $\pm$ 23,3	0,98 $\pm$ 38,1	0,7 $\pm$ 31,3
80	1074,77	3,37	< 0.000001	2,36 $\pm$ 50,1	4,41 $\pm$ 61,3	1,04 $\pm$ 28,9	2,53 $\pm$ 42,6	1,18 $\pm$ 26,4	1,13 $\pm$ 22	1,13 $\pm$ 39,2
81	1102,98	8,63	< 0.000001	4,28 $\pm$ 48,2	9,37 $\pm$ 61,1	0,74 $\pm$ 36,1	3,74 $\pm$ 58,8	1,3 $\pm$ 40,1	1,06 $\pm$ 37,8	1,66 $\pm$ 71,2
82	1119,04	5,37	< 0.000001	3,61 $\pm$ 60,8	6,09 $\pm$ 56,2	0,72 $\pm$ 33,9	3,18 $\pm$ 56,9	1,23 $\pm$ 34,4	1,02 $\pm$ 44	1,39 $\pm$ 57
83	1147,22	13,34	< 0.000001	6,72 $\pm$ 79,2	14,7 $\pm$ 55,5	1,4 $\pm$ 30,7	6,48 $\pm$ 66,8	1,81 $\pm$ 41,5	1,37 $\pm$ 25,9	2,28 $\pm$ 76,3
84	1163,32	8,34	< 0.000001	5,09 $\pm$ 55,2	9,53 $\pm$ 53,3	1,41 $\pm$ 36,5	4,82 $\pm$ 59,5	1,6 $\pm$ 31	1,19 $\pm$ 31,8	1,74 $\pm$ 69,3
85	1191,46	20,97	< 0.000001	9,47 $\pm$ 60,6	21,8 $\pm$ 54,8	0,84 $\pm$ 32,7	10,1 $\pm$ 70,3	1,84 $\pm$ 48,2	1,28 $\pm$ 35,8	2,96 $\pm$ 87,5
86	1207,56	12,2	< 0.000001	7,59 $\pm$ 56	13,1 $\pm$ 54,6	0,91 $\pm$ 27,5	8,23 $\pm$ 70,5	1,77 $\pm$ 37,8	1,32 $\pm$ 26,3	2,49 $\pm$ 75,5
87	1235,74	32,39	< 0.000001	13,5 $\pm$ 56,8	33,1 $\pm$ 54,2	0,71 $\pm$ 30,9	12,3 $\pm$ 71,8	2,21 $\pm$ 50,3	1,35 $\pm$ 43	3,86 $\pm$ 91,2
88	1251,81	23,58	< 0.000001	10,8 $\pm$ 57	24,3 $\pm$ 59,5	0,68 $\pm$ 44,3	10,5 $\pm$ 60	1,94 $\pm$ 45,7	1,16 $\pm$ 43,8	2,76 $\pm$ 81,9

(**D Ave**: Difference between the maximal and minimal average feature intensity for healthy tissue and all six time points (arb. u.); **PKW**: P-value calculated by the Kruskal-Wallis test; **Ave N $\pm$ % CV**: Feature intensity average of class N where 'H'= healthy tissue and 'D'= Day post-wounding; $\pm$ coefficient of variation in % of class N)

Appendix VIII: List of proteins identified in Day 3, 5, 8, 10, 12 and 16 porcine acute wound tissue

(All proteins were identified with 100% confidence and ≥ 2 unique peptides)

Main Accession #	Protein Description
Q29599	2'-5'-oligoadenylate synthase 1
P62197	26S protease regulatory subunit 8
P46405	40S ribosomal protein S12
P62279	40S ribosomal protein S13 (Fragment)
P62844	40S ribosomal protein S15
Q29201	40S ribosomal protein S16
Q6QAP7	40S ribosomal protein S17
P62272	40S ribosomal protein S18
Q29308	40S ribosomal protein S19 (Fragment)
A1XQU9	40S ribosomal protein S20
P63221	40S ribosomal protein S21
Q6SA96	40S ribosomal protein S23
P49171	40S ribosomal protein S26
Q6QAP6	40S ribosomal protein S29
Q0Z8U2	40S ribosomal protein S3
P62863	40S ribosomal protein S30
Q29197	40S ribosomal protein S9 (Fragment)
Q4GWZ2	40S ribosomal protein SA
Q29228	4-trimethylaminobutyraldehyde dehydrogenase (Fragment)
Q09136	5'-AMP-activated protein kinase catalytic subunit alpha-1 (Fragments)
Q29214	60S acidic ribosomal protein P0

