

**Development and Validation of a High-Throughput Mass
Spectrometric Platform for the Molecular Characterisation of Cells**

A thesis submitted by:

Stefania Maneta-Stavrakaki

For the degree of Doctor of Philosophy (PhD)

Imperial College London

Department of Metabolism Digestion and Reproduction

To all my family, friends, and colleagues, who supported me through this journey.

Statement of Originality

I certify that this thesis and the research reported herein are the products of my own work. Information derived from published research and any contribution from the work of other people has been acknowledged in the text and acknowledgments, and the references are given in accordance with standard referencing practices of this academic discipline.

Stefania Maneta-Stavrakaki

Copyright declaration

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non-Commercial 4.0. International Licence (CC BY-NC). Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose. When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes. Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Research ethics and animal research

All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.

Abstract

Understanding the dynamic cellular metabolism is crucial for gaining deeper knowledge of the intracellular and intercellular functions, as well as the influence of microenvironment on cells.

Mass Spectrometry has been used extensively for metabolomic and lipidomic analysis of cells, especially when hyphenated with separation techniques such as liquid chromatography (LC-MS). These methods are very sensitive and can give vital information regarding the lipidome and metabolome of cells. However, the sample preparation needed for these techniques is laborious and time-consuming and does not allow for real-time analysis of cells.

Laser-Desorption Rapid Evaporative Ionisation Mass Spectrometry (LD-REIMS) is an ambient ionisation technique utilising infrared (IR) lasers for the generation of molecule-rich aerosol, which can be analysed with mass spectrometry methods.

This PhD thesis aims to expand the application of the automated LD-REIMS platform for the molecular characterisation of cells with minimal or no sample preparation. A systematic comparison was performed between REIMS modalities that have been used previously for cell pellet analysis – bipolar forceps and cell probe REIMS –, and the LD-REIMS autosampler, – while a protocol for the preparation-free analysis of living or frozen cells was also developed and validated. LD-REIMS outperformed other REIMS modalities in terms of classification, molecular coverage, and repeatability when used for the analysis of 5 breast and 5 colorectal cancer cell lines.

The applicability of LD-REIMS was demonstrated for the analysis of cells producing biopharmaceutical compounds, where metabolic differences were demonstrated between high and low producing cell lines. LD-REIMS was also used for the detection of significant differences in the lipid metabolism of phospholipidosis-induced cells, revealing upregulation of polyunsaturated fatty acids (PUFA) and the presence of Bis(Monoacylglycerol)Phosphate (BMP) lipids.

In conclusion, this PhD thesis demonstrates the potential of LD-REIMS for the high-throughput analysis of cells with minimal sample preparation, and the preparation-free analysis of living cells.

Acknowledgements

First of all, I would like to thank my PhD supervisor, Prof. Zoltan Takats, for all the support and guidance he has provided me over the past 4 years, and for all the valuable advice and lessons I have received from him.

Special thanks to my Early stage and Late stage assessment examiners, Dr. George Poulogiannis and Prof. Josephine Bunch, for their feedback and advice on my PhD project, and Prof. Jules Griffin for his helpful advice.

I would also like to acknowledge my colleagues – Aurelien Tripp, Vincen Wu, Yuchen Xiang, James McKenzie, and Stathis Elia – and thank them all for their help and contributions to this thesis.

Special thanks to Dan McGill, who had the patience to proof-read my thesis and give very constructive and insightful feedback and advice.

Very special thanks to Daniel Simon for all his help, and for the development of the instrumentation used during my PhD.

I would like to thank my dear friend and colleague Alvaro Perdones-Montero, who supported me with his help and friendship, and who had the patience and the kindness to guide me during my PhD. He will always be remembered, and he is greatly missed.

I would also like to say a big thank you to Lauren Ford, Toma Ramonaite, Eftychios Manoli, Anna Mroz, Riad Yagoubi, Erika Dorado and the many more colleagues and friends who transformed my PhD years into a very beautiful, educational, and fun adventure.

A very special thank you to Maria Sani and Petra Paizs, who were and are always there for me – for support through the difficult times, and to celebrate during the happy ones. Their contributions and help have been priceless.

Furthermore, I would like to thank my GSK collaborators, Carla Newman, Gary Finka, and Paloma Diaz Fernandez for their help and guidance, and everyone from GSK who supported and helped me in this project.

I would like to thank GSK and BBSRC who funded this PhD project, and Waters Corporation for the instrumentation I used during the past 4 years.

Finally, words are not enough for the gratitude and appreciation I feel for my family, who have always supported me in my endeavours, and especially my mother Eleni and my brother Nikos, who are always there for me, and who continue to support me in any way possible.

Table of Contents

Statement of Originality	3
Copyright declaration.....	4
Research ethics and animal research	5
Abstract.....	6
Acknowledgements.....	7
List of Figures	13
List of Tables	20
List of Abbreviations.....	22
Chapter 1: Introduction	28
1.1. Cellular systems.....	28
1.2. “Omics” technologies	29
1.2.1. Metabolomics.....	30
1.2.2. Lipidomics	32
1.2.2.1. Lipid classes and functions	33
1.2.2.2. Lipid Nomenclature	39
1.3. Characterisation of cell cultures using mass spectrometry	41
1.3.1. Liquid Chromatography-Mass Spectrometry (LC-MS).....	41
1.3.1.1. Fundamentals of LC-MS.....	41
1.3.1.2. Applications of LC-MS to cell analysis	48
1.3.2. Direct Infusion (DI) and Flow injection (FI) Mass Spectrometry	49
1.3.3. Matrix Assisted Laser Desorption Ionisation (MALDI).....	50
1.3.4. Secondary Ion Mass Spectrometry (SIMS)	51
1.3.5. Ambient ionisation mass spectrometry	52
1.3.5.1. Desorption Electrospray Ionisation (DESI)	52
1.3.5.2. Acoustic mist ionisation (AMI)	52
1.3.5.3. Rapid Evaporative Ionisation Mass Spectrometry (REIMS)	53
1.4. Statistical analysis and data interpretation of MS data.....	57
1.4.1. Raw MS spectra pre-processing.....	57
1.4.2. Statistical analysis and biomarker discovery	59
1.4.3. Metabolite and Lipid tentative annotation and identification	61

Chapter 2: Hypothesis and Aims.....	64
2.1. Hypothesis	64
2.2. Aims	64
Chapter 3: Method development, Optimisation, and Validation of LD-REIMS cell analysis	67
3.1. Introduction	67
3.2. Materials and Methods	69
3.2.1. Cell Culture	69
3.2.1.1. Human breast and colorectal cancer cell lines	69
3.2.1.2. PIK3CA mutant and wild type MCF10A breast cancer cell lines	70
3.2.2. Mass Spectrometry sample preparation	70
3.2.2.1. Cell pellets	70
3.2.2.2. Cell monolayer.....	71
3.2.3. Mass Spectrometer Cleaning and Calibration.....	71
3.2.4. Sampling techniques	72
3.2.4.1. Bipolar forceps.....	72
3.2.4.2. Cell probe.....	72
3.2.4.3. Laser Desorption.....	73
3.2.5. REIMS interface.....	73
3.2.6. Mass spectrometry	74
3.2.7. Data processing and statistical analysis	74
3.2.9. LC-MS Lipidomics assay.....	78
3.3. Results and Discussion	80
3.3.1. Comparison of the different REIMS modalities for the analysis of cell pellets	80
3.3.2. LD-REIMS on cell monolayer.....	92
3.3.2.1. Optimisation of the sample preparation process	93
3.3.2.2. LD-REIMS cell monolayer – Classification performance and intermediate precision	100
3.3.3. Comparison of LD-REIMS cell monolayer analysis against the LD- REIMS, bipolar forceps, and cell probe REIMS on cell pellets.....	104
3.3.4. LD-REIMS on cell monolayer treated with cPLA2 inhibitors	107
3.4. Conclusion	108
Chapter 4: LD-REIMS on genetically modified CHO cells producing therapeutic monoclonal antibodies.....	111