Main Accession #	Protein Description
Q29315	60S acidic ribosomal protein P2
Q29195	60S ribosomal protein L10
P53027	60S ribosomal protein L10a (Fragment)
Q29205	60S ribosomal protein L11
Q95307	60S ribosomal protein L13a (Fragment)
A1XQU3	60S ribosomal protein L14
P79324	60S ribosomal protein L15 (Fragment)
Q95342	60S ribosomal protein L18 (Fragment)
P49666	60S ribosomal protein L21 (Fragment)
P67985	60S ribosomal protein L22
P62831	60S ribosomal protein L23
A1XQU5	60S ribosomal protein L27
Q29293	60S ribosomal protein L3 (Fragment)
P62901	60S ribosomal protein L31
Q29223	60S ribosomal protein L34
Q29361	60S ribosomal protein L35
Q95276	60S ribosomal protein L5 (Fragment)
Q2YGT9	60S ribosomal protein L6
Q29375	60S ribosomal protein L7a (Fragment)
A5GFQ0	60S ribosomal protein L7-like 1
P14332	6-phosphogluconate dehydrogenase, decarboxylating (Fragment)
P34935	78 kDa glucose-regulated protein (Fragment)
P16276	Aconitate hydratase, mitochondrial
P68137	Actin, alpha skeletal muscle
Q6QAA1	Actin, cytoplasmic 1

Main Accession #	Protein Description
P19205	Acylamino-acid-releasing enzyme
O02858	Acyl-CoA desaturase (Fragment)
P12026	Acyl-CoA-binding protein
Q710C4	Adenosylhomocysteinase
P00571	Adenylate kinase isoenzyme 1
A4Z6H0	Adenylosuccinate synthetase isozyme 1
P40125	Adenylyl cyclase-associated protein 1 (Fragments)
Q6QRN9	ADP/ATP translocase 3
Q007T5	ADP-ribosylation factor 6
P50578	Alcohol dehydrogenase [NADP(+)]
Q2XQV4	Aldehyde dehydrogenase, mitochondrial
Q9GKX6	Aldose 1-epimerase
P80276	Aldose reductase
P50447	Alpha-1-antitrypsin
P29700	Alpha-2-HS-glycoprotein (Fragment)
Q7M2W6	Alpha-crystallin B chain
Q8MJ76	Alpha-fetoprotein
P37111	Aminoacylase-1
P15145	Aminopeptidase N
P19619	Annexin A1
P19620	Annexin A2
P08132	Annexin A4
P49930	Antibacterial peptide PMAP-23
P49932	Antibacterial peptide PMAP-37
P80054	Antibacterial protein PR-39

Main Accession #	Protein Description
P22298	Antileukoproteinase (Fragment)
P18648	Apolipoprotein A-I
O46409	Apolipoprotein A-IV
P27917	Apolipoprotein C-III
P18650	Apolipoprotein E
Q2LE37	Apolipoprotein M
Q03472	Apolipoprotein R
P16469	Arachidonate 15-lipoxygenase
P30356	Arachidonate 5-lipoxygenase-activating protein (Fragment)
Q95JC8	Arginase-1
P00503	Aspartate aminotransferase, cytoplasmic
P00506	Aspartate aminotransferase, mitochondrial
Q35914	ATP synthase protein 8
P80021	ATP synthase subunit alpha, mitochondrial
Q9MYT8	ATP synthase subunit e, mitochondrial
Q95339	ATP synthase subunit f, mitochondrial
Q2EN81	ATP synthase subunit O, mitochondrial
Q2HYU2	ATP-dependent 6-phosphofructokinase, muscle type
P80015	Azurocidin
Q1KYT0	Beta-enolase
Q4FAT7	Beta-glucuronidase
Q29548	Beta-hexosaminidase subunit beta
Q6Q2C2	Bifunctional epoxide hydrolase 2
Q9GKQ6	Biglycan (Fragments)
P35750	Calpain-1 catalytic subunit

Main Accession #	Protein Description
P43367	Calpain-2 catalytic subunit (Fragment)
Q08092	Calponin-1
Q08094	Calponin-2 (Fragment)
P28491	Calreticulin
P36887	cAMP-dependent protein kinase catalytic subunit alpha
P05383	cAMP-dependent protein kinase catalytic subunit beta
Q5S1S4	Carbonic anhydrase 3
Q28960	Carbonyl reductase [NADPH] 1
O62839	Catalase
Q99028	Catechol O-methyltransferase (Fragment)
A1E295	Cathepsin B
P00795	Cathepsin D
Q6RVA9	Caveolin-1
Q007T2	Cell division control protein 42 homolog
Q29238	Chloride intracellular channel protein 1 (Fragment)
P00889	Citrate synthase, mitochondrial
Q29549	Clusterin
P16293	Coagulation factor IX (Fragment)
Q9GLP1	Coagulation factor V
O97507	Coagulation factor XII
D2SW95	Coatomer subunit beta
P10668	Cofilin-1
Q5G6V9	Cofilin-2
P04258	Collagen alpha 1(III) chain
Q69DL0	Complement C1q subcomponent subunit A

Main Accession #	Protein Description
Q69DK8	Complement C1s subcomponent
P01025	Complement C3
Q9TUQ3	Complement component C7
P51779	Complement factor D
A7Y521	COP9 signalosome complex subunit 4
A7TX81	COP9 signalosome complex subunit 6
Q9GK37	Corticosteroid-binding globulin
O19062	C-reactive protein
Q29594	Creatine kinase B-type (Fragment)
Q5XLD3	Creatine kinase M-type
Q29577	Creatine kinase U-type, mitochondrial
Q28988	Cystatin-A1
Q29290	Cystatin-B
P62895	Cytochrome c
P50667	Cytochrome c oxidase subunit 2
Q95283	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial (Fragment)
Q5S3G4	Cytochrome c oxidase subunit 5B, mitochondrial
A1XQT2	Cytochrome c oxidase subunit 6C
A5GFY8	D-3-phosphoglycerate dehydrogenase
Q9XSD9	Decorin
Q8WNV7	Dehydrogenase/reductase SDR family member 4
P45846	Dermatopontin
P02540	Desmin
Q3BDI7	Desmoglein-1
P60982	Destrin

Main Accession #	Protein Description
Q9N0F1	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex
Q8MJ30	Dihydropteridine reductase
O46374	DNA topoisomerase 2-alpha
A5GFZ5	Dolichol-phosphate mannosyltransferase subunit 1
Q29381	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit
Q9GMB0	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1
Q9GL01	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2
Q29036	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1
Q5GN48	Dystrophin
Q9MYU4	Ectonucleoside triphosphate diphosphohydrolase 1
Q6UAQ8	Electron transfer flavoprotein subunit beta
Q29387	Elongation factor 1-gamma (Fragment)
Q29092	Endoplasmin
B6CVD7	ERO1-like protein alpha (ERO1A_PIG)
A6M931	Eukaryotic initiation factor 4A-III
Q29221	F-actin-capping protein subunit alpha-2
A0PFK7	F-actin-capping protein subunit beta
B2MVP8	Fat storage-inducing transmembrane protein 1
O97788	Fatty acid-binding protein, adipocyte
P19130	Ferritin heavy chain
P19133	Ferritin light chain (Fragment)
Q9TV36	Fibrillin-1
P14460	Fibrinogen alpha chain (Fragment)
Q9TTB4	Fibromodulin (Fragment)