4.1. Introduction	111
4.2. Methods	113
4.2.1. GM CHO cell lines producing adalimumab	113
4.2.2. CHO cell culturing protocol.....	114
4.2.2.1. Cell recovery.....	115
4.2.2.2. Subculturing and generation of biological replicates.....	116
4.2.2.3. ‘Fed-batch’ CHO cell culturing	116
4.2.2.4. Cell harvesting and sample preparation for the LD-REIMS analysis.....	117
4.2.2.5. LD-REIMS analysis.....	117
4.2.3. LC-MS Lipidomics assay.....	118
4.2.4. Data analysis	119
4.3. Results and Discussion	120
4.3.1. Lipidomic differences between “non-production” and “fed-batch” CHO cells.....	120
4.3.2. Classification of high producers and low producers in the different cell culture conditions.....	123
4.3.2.1. PE ether lipids in high producing “non-production” CHO cells.....	124
4.3.2.2. PC and PI lipids in “fed-batch” CHO cells	125
4.3.2.3. Incorporation of polyunsaturated fatty acids in the phospholipids detected in the low producing CHO cells	127
4.3.3. Amino acids and small metabolites in the low producing cell lines..	127
4.4. Conclusion	129
Chapter 5: Investigation of Drug Induced Phospholipidosis in alveolar macrophages using LD-REIMS.....	131
5.1. Introduction	131
5.2. Materials and Methods	135
5.2.1. Sample preparation – rat alveolar macrophages	135
5.2.2. Sample preparation – rat lung tissue imaging.....	136
5.2.3. High-throughput LD-REIMS cell pellet analysis	136
5.2.4. LD-REIMS Imaging	136
5.2.5. DESI MS Imaging.....	137
5.2.6. Oxylin quantification	137
5.2.7. Untargeted Lipidomics LC-MS analysis.....	137
5.2.8. Western blot of rat alveolar macrophages.....	138
5.2.9. Data analysis	138

5.2.9.1.	LD-REIMS analysis of alveolar macrophages.....	138
5.2.9.2.	DESI MSI and LD-REIMS Imaging.....	139
5.2.9.3.	LC-MS and LC-MS/MS Lipidomics	139
5.2.9.4.	LC-MS Oxylin assay	139
5.3.	Results and Discussion	140
5.4.	PUFAs and eicosanoid upregulation.....	140
5.4.1.	BMP lipids in phospholipidosis and their differentiation from their PG counterparts.....	146
5.4.2.	Sphingomyelin accumulation in the CAD treated macrophages	151
5.4.3.	Presence of PG ether lipids in the lung tissue.....	152
5.5.	Conclusion	157
	Chapter 6: Conclusion and Future Work.....	159
6.1.	Conclusion	159
6.2.	Future Work	161
	Bibliography	163
	Appendix	190
	List of conference presentations	201
	Podium presentations.....	201
	Poster presentations	201
	Permissions to reprint.....	202

List of Figures

Figure 1. Phospholipid structure, consisting of the hydrophilic headgroup – with a class specific group, a phosphate, and a glycerol moiety – and a hydrophobic tail – consisting of two fatty acyl chains, either saturated or unsaturated.....	28
Figure 2. Cell membrane illustration. Figure adapted from Escalante-Martinez et al. (2018), Reprinted, with permission, from © 2018 IEEE	29
Figure 3. Structure of glycerophospholipids. A glycerol moiety is attached to two fatty acyl chains in the sn-1 and sn-2 positions, with a phosphate group in the sn-3 position. The phosphate group is attached to glycerophospholipid class specific group “X”..	33
Figure 4. Illustration of PE lipids forming hexagonal phase in the lipid bilayer. Figure adapted from Hamai et al. (2006), Reprinted, with permission, from © 2006 The Biophysical Society. Published Elsevier Inc.....	36
Figure 5. Glycerophospholipid classes and their characteristic headgroups attached to the phosphate moiety.....	38
Figure 6. A) Ether lipid structure, with an alkyl ether bond in the sn-1 position of the glycerol moiety. B) Plasmalogen structure, with an alkenyl ether bond in the sn-1 position of the glycerol moiety	39
Figure 7. Illustration of a hybrid QToF mass analyser, using orthogonal acceleration (ion pusher diverts the ion beam an orthogonal direction) and the reflectron. Figure adapted from Lucini et al. (2015), reproduced with permission from Elsevier.	47
Figure 8. Ionisation of analytes in MALDI MS. Figure adapted from de Hoffmann et al. (1996), reproduced with permission from John Wiley and Sons Ltd. © 2003.	50
Figure 9. Schematic of cell probe and bipolar forceps REIMS sampling techniques used for the direct analysis of cell pellets	55
Figure 10. High-throughput LD-REIMS platform.....	56
Figure 11. Basic steps for the development and validation of an analytical method.	67

Figure 12. MS data preprocessing pipeline.....	75
Figure 13. Example of confusion matrix, describing true positive (TP), true negative (TN), false positive (FP), and False Negative (FN) values.....	76
Figure 14. PCA scores plot of the 5 breast and 5 colorectal cancer cell lines analysed with bipolar forceps REIMS.	81
Figure 15. MCF7 cells seem to have increased monounsaturated and saturated fatty acids and decreased complex lipids containing these fatty acyl chains.	82
Figure 16. Confusion matrix based on the LR classification model built using the RFECV features of the bipolar forceps REIMS.....	83
Figure 17. PCA scores plot of the 5 breast and 5 colorectal cancer cell lines analysed with cell probe REIMS.....	85
Figure 18. Confusion matrix based on the LR classification model built using the RFECV features for the cell probe REIMS.....	86
Figure 19. PCA scores plot of the 5 breast and 5 colorectal cancer cell pellets analysed with LD-REIMS.....	87
Figure 20. Confusion matrix based on the LR classification model built using the RFECV features for the LD-REIMS on cell pellets.	88
Figure 21. Venn diagram (left) showing the overlap of the significant lipids/metabolites detected in each modality. The occurrence of the overlapping features is shown in the table on the right with the respective adduct ions for each sampling method.....	90
Figure 22. Representative mass spectra (1 scan) of the BT549 (breast cancer) cell line, analysed as cell pellets with LD-REIMS (top) and bipolar forceps (bottom)..	90
Figure 23. Workflow of cell pellet and cell monolayer preparation for the LD-REIMS analysis.....	92
Figure 24. Representative spectra obtained from MCF7 breast cancer cell line. Top, LD-REIMS analysis was performed in the cell monolayer after the discard of the	

growth medium, without washing. Bottom, spectrum acquired on the cell monolayer after the growth medium was discarded and the cells washed with 150 mM ammonium acetate.....93

Figure 25. Mass spectra obtained to test the different laser energies. With increasing laser energy, signals in the phospholipid region between 600-900 Da increases in intensity 95

Figure 26. Average spectra of the 5 breast and 5 colorectal cancer cell lines produced by LD-REIMS. Top mass spectrum, cells analysed after they underwent flash freezing in liquid nitrogen and cryopreservation in -80°C (after washing the cells with ammonium acetate). Bottom mass spectrum, cell analysed directly after washing with ammonium acetate – living cells.....96

Figure 27. Representative spectra (1 scan) of the MCF7 cell line, analysed as cell pellet (top) and as cell monolayer (living cells in this case) (bottom) with LD-REIMS. 98

Figure 28. PCA scores plot of the 5 breast and 5 colorectal cancer cell lines analysed with LD-REIMS as cell monolayer..... 100

Figure 29. Confusion matrix based on the LR classification model built using the RFECV features for the LD-REIMS on cell monolayer..... 101

Figure 30. Confusion matrix based on the LR classification model built using the RFECV features for the LD-REIMS on cell monolayer..... 102