Main Accession #	Protein Description
Q29041	Ficolin-2
Q767L6	Flotillin-1
P10173	Fumarate hydratase, mitochondrial
Q49I35	Galectin-1
B3SP85	Gamma-interferon-inducible-lysosomal thiol reductase
P20305	Gelsolin (Fragment)
P08059	Glucose-6-phosphate isomerase
Q70KH2	Glucosylceramidase
P46410	Glutamine synthetase
P12309	Glutaredoxin-1
Q8MJ14	Glutathione peroxidase 1
P51781	Glutathione S-transferase alpha M14
Q9N1F5	Glutathione S-transferase omega-1
P80031	Glutathione S-transferase P
P00355	Glyceraldehyde-3-phosphate dehydrogenase
P68266	Glycolipid transfer protein
Q2MJK3	GTPase NRas
Q52NJ3	GTP-binding protein SAR1a
Q5PYH3	GTP-binding protein SAR1b
Q2PKF4	Guanine nucleotide-binding protein G(q) subunit alpha
P29797	Guanine nucleotide-binding protein G(s) subunit alpha
Q8SPS7	Haptoglobin
Q6S4N2	Heat shock 70 kDa protein 1B
A5A8V7	Heat shock 70 kDa protein 1-like
Q04967	Heat shock 70 kDa protein 6

Main Accession #	Protein Description
Q5S1U1	Heat shock protein beta-1
O02705	Heat shock protein HSP 90-alpha
P32394	Heme oxygenase 1
P01965	Hemoglobin subunit alpha
P02067	Hemoglobin subunit beta
P50828	Hemopexin
Q1W674	Hexokinase-2
P12682	High mobility group protein B1
P17741	High mobility group protein B2
P06348	Histone H1t
Q71LE2	Histone H3.3
P62802	Histone H4
Q68J42	Hormone-sensitive lipase
P00348	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial
Q45FY6	Hypoxanthine-guanine phosphoribosyltransferase
P01846	Ig lambda chain C region
Q29545	Inhibitor of carbonic anhydrase
Q9GLP0	Integrin beta-1
P53714	Integrin beta-2
Q29052	Inter-alpha-trypsin inhibitor heavy chain H1
O02668	Inter-alpha-trypsin inhibitor heavy chain H2
P79263	Inter-alpha-trypsin inhibitor heavy chain H4
P27594	Interferon-induced GTP-binding protein Mx1
A7VK00	Interferon-induced GTP-binding protein Mx2
P33198	Isocitrate dehydrogenase [NADP], mitochondrial (Fragment)

Main Accession #	Protein Description
P02535-1	Isoform 1 of Keratin, type I cytoskeletal 10
Q8WNW3	Junction plakoglobin
P13645	Keratin, type I cytoskeletal 10
P02533	Keratin, type I cytoskeletal 14
Q9QWL7	Keratin, type I cytoskeletal 17
P05784	Keratin, type I cytoskeletal 18
P19001	Keratin, type I cytoskeletal 19
P35527	Keratin, type I cytoskeletal 9
P04264	Keratin, type II cytoskeletal 1
Q6IFZ6	Keratin, type II cytoskeletal 1b
P35908	Keratin, type II cytoskeletal 2 epidermal
P13647	Keratin, type II cytoskeletal 5
P50446	Keratin, type II cytoskeletal 6A
P04259	Keratin, type II cytoskeletal 6B
P08729	Keratin, type II cytoskeletal 7
O95678	Keratin, type II cytoskeletal 75
P79385	Lactadherin
P14632	Lactotransferrin
Q8SQ26	Lambda-crystallin homolog
P80229	Leukocyte elastase inhibitor
B0KYV5	LIM domain and actin-binding protein 1
Q29550	Liver carboxylesterase
P00339	L-lactate dehydrogenase A chain
P00336	L-lactate dehydrogenase B chain
P79274	Long-chain specific acyl-CoA dehydrogenase, mitochondrial

Main Accession #	Protein Description
Q28942	Low affinity immunoglobulin gamma Fc region receptor III
P81693	Low molecular weight phosphotyrosine protein phosphatase
P12067	Lysozyme C-1
P12068	Lysozyme C-2
P80928	Macrophage migration inhibitory factor
P11708	Malate dehydrogenase, cytoplasmic
P00346	Malate dehydrogenase, mitochondrial
I3LUP1	Mannose-1-phosphate guanylttransferase alpha
P0C5I2	Mannose-1-phosphate guanylttransferase beta
Q8MJ39	Matrix Gla protein
Q9XT90	Matrix metalloproteinase-14
Q767L8	Mediator of DNA damage checkpoint protein 1
P41367	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial
Q95250	Membrane-associated progesterone receptor component 1
P79382	Microsomal glutathione S-transferase 1
P26042	Moesin
A5GFN6	mRNA export factor
P60662	Myosin light polypeptide 6
P29269	Myosin regulatory light polypeptide 9
P81271	Myosin-11 (Fragments)
P79293	Myosin-7
Q8WNQ7	N-acetylgalactosamine-6-sulfatase
Q866Y3	N-acetylmuramoyl-L-alanine amidase
P83686	NADH-cytochrome b5 reductase 3 (Fragment)
Q9TDR1	NADH-ubiquinone oxidoreductase chain 5

Main Accession #	Protein Description
Q29558	NADP-dependent malic enzyme (Fragment)
P04175	NADPH--cytochrome P450 reductase
P79403	Neutral alpha-glucosidase AB
P21999	Nuclear factor 1 C-type
Q2EN76	Nucleoside diphosphate kinase B
Q8MJ49	Osteoclast-stimulating factor 1
P14287	Osteopontin
Q9TTK7	Oxidized low-density lipoprotein receptor 1
Q2MJV8	Paralemmin-1
P62936	Peptidyl-prolyl cis-trans isomerase A
Q5BLZ2	Perilipin-3
P52552	Peroxiredoxin-2 (Fragment)
Q9TSX9	Peroxiredoxin-6
F1RQM2	Phosphoacetylglucosamine mutase
Q7SIB7	Phosphoglycerate kinase 1
P06867	Plasminogen
P30034	Platelet factor 4
Q29099	Polypyrimidine tract-binding protein 1
Q56P28	PRA1 family protein 3
Q3ZD69	Prelamin-A/C
Q9GLV6	Probable ATP-dependent RNA helicase DDX58
O46427	Pro-cathepsin H
P23687	Prolyl endopeptidase
P51524	Prophenin and tritrypticin precursor (Fragment)
P51525	Prophenin-2

Main Accession #	Protein Description
Q29073	Prostaglandin reductase 1
Q64L94	Proteasome activator complex subunit 1
Q29384	Proteasome subunit beta type-4 (Fragment)
P32194	Protegrin-1
P32196	Protegrin-3 (PG3_PIG)
P04366	Protein AMBP (Fragment)
P04163	Protein S100-A10
P31950	Protein S100-A11
P80310	Protein S100-A12
Q19AZ8	Prothrombin
O46560	Pyridoxal kinase
P29804	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial (Fragment)
Q6Q7J2	Rab GDP dissociation inhibitor beta
P26044	Radixin
Q52NJ1	Ras-related protein Rab-11A
Q52NJ6	Ras-related protein Rab-14
Q52NJ2	Ras-related protein Rab-1A
Q06AU7	Ras-related protein Rab-1B
Q06AU3	Ras-related protein Rab-3A
Q06AU6	Ras-related protein Rab-5A
Q06AU2	Ras-related protein Rap-2a
P63246	Receptor of activated protein C kinase 1
P27485	Retinol-binding protein 4
M3TYT0	Rho-associated protein kinase 2