Figure 31. Scatter plot demonstrating the repeatability performance of the individual cell lines using LD-REIMS cell monolayer analysis and LD-REIMS, bipolar forceps, and “cell” probe REIMS analysis on cell pellets. 104

Figure 32. Scatter plot demonstrating the average repeatability performance of the cell lines using LD-REIMS cell monolayer analysis and LD-REIMS, bipolar forceps, and “cell” probe REIMS analysis on cell pellets. 105

Figure 33. Box plots of the normalised ion intensity PUFAs, saturated fatty acids and PI lipids containing both saturated and polyunsaturated fatty acyl chains. In the box

plots, the intensities of the 2 isogenic PIK3CA mutant (MT1 and MT2) cell lines are compared to the wild type (WT) cell lines, untreated and treated with the cPLA2 inhibitor ASB14780. 107

Figure 34. Schematic to describe the two culturing protocols used. Left – CHO cell culturing under “non-production” conditions and the generation of 3 biological replicates from the original cell bank cryovial. Right – CHO cell culturing using ‘fed-batch’ production protocol, where the cells are stressed to produce more product. 115

Figure 35. PCA scores plot showing the differentiation between the “non-production” and “fed-batch” Day 12 cell culturing and the respective groups of the high and the low producing cell lines, based on their mass spectrometric profile of the biological and technical replicates of each condition acquired with LD-REIMS. B) PCA loadings plot showing the features mainly contributing in the PCA. 120

Figure 36. Representative mass spectra of the WT2 cell line, which is a high-producing cell line for each culturing condition. 121

Figure 37. Representative mass spectra of the WT4 cell line, which is a low-producing cell line for each culturing condition. 122

Figure 38. Confusion matrices of the test set cell lines used to test the classification performance of the models and the ability of the models to predict high and low producing cells 123

Figure 39. Venn diagram of the RFECV features used for generation of the classification models used. The tables are showing the main molecular classes observed in each group and if they were abundant in the high or low producing cell line of each condition. (HP: high producer and LP: low producer) 124

Figure 40. PC and PI lipids relative intensities in high and low producing CHO cells, cultures under “fed batch” conditions (harvested at Day 12) 126

Figure 41. PEs are main components of the cellular membrane, heavier unsaturated fatty acyl chains could alter the permeability of the plasma membrane, creating a more fluid membrane, with low protection against peroxidation ROS..... 127

Figure 42. Relative abundance of amino acids and small metabolites in high and low producing CHO cell lines cultured under “non-production” and "fed-batch" conditions.	128
Figure 43. Three commonly prescribed CADs – amiodarone, chlorcyclizine, and tamoxifen. CADs share common structural features, the hydrophobic aromatic rings (green) and the hydrophilic cationic amine groups (red)	131
Figure 44. Illustration of the CID MS/MS fragmentation pattern of PG (top) and BMP (bottom) lipids in negative ion mode. The MS/MS spectra produced by these lipid isomers are identical, with the base peaks being the respective fatty acyl ions.....	133
Figure 45. Illustration of the CID MS/MS fragmentation pattern of PG (top) and BMP (bottom) lipids in positive ion mode. The MS/MS patterns are visibly different....	134
Figure 46. PUFA upregulation in alveolar macrophages treated with 5 µg/µL of Amiodarone (green), Chlorcyclizine (blue) and Tamoxifen (pale blue), in comparison to control – untreated – cells (red).	140
Figure 47. Single ion images of DESI MSI (top) and REIMS Imaging (bottom) of and control rat lung tissue	141
Figure 48. Overlaid chromatograms of treated and control alveolar macrophages (top) and treated and control lung tissue (bottom).....	142
Figure 49. DESI MSI of amiodarone-treated rat lung tissue, revealing the presence of eicosanoids identified by LC-MS/MS as members of the 5-LOX pathway (See Table 16 in Appendix).	143
Figure 50. 5-LOX pathway for the generation of 5HpETE, 5-HETE, and 5-oxoETE.	144
Figure 51. Western blot of calcium-dependent cytoplasmic cPLA2, phospho-PLA2 and calcium independent iPLA2 in control alveolar macrophages and macrophages dosed with 2.5 and 5 µg/mL of amiodarone. GADPH was used a control protein..	145

Figure 52. BMP lipids detected with LD-REIMS in amiodarone, chlorcyclizine, and tamoxifen alveolar macrophages. In the control cells these lipids are either not detected or detected in much lower abundance than the phospholipidosis cells.....	146
Figure 53. Representative positive ion mode LC-MS/MS spectra of BMP(18:1/18:1) and PG(18:1/18:1).....	148
Figure 54. Glycerophosphoglycerol (GPG) detected in the phospholipidosis induced cells, potentially originating from the biosynthesis or catabolism of BMPs and/or PG lipids.....	149
Figure 55. Rat lung tissue treated with amiodarone does not seem to exhibit upregulation in BMP lipids when compared to the control lung tissue, when analysed with DESI MSI and LD-REIMS imaging.....	150
Figure 56. Sphingomyelins (SM) detected as chlorinated adducts in the alveolar macrophages treated with CADs and untreated (control).	151
Figure 57. PG ether lipids detected in the amiodarone treated rat lungs with DESI MSI. Fatty alkyl and fatty acyl chain length confirmed with LC-MS/MS (Table 13).	152
Figure 58. PG ether lipids detected in the amiodarone treated rat lungs with LD-REIMS -imaging. Fatty alkyl and fatty acyl chain length was confirmed with LC-MS/MS (Table 2).	153
Figure 59. Extracted chromatogram of m/z 731.5 in amiodarone treated rat lungs and control lung tissue. The high abundance of the lipid in amiodarone treated tissue, not present in the control.	154
Figure 60. MS/MS spectrum of m/z 729.507, which produces the LPG ether backbone, m/z 467.277 matching the mass of LPG(O-16:1) with the loss of the fatty acyl chain from the sn-2 position of the glycerol moiety, and the fatty acyl anion at m/z 279.233 corresponding to the fatty acyl chain 18:2.	156
Figure 61. MS/MS spectrum of m/z 781.538, which produces the LPG ether backbone, m/z 495.308 matching the mass of LPG(O-18:1) with the loss of the fatty	

acyl chain from the sn-2 position of the glycerol moiety, and the fatty acyl anion at m/z 303.233 corresponding to the fatty acyl chain 20:4, i.e., the arachidonic acid. 156

Figure 62. PCA scores plots of the first 5 principal components of the 5 breast and 5 colorectal cancer cell pellets analysed with bipolar forceps REIMS..... 190

Figure 63. PCA scores plots of the first 5 principal components of the 5 breast and 5 colorectal cancer cell pellets analysed with cell probe REIMS. 191

Figure 64. PCA scores plots of the first 5 principal components of the 5 breast and 5 colorectal cancer cell pellets analysed with LD-REIMS 192

Figure 65. PCA scores plots of the first 5 principal components of the 5 breast and 5 colorectal cancer cell lines analysed with LD REIMS as cell monolayer. 193

List of Tables

Table 1. Mass spectrometer settings used for the bipolar forceps REIMS, cell probe REIMS and LD-REIMS for the analysis of the 5 colorectal and 5 breast cancer cell lines, and the PIK3CA mutant and wild type cell lines.	74
Table 2. List of the lipids used as internal standards for the LC-MS lipidomic assay and their final concentrations.	78
Table 3. LC-MS lipidomics, gradient profile	79
Table 4. MS parameters used for the LC-MS lipidomic analysis	80
Table 5. List of the RFECV features and the annotations based on the accurate mass and isotopic pattern from the data acquired with bipolar forceps REIMS.....	84
Table 6. List of the RFECV features of the cell probe REIMS and their lipid and metabolite annotations.	86
Table 7. List of the RFECV features of the LD-REIMS on cell pellets and their lipid and metabolite annotations.....	89
Table 8. List of the metabolites detected in the cell monolayer, including glucose, glutamate, and amino acids, all taking part in important cellular metabolic pathways.	99
Table 9. List of the RFECV features of the LD-REIMS on cell monolayer and their lipid and metabolite annotations.	103
Table 10. Cell lines producing < 25 mg/L classified as low producers and cell lines producing > 25 mg/L classified as high producers	114
Table 11. Cell pellets analysed by LD-REIMS, including cell line ID and biological replicates ID used.....	118
Table 12. BMP lipids identified with LC-MS/MS in negative and positive ion mode, and the BMP lipid class characteristic fragment ions in positive ion mode.	147

Table 13. Fatty alkyl and fatty acyl chain length of PG ether lipids, confirmed with LC-MS/MS.....	155
Table 14. Supplementary material of section 3.3.2.2., Figure 27. Accuracy, Sensitivity, Specificity, Precision, and F1 score of each cell line of the testing dataset used to assess the intermediate precision of the LD-REIMS analysis on cell monolayer.	190
Table 15. Complete list of the RFECV feature annotation of all three CHO culturing conditions, excluding isotope peaks and unidentified features.....	199
Table 16. Oxylin quantification results. LLOQ: Lower Limit of Quantification.	200

List of Abbreviations

AA	Arachidonic Acid
AMI	Acoustic Mist Ionisation
BMP	Bis(Monoacylglycero)Phosphate
BSA	Bovine Serum Albumin
CAD	Cationic Amphiphilic Drug
Cer	Ceramide
CHO	Chinese Hamster Ovaries
CL	Cardiolipins
COX	Cyclooxygenase
cPLA2	cytosolic Phospholipase 2
CYP	Cytochrome
DC	Direct Current
DDA	Data Dependent Acquisition
DESI	Desorption Electrospray Ionisation
DG	Diacylglycerol
DHA	Docosahexaneic acid
DI	Direct Injection
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DPPC	Dipalmitoylphosphatidylcholine
EGF	Epidermal Growth Factor
EI	Electron Ionisation
ELISA	Enzyme-Linked ImmunoSorbent Assay
EM	Electron Microscopy
EPA	Eicosapentaenoic acid
ESI	Electrospray Ionisation
FA	Fatty Acid

FBS	Foetal Bovine Serum
FFT	Fast Fourier Transform
FI	Flow Injection
FN	False Negative
FP	False Positive
FT	Fourier Transform
FT-ICR	Fourier Transform-Ion Cyclotron Resonance
FWHM	Full Width at Half Maximum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLP	Good Laboratory Practice
GM	Genetically Modified
GPG	Glycerophosphoglycerol
HETE	Hydroxyeicosatetraenoic acid
HILIC	Hydrophilic Interaction Liquid Chromatography
HpETE	Hydroperoxyeicosatetraenoic acid
HPLC	High-Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometer
IPA	Isopropanol or propan-2-ol
iPLA2	calcium-independent phospholipase A2
IR	Infrared
LC	Liquid Chromatography
LCFA	Long Chain Fatty Acid
LC-MS	Liquid Chromatography-Mass Spectrometry
LDI	Laser Desorption Ionisation
LD-REIMS	Laser Desorption Rapid Evaporative Ionisation Mass Spectrometry
LOOCV	Leave-one-out Cross Validation
LOX	Lipoxygenase
LPE	Lysophosphatidylethanolamine
LPG	Lysophosphatidylglycerol

LPI	Lysophosphatidylinositol
LR	Logistic Regression
MALDI	Matrix Assisted Laser Desorption Ionisation
MCFA	Medium Chain Fatty Acid
MS	Mass spectrometry
MS/MS	Tandem Mass Spectrometry
MSI	Mass Spectrometry Imaging
MT	Mutant
NMR	Nuclear Magnetic Resonance
OPO	Optical Parametric Oscillator
oxo-ETE	oxo-eicosatetraenoic acid
PA	Phosphatidic Acid
PBS	Phosphate-Buffered Saline
PC	Phosphatidylcholine
PCA	Principal Component Analysis
PE	Phosphatidylethanolamine
PEEK	Polyether Ether Ketone
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha
PS	Phosphatidylserine
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acid
QToF	Quadrupole Time of Flight
REIMS	Rapid Evaporative Ionisation Mass Spectrometry
RF	Radiofrequency
RFECV	Recursive Feature Elimination, Cross-Validated
RNA	ribonucleic acid
ROS	Radical Oxygen Species

S/N	Signal-to-noise ratio
SD	Standard Deviation
SIMS	Secondary Ion Mass Spectrometry
SCFA	Short Chain Fatty Acid
TG	Triacylglycerol
TIC	Total Ion Current
TN	True Negative
TP	True Positive
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet
VLCFA	Very Long Chain Fatty Acid
WT	Wild Type

Chapter 1

Introduction

Chapter 1: Introduction

1.1. Cellular systems

Cells are the basic building blocks of every living organism. Different types of cells exist to serve different purposes, but they all share some common features¹. The two main groups of cells are eukaryotic – present in animals, plants, fungi, and protozoa – and prokaryotic – comprising bacteria and archaea. This PhD thesis will be focusing on mammalian cells and their molecular characterisation using recently developed mass spectrometry-based analytical methods.

All cells are surrounded by a cell membrane (also referred to as plasma membrane) that serves as a barrier between the external environment and the cellular contents. The basic structure of the cell membrane is formed by the lipid bilayer, built – as the name suggests – by two layers of phospholipids. Phospholipids are a class of lipids containing a hydrophilic polar headgroup, which contains a phosphate group, and a hydrophobic tail consisting of two hydrocarbon-based fatty acyl chains, typically

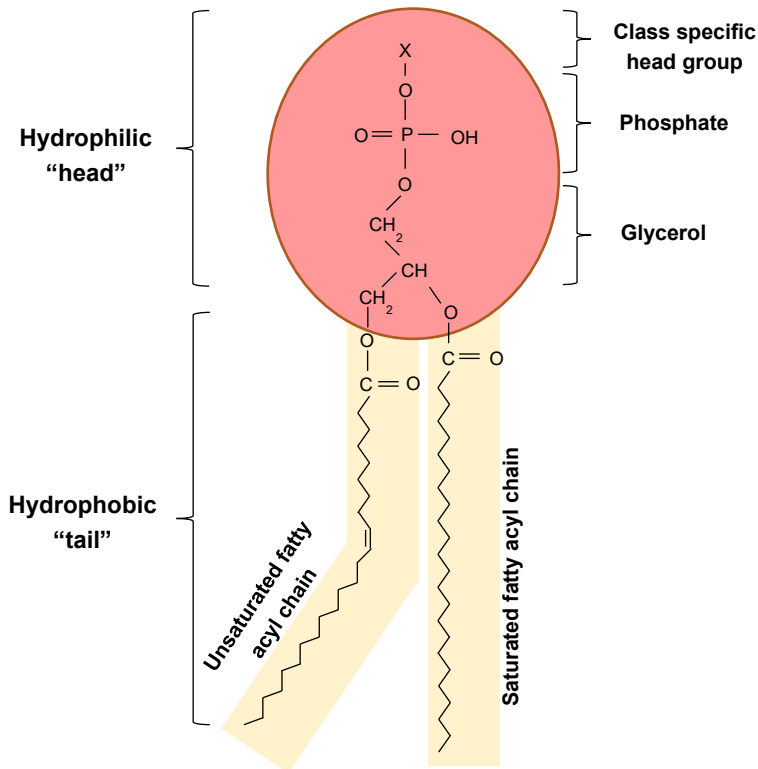


Figure 1. Phospholipid structure, consisting of the hydrophilic headgroup – with a class specific group, a phosphate, and a glycerol moiety – and a hydrophobic tail – consisting of two fatty acyl chains, either saturated or unsaturated.

attached to a glycerol moiety (Figure 1). In the lipid bilayer the hydrophilic headgroups of the phospholipids interact with the aqueous environment of the cytoplasm in the cell (inner lipid layer) and the aqueous environment of the intra-cellular space (outer lipid layer), while the hydrophobic “tails” of the phospholipids are trapped in the centre of the two layers, as shown in Figure 2^{1,2}. Apart from the lipid bilayer, proteins, cholesterol, and saccharides are important features of cell membranes, affecting their structure and functions³.

Monitoring cellular processes and metabolism is useful in many ways, such as investigating the underlying mechanisms and function of biological systems, help in the elucidation of pathophysiological conditions, or assessing drug toxicity *in vitro*^{4,5}.

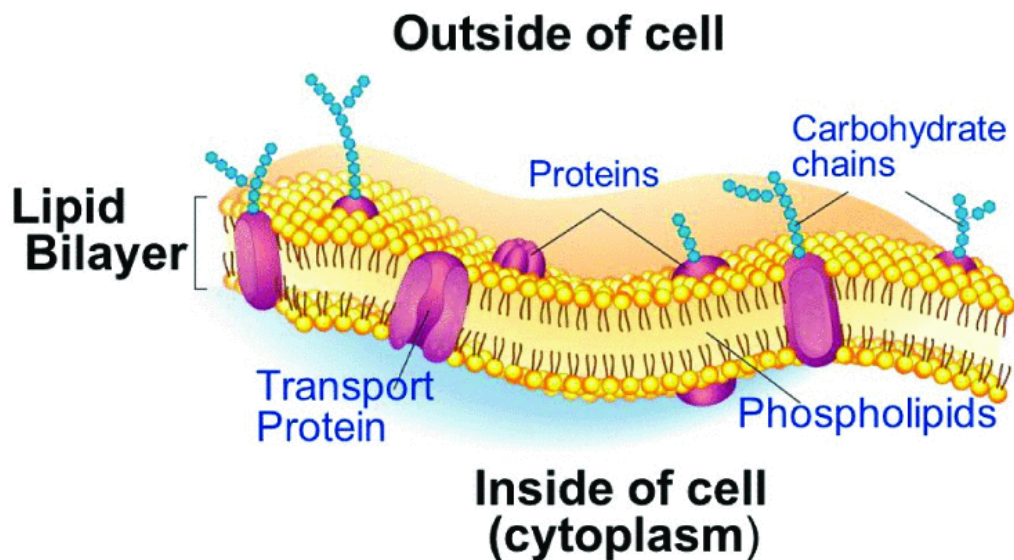


Figure 2. Cell membrane illustration. Figure adapted from Escalante-Martinez et al. (2018), Reprinted, with permission, from © 2018 IEEE

1.2. “Omics” technologies

The term “omics” is referred to the collection of technologies available that can be used to explore the roles and interactions, of the different types of molecules found in biological systems⁶. “Omics” include the disciplines of science investigating different aspects of a biological system, with the main targets being genes (genomics), mRNA (transcriptomics), proteins (proteomics), metabolites (metabolomics), and lipids (lipidomics). “Omics” technologies utilise a holistic and multidimensional approach to understand the underlying processes in a biological system – measuring and characterising the genome, transcriptome, metabolome, lipidome, and proteome⁷. The

applications of “omics” technologies vary, and can include understanding of physiological and pathophysiological conditions, disease diagnosis and prognosis, and biomarker discovery⁸.

1.2.1. Metabolomics

Metabolites are molecules taking part in biochemical reactions in biological systems. Some of the main metabolite classes include amino acids, carbohydrates, and metabolites belong to the core carbon metabolism, which are produced during the anabolic and catabolic processes of a biological system. In cellular systems, the building or breaking down of cellular components, based on the cell’s needs to function and survive, forms metabolic pathways. Metabolomics is the field studies the global metabolome of a given living organism, and the alterations caused by exogenous or endogenous triggers and can be very useful to gain in-depth understanding of cellular metabolism and the biochemical reactions occurring^{1,9–13}.

Metabolomics has been developed significantly in the recent years, mainly due to the development of advanced analytical techniques in combination with statistical methodologies. Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) are the most common analytical platforms used in metabolomics. Metabolomics can be divided into two smaller categories – untargeted metabolomics, focusing on the global profiling of the metabolome, and targeted metabolomics, focusing on the identification, and often quantification of selected metabolites¹⁴.

NMR is an analytical technique used to detect and determine the chemical structure of molecules. The nuclei of certain atoms exhibit spin, and when an external magnetic field is applied, the nuclei can either align with – lower energy, ground state – or against – higher energy, excited state – the orientation of the magnetic field. Radiofrequency energy is required to move the nuclei from the ground to excited state, proportional to the strength of the external magnetic field applied. The relaxation of the nuclei from the excited to ground state emits radiofrequency energy – also known as the Free Induction Decay (FID) – which is Fourier transformed, generating a spectrum with signal intensity peaks of the resonant frequency of the nuclei – represented as chemical shift in *ppm* units – relative to a reference peak. The magnetic field a nucleus experiences – the effective magnetic field – is different than the applied

external magnetic field and depends on the chemical and electronic environment of the nucleus. Some nuclei are shielded, i.e., experiencing lower effective magnetic field than the external field applied with the main factors affecting the degree of the shielding of the nuclei being electron density (the electrons present in the chemical environment of the nucleus), magnetic anisotropy (magnetic fields of opposing directions generated by π -electron systems), and hydrogen bonding¹⁵. The NMR spectrum provides information about the chemical environment of each NMR-visible nucleus, which allows for the elucidation of the chemical structure of the analyte¹⁶. NMR presents certain advantages – mainly its non-destructive nature and the minimal sample preparation needed, its inherently quantitative nature, and its high reproducibility¹⁴ – which make its use appealing for metabolomics, mainly on biofluids, but also on tissue extracts and cell cultures¹⁷. NMR is primarily applied in liquid samples, thus the analysis of biofluids – such as saliva, serum, and urine – is one of its main applications, as it usually requires minimal sample preparation, consisting of mixing the samples with appropriate buffers, such as phosphate buffer saline (PBS), followed by centrifugation, and the analysis of the supernatant using NMR. Cells and tissue require more extensive sample preparation, which includes additional steps such as homogenisation in water/methanol mixture prior to the addition of the buffers¹⁸. A major disadvantage of NMR is the lack of sensitivity (the ability of an analytical technique to detect the target analyte, expressed usually as the minimum concentration of the analyte measured¹⁹), with the technique only able to give accurate measurements in the 1-100 mM concentration range²⁰.

Mass spectrometry, which is widely used in metabolomics, can measure the mass-to-charge ratio (m/z) of positive and negative ions, which can be used for the detection of metabolites. However, due to the presence of isobaric compounds (compounds with the same nominal mass but different elemental composition²¹) and isomers (molecules with the same elemental composition, but different arrangement of the atoms²²), it is not always possible to identify a metabolite only from the m/z value. This is where tandem MS (MS/MS) – a two-step mass spectrometric analysis²³, where an ion of chosen m/z is selected in the first step, undergoes fragmentation, and then its characteristic product ions are measured and detected in the second step – can be useful revealing further information about the ion's identity²⁴. MS and MS/MS in metabolomics are commonly coupled to separation techniques, such as liquid

chromatography (LC), which reduces the complexity of a mixture by separating metabolites prior to their detection by mass spectrometry. Mass spectrometry's high sensitivity – with detection levels at picomolar to femtomolar concentration ranges – and its ability to detect a large number of different metabolites makes it well suited for metabolomic studies^{1,9–13}. However, as opposed to NMR, mass spectrometry is a destructive technique, and usually requires extensive sample preparation. In addition, mass spectrometry is highly dependent on the ionisation efficiency of the analytes, i.e., the number of ions produced by a given compound, which depends on the molecule's chemistry and matrix effects, such as ion suppression^{25–27}. Ion suppression describes the decrease in the analyte MS signal response due to matrix effects, such as the presence of inorganic electrolytes and salts, or highly polar compounds. This phenomenon creates bias in the results and can affect the mass spectrometric data, in terms of ion ratio and quantitation²⁸ (see section 1.3.1. for a more detailed description of mass spectrometry and liquid chromatography).

1.2.2. Lipidomics

Lipids are a special class of biomolecules sharing several attributes – including common physicochemical properties, a role as building blocks of the plasma membrane and the membranes of intracellular organelles, like mitochondria and lysosomes, while they can also act as signalling molecules critical for cell survival and health^{29,30}. Lipidomics, i.e., the systems-level investigation of the global population of lipids in a biological system – the lipidome –, can provide an accurate insight into cellular functions and reveal the roles of different lipid classes and subclasses in biological systems³¹. Lipid metabolism has a role in the aetiology of several human diseases, such as cancer and Alzheimer's disease³². Thus, the understanding of lipid metabolism can be vital for the elucidation and potential therapy of pathophysiological conditions.

One of the main subcategories of lipids are the glycerophospholipids, consisting of a glycerol backbone attached to two fatty acyl chains in the sn-1 and sn-2 positions of the glycerol group and to a phosphate moiety in the sn-3 position, which is attached to a unique hydrophilic polar group, characteristic for each glycerophospholipid class (Figure 3).

In lipidomics, the method of choice is mass spectrometry – due to its high sensitivity – usually coupled to a separation technique, mainly LC-MS³³. As well as “traditional” mass spectrometric methods, mass spectrometry imaging (MSI) has also been used in the recent years for the lipidomic analysis of biological surfaces, mainly tissue sections, which gives information of the spatial distribution and localisation of the detected lipids³⁴. NMR is not often used in lipidomics, due to the lack of sensitivity, but also because of overlapping signals of the various lipids, which result in “crowded” spectra that are not easily interpretable³⁵.

1.2.2.1. Lipid classes and functions

Lipids can be assigned to several lipid classes based on their chemical structure, physicochemical properties, and biological function. Some of the main lipid classes are fatty acids, oxylipins, glycerolipids, sphingolipids, sterol lipids, and glycerophospholipids, each with a unique and important role in cellular metabolism³⁶. This PhD thesis mainly focuses on glycerophospholipids as the main components of the cell membrane, as well as fatty acids and oxylipins, since these are important bioactive and signalling molecules.

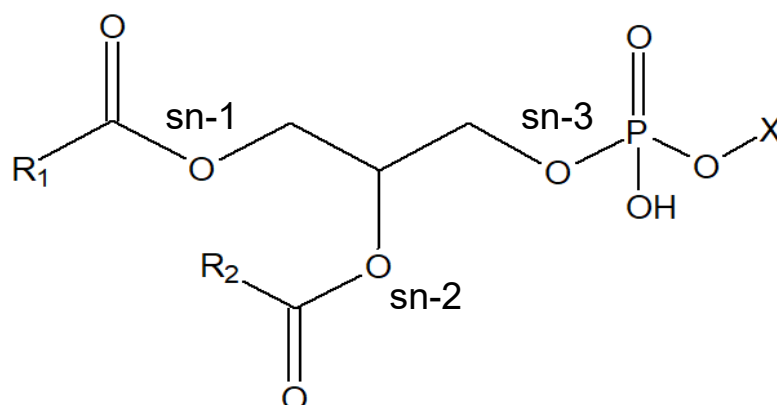


Figure 3. Structure of glycerophospholipids. A glycerol moiety is attached to two fatty acyl chains in the sn-1 and sn-2 positions, with a phosphate group in the sn-3 position. The phosphate group is attached to glycerophospholipid class specific group “X”.

Fatty acids

Fatty acids consist of a carboxylic group attached to an aliphatic chain. Subclasses of the fatty acids are unsaturated fatty acids and saturated fatty acids, depending on the presence or absence of double bonds in the aliphatic chain, respectively³⁷. Fatty acids can be further categorised based on the length of the acyl chain, into short-chain fatty acids (SCFA) with 6 or less carbon atoms in the aliphatic chain, medium-chain fatty acids (MCFA) of 7-12 atom carbons³⁸, long-chain fatty acids (LCFA) with 13-21 carbons³⁹, and very long chain fatty acids (VLCFA) with aliphatic chain of 22 or more carbon atoms⁴⁰.

A special category of the unsaturated fatty acids is the polyunsaturated fatty acids (PUFA)³⁷. PUFAs are fatty acids with more than one double bond in their hydrocarbon backbone. These fatty acids participate in different biological processes, serving as precursors for a class of signalling molecules, the oxylipins. Arachidonic acid (AA) is one of the most important PUFAs – with 20 carbon atoms and 4 double bonds (20:4) in the alkyl chain. Oxylipins derived from arachidonic acid are called eicosanoids (although eicosanoids can also derive from other PUFAs of 20 carbon atoms), and they act as regulators for many biological functions, including inflammation. Other PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been suggested to alter the fluidity of cell membranes after their incorporation in membrane phospholipids, which leads to changes in membrane protein functions^{37,41}. PUFAs can be further categorised into two main categories, omega-3 (n-3), and omega 6 (n-6) PUFAs, depending on the position of the first double bond from the methyl terminal. Typical examples of n-3 PUFAs are the EPA and α -linolenic acid (FA(18:3), n-3), while characteristic n-6 PUFAs are the linoleic acid (FA(18:2), n-6) and arachidonic acid⁴². Omega-6 PUFAs are believed to be involved in inflammatory processes, while omega-3 PUFAs present anti-inflammatory and inflammatory-resolving effects⁴³.

Oxylipins

Oxylipins are bioactive lipid metabolites generated from the oxidative metabolism of PUFAs, often created as response to cellular stress⁴⁴. Oxylipins have a wide variety of molecular structures, including hydroxy-, oxo-, or keto-fatty acids, or the volatile aldehydes. With such a wide structural variety, the oxylipins cover a wide range of biological roles, include acting as intercellular messengers or as inflammation mediators, either amplifying or reducing inflammation. The structural variety observed among oxylipins is a result of the various enzymatic pathways that take place in PUFA oxidation. The main enzymes governing oxylipin synthesis are lipoxygenase (LOX), cyclooxygenase (COX) and cytochrome (CYP) P450^{45–47}.

Eicosanoids are a subcategory of oxylipins, mainly referring to the oxylipins derived from arachidonic acid but also from other PUFAs. The eicosanoids can be further divided into three categories: prostanoids, synthesised via the COX pathway; leukotrienes and certain hydroxy acids, through the LOX pathway; and epoxides, formed via the CYP P450 pathway. Eicosanoids have either pro- or anti-inflammatory roles, mediating the immune response to cellular stress. While arachidonic acid generally gives rise to pro-inflammatory eicosanoids, the other major C₂₀ PUFA, EPA, produces anti-inflammatory eicosanoids⁴⁸.

Glycerophospholipids

Glycerophospholipids are derivatives of glycerophosphoric acid containing at least one acyl, alkyl, or alkenyl group attached to the glycerol residue. Cell membranes contain more than 1,000 different glycerophospholipids, with different fatty acyl chains esterified in positions sn-1 and sn-2 of the glycerol backbone, and different polar headgroups attached at the sn-3 position. The hydrophilic headgroups of the glycerophospholipids is what defines their sub-classes⁴⁹. Glycerophospholipids account for approximately 70% of the total lipid content of mammalian cells⁵⁰.

Phosphatidic acid (PA) is the simplest glycerophospholipid class and can be found only in small amounts (less than a few % of the total glycerophospholipid pool)

in cell membranes; however, their presence is vital for cell survival since it is a key component in lipid metabolism, and more specifically in glycerophospholipid and triacylglycerol biosynthesis, serving as a substrate for the enzymes involved. In addition, PA lipids are generated in the initial phases of cell signalling, acting as a lipid messenger in intracellular and extracellular functions^{51–53}.

Phosphatidylcholine's (PC) headgroup is a choline moiety. PC is the most abundant class of phospholipids in mammalian cells, comprising approximately 40-50% of total cellular phospholipids⁵⁴. PC lipids are one of the major components of the cell membrane, while also holding cellular signalling roles. Disruption of PC homeostasis in cells can lead to apoptosis⁵⁵.

Phosphatidylethanolamine (PE) is an aminophospholipid class containing an ethanolamine group and is the second most abundant class in mammalian cell membranes, constituting 20-50% of total phospholipids. Apart from their role as membrane components, PEs are also important signalling molecules for various cellular functions, such as cell apoptosis⁴⁹. PE lipids can play a role in membrane fusion events through their ability to form a hexagonal phase (Figure 4), where the ends of the lipids “tails” form hexagons in the lipid bilayer⁵⁶, thus influencing the membrane's structure and function⁵⁷.

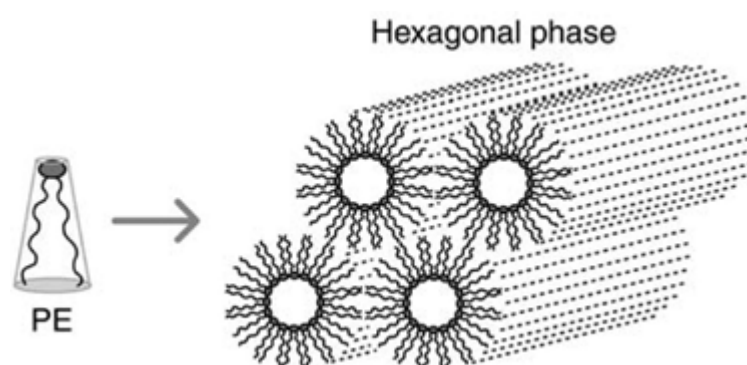


Figure 4. Illustration of PE lipids forming hexagonal phase in the lipid bilayer.

Figure adapted from Hamai et al. (2006), Reprinted, with permission, from ©
2006 The Biophysical Society. Published Elsevier Inc.

Phosphatidylserine (PS) is not a very abundant class in mammalian membranes, accounting for 2-10% of total phospholipids. Most commonly, the sn-1 position of the PS lipids is covered by saturated fatty acids of 16 or more carbons, while the sn-2 position usually by unsaturated fatty acids. The phosphate group is linked to a serine moiety, making PS lipids anionic molecules. PS hold an important role as a PE precursor in mitochondria. In the cell membrane PS lipids are almost entirely components of the inner leaflet of the lipid bilayer, while during apoptosis they externalize on the outside of cells^{49,50}.

Phosphatidylglycerol (PG) lipids comprise only 1-2% of total phospholipids in mammalian cells and tissue, however the lipid class is the second most abundant in pulmonary surfactant, consisting of approximately 10% of total phospholipids^{58,59}. The PG polar head group comprises of a second glycerol moiety attached to the phosphate, and under physiological conditions PGs have a negative charge⁶⁰.

Phosphatidylinositol (PI) is an acidic lipid class, like PG, with an inositol moiety bonded to the phosphate group. PI lipids carry a negative charge under physiological conditions, and therefore mass spectrometric analysis is easier to be conducted in negative ion mode, as it is the case for other acidic phospholipids.⁵⁸.

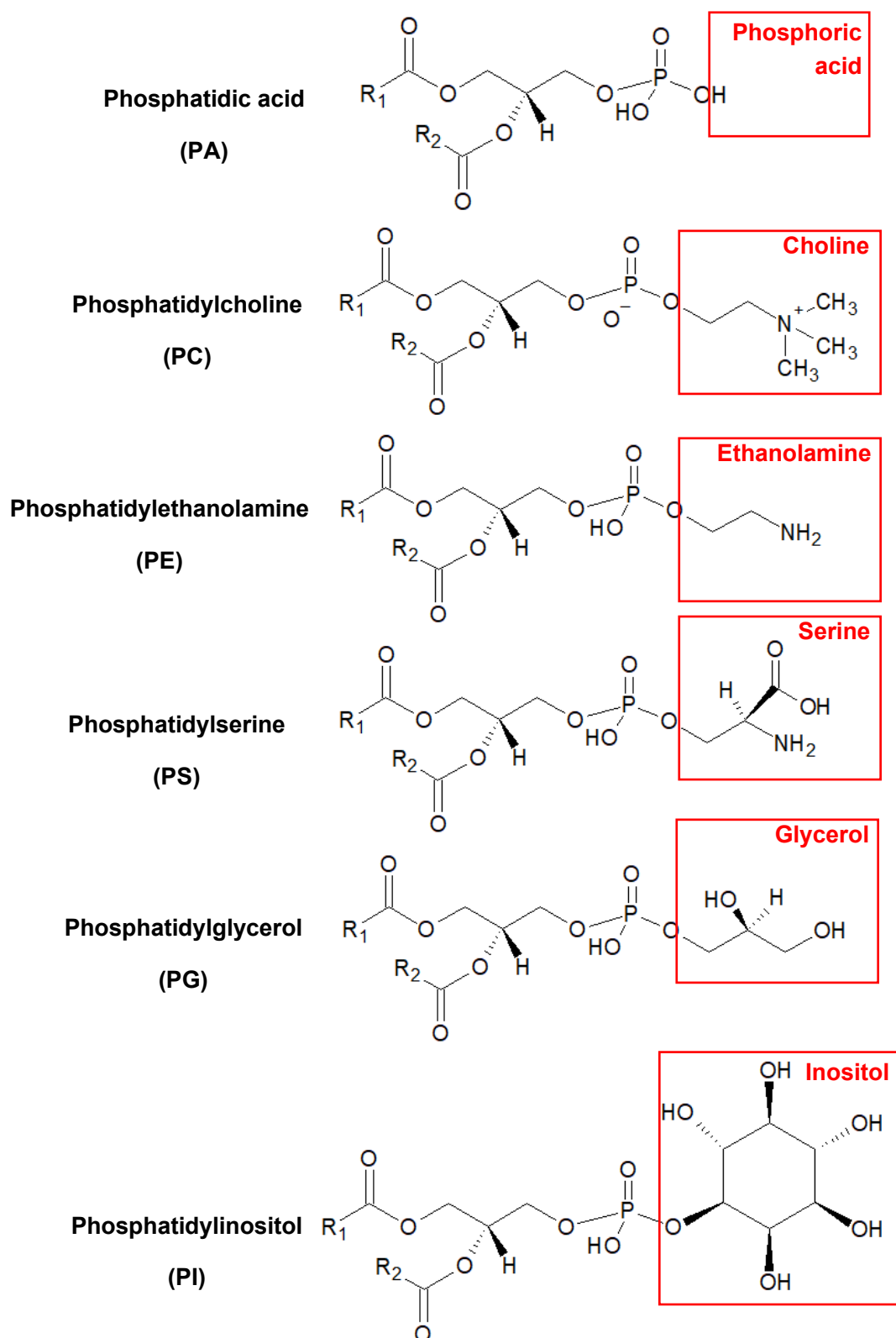


Figure 5. Glycerophospholipid classes and their characteristic headgroups attached to the phosphate moiety.

Finally, ether lipids are a unique group of glycerophospholipids, having an ether bond linked to an alkyl hydrocarbon chain instead of an ester bond in the sn-1 position of the glycerol moiety. Glycerol is attached to a fatty acyl chain in the sn-2 position as in the case of the diacyl glycerophospholipids, and the phosphate group is attached to a class-specific headgroup in the sn-3 position of the glycerol

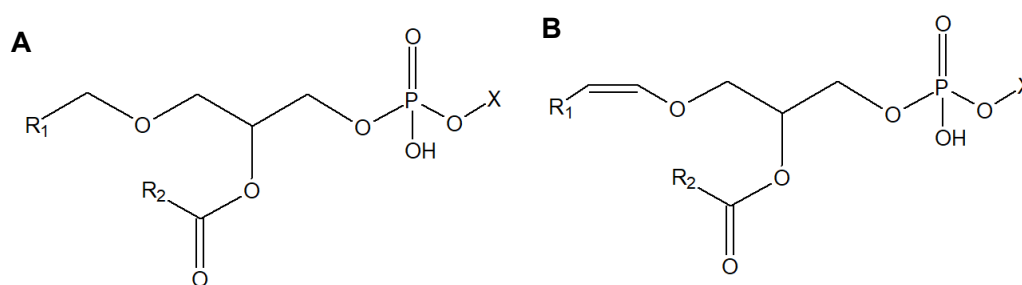


Figure 6. A) Ether lipid structure, with an alkyl ether bond in the sn-1 position of the glycerol moiety. B) Plasmalogen structure, with an alkenyl ether bond in the sn-1 position of the glycerol moiety. In both cases, a fatty acyl chain is attached via an ester bond in the sn-2 position, while as in the case of glycerophospholipids, the phosphate group is attached to a class specific hydrophilic headgroup.

group. Plasmalogens are a subcategory of the ether lipids, where the ether bond is attached to an alkenyl chain, forming a vinyl ether bond at the sn-1 position of the glycerol (Figure 6). Ether lipids constitute approximately 20% of the total phospholipids in mammalian cells, though this percentage can vary depending on the cell type. The phospholipid classes that can be usually found as ether lipids in the mammalian systems are PEs and PCs, but PIs and PSs ether lipids have also been detected at trace levels⁶¹.

1.2.2.2. Lipid Nomenclature

Fatty acyl nomenclature includes the number of carbon atoms comprising the acyl chain, followed by the number of the double bonds contained in the hydrocarbon chain, e.g., a fatty acyl chain of 16:0 means that there are 16 carbon atoms in the acyl chain and 0 double bonds, while a fatty acyl chain of 18:1 means that there are 18 carbon atoms and 1 double bond. The position of the double bond in the

fatty acyl chain can be expressed either using the delta system, in which the position of the double bond is expressed as Δ^x , where x is the first unsaturated carbon, counting from the carboxylic group terminal or with the omega system, in which the position of the double bond is expressed as ω -y or n-y, where y is the first unsaturated carbon, counting from the methyl terminal^{62,63}. Conventional MS/MS methods based on low energy CID cannot unambiguously establish the position of the double bonds in the fatty acyl chains. However, there are ways to establish the localisation of the double bonds using mass spectrometry. High-energy CID can provide more structurally informative MS/MS spectra, which can be used to determine the position of the double bond. Other methods, such as low-energy CID on metallated lipids (derivatised with metal ligands such as Li, Ag, etc.) have shown promising results. Ozone-induced dissociation (OzID) – based on gas-phase reactions with ozone –, and ultraviolet photodissociation (UVPD) have also been used successfully to localise the double bond in the fatty acyl chains⁶⁴.

Glycerophospholipid nomenclature has been established based on the glycerophospholipid class and the composition of the fatty acyl chains attached to the sn-1 and sn-2 position of the glycerol. The name of a glycerophospholipid includes its class abbreviation, e.g., PA, PE, PC etc., followed by information on its fatty acyl composition in the sn-1 and sn-2 positions of the glycerol, e.g., PE(16:0/18:1) indicates that the phospholipid's class is phosphatidylethanolamine, with a 16:0 fatty acyl chain in the sn-1 position and 18:1 fatty acyl chain in the sn-2 position of the glycerol group. MS/MS analysis of glycerophospholipids allows us to gather information on the lipid class and its fatty acyl chain composition. However, the technique cannot distinguish which fatty acyl chain is attached in the sn-1 and sn-2 position of the glycerol group – therefore in this PhD thesis, glycerophospholipids will be referred to as PX(FA₁_FA₂) to symbolise that the fatty acyl chains can be in either of the two positions. For example, PE(16:0_18:1) indicates that the lipid has been identified as a phosphoethanolamine, with a 16:0 and a 18:1 fatty acyl chain, which can be esterified in either of the sn-1 and sn-2 position. Without MS/MS analysis, we cannot be certain of the fatty acyl composition of the lipid, however, based on accurate mass we can assign the lipid class and the total number of carbon atoms

and double bonds contained in the two fatty acyl chains, e.g., PE(34:1). In this PhD thesis, lipids that have been identified by accurate mass and MS/MS will be stated as PX(FA₁_FA₂) – e.g., PE(16:0_18:1) – while lipids annotated only by the accurate mass will be stated the total number of carbon atoms and the total number of double bonds in both fatty acyl chains – e.g., PE(34:1)^{65–67}.

For ether glycerophospholipids, the “O-” prefix after the lipid class is used to indicate the ether bond, PX(O-FA₁_FA₂), e.g., PE(O-16:0_18:1) indicates the presence of an ether bond connecting the glycerol group to a 16:0 alkyl chain. However, as in the case of diacyl glycerophospholipids, mass spectrometry cannot distinguish if the alkyl chain is in the sn-1 or sn-2 position. For plasmalogens, the prefix “P-” is used after the lipid class, stating the presence of a vinyl ether bond. In this case the prefix “P-” already includes the double bond in the alkenyl chain, and thus there is no need to state it as a double bond in alkenyl chain composition – e.g., PE(P-16:0_18:1) indicates a PE plasmalogen with the double bond of the vinyl ether linkage already included in the “P-” prefix. However, common mass spectrometric methods are not able to distinguish the position of the double bond, i.e., if it is next to the ether linkage, making it a plasmalogen, or if it is further down in the alkyl chain – therefore, in this PhD thesis, only the “O-” prefix will be used to indicate the general class of ether glycerophospholipids, without indicating if the lipid is a plasmalogen or not⁶⁷.

1.3. Characterisation of cell cultures using mass spectrometry

1.3.1. Liquid Chromatography-Mass Spectrometry (LC-MS)

1.3.1.1. Fundamentals of LC-MS

Liquid chromatography (LC) is a well-established analytical technique, used for the separation of a mixture to reduce its complexity. LC can be applied to a range of biomolecular classes, from carbohydrates and amino acids to lipids and proteins. In LC, the mixture is separated by the distribution of its components between two phases; the mobile phase, a solvent system that carries the analyte through a chromatographic

column containing the stationary phase. The stationary phase consists of particles of a specific chemistry – usually an adsorbent solid, often silica-based – which retain the analyte based on its affinity to the stationary phase for a certain amount of time, characteristic for each compound (retention time)^{68–70}. Reversed phase liquid chromatography-mass spectrometry (RPLC-MS) is used commonly for the analysis of biological samples and the coverage of the metabolome and lipidome. RPLC utilises a hydrophobic/non-polar stationary phase and a hydrophilic/polar mobile phase. The separation of the metabolites and lipids is based on hydrophobic interactions between the molecules and the stationary phase^{71,72}.

Different detectors can be used to analyse the eluted components of the mixture after separation in the chromatographic column, such as ultraviolet, fluorescence, or mass spectrometry⁷³. Mass spectrometry is a very sensitive analytical technology that measures the mass-to-charge ratio (m/z) of gas-phase positive or negative ions. A typical mass spectrometer consists of an ionisation source, which ionises the neutral analyte molecules; the mass analyser, where the ions are sorted and separated based on their m/z ; and the ion detection system, where ions are separated in time or space, are measured and the mass spectra, featuring the m/z values on the X axis and their intensities on the Y axes, are acquired⁷⁴.

The ionisation source is a very important element during a mass spectrometric analysis, since it is the part converting neutral molecules into positive or negative ions and allowing their detection by the mass spectrometer. A variety of ionisation techniques exist, and they can be categorised into two groups, “hard” and “soft” ionisation sources, based on how energetic the ionisation is and the type of ions generated. Hard ionisation techniques, such as Electron Ionisation (EI