Main Accession #	Protein Description
P10775	Ribonuclease inhibitor
Q7M329	Ribonuclease T2
A2TLM1	Ribose-5-phosphate isomerase
P16960	Ryanodine receptor 1
P11607	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
Q2VL90	Scavenger receptor cysteine-rich type 1 protein M130
Q711S8	Secreted phosphoprotein 24
Q3YLA6	Serine/arginine-rich splicing factor 1
Q06A98	Serine/arginine-rich splicing factor 2
Q29090	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform (Fragment)
P54612	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform
P54613	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform (Fragment)
P11493	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform (Fragment)
P61292	Serine/threonine-protein phosphatase PP1-beta catalytic subunit
Q6PQD5	Serine-protein kinase ATM
P09571	Serotransferrin
P08835	Serum albumin
O19063	Serum amyloid P-component
Q9GJT2	S-formylglutathione hydrolase
P79273	Short-chain specific acyl-CoA dehydrogenase, mitochondrial
Q764M5	Signal transducer and activator of transcription 1
Q19S50	Signal transducer and activator of transcription 3
P15981	SLA class II histocompatibility antigen, DQ haplotype D alpha chain
P15983	SLA class II histocompatibility antigen, DQ haplotype D beta chain

Main Accession #	Protein Description
A1XQR9	Small nuclear ribonucleoprotein E
P61958	Small ubiquitin-related modifier 2
P05024	Sodium/potassium-transporting ATPase subunit alpha-1
P05027	Sodium/potassium-transporting ATPase subunit beta-1
P20303	Solute carrier family 2, facilitated glucose transporter member 1
P20112	SPARC
Q29024	Spliceosome RNA helicase DDX39B
Q6DUB7	Stathmin
I3L5V6	Sterile alpha and TIR motif-containing protein 1
Q0QF01	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
Q007T0	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial
O19069	Succinate--CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial
P53590	Succinate--CoA ligase [GDP-forming] subunit beta, mitochondrial (Fragment)
Q29551	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial
P04178	Superoxide dismutase [Cu-Zn]
P28768	Superoxide dismutase [Mn], mitochondrial (Fragment)
Q29068	T-complex protein 1 subunit gamma (Fragment)
Q29116	Tenascin
P82460	Thioredoxin
Q9MYY8	Thioredoxin reductase 1, cytoplasmic
P47787	Thromboxane-A synthase
P21753	Thymosin beta-10
Q95274	Thymosin beta-4
Q9TV69	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase
P17630	Transcobalamin-1

Main Accession #	Protein Description
Q5D144	Transcription factor A, mitochondrial
Q8HZV3	Transferrin receptor protein 1
O11780	Transforming growth factor-beta-induced protein ig-h3
P03974	Transitional endoplasmic reticulum ATPase
P61288	Translationally-controlled tumor protein
P50390	Transthyretin
Q29554	Trifunctional enzyme subunit alpha, mitochondrial
F1RKQ4	Triokinase/FMN cyclase
Q29371	Triosephosphate isomerase (TPIS_PIG)
Q19PY3	tRNA-splicing ligase RtcB homolog
P42639	Tropomyosin alpha-1 chain
A1XQV4	Tropomyosin alpha-3 chain
P67937	Tropomyosin alpha-4 chain
P00761	Trypsin
P02550	Tubulin alpha-1A chain
Q2XVP4	Tubulin alpha-1B chain
P02554	Tubulin beta chain
A1Y2K1	Tyrosine-protein kinase Fyn
Q06AA4	U1 small nuclear ribonucleoprotein A
P63053	Ubiquitin-60S ribosomal protein L40
Q06AA9	Ubiquitin-conjugating enzyme E2 D2
Q29561	UMP-CMP kinase
Q29122	Unconventional myosin-VI
P79303	UTP--glucose-1-phosphate uridylyltransferase
A5GFS8	Vesicle-associated membrane protein-associated protein B

Main Accession #	Protein Description
P02543	Vimentin
P26234	Vinculin (VINC_PIG)
Q9GLP2	Vitamin K-dependent protein C
P48819	Vitronectin
Q9MZ16	Voltage-dependent anion-selective channel protein 1
Q9MZ15	Voltage-dependent anion-selective channel protein 2
Q29380	Voltage-dependent anion-selective channel protein 3
Q28833	von Willebrand factor (Fragment)
Q29048	V-type proton ATPase catalytic subunit A
Q9TSV6	V-type proton ATPase subunit G 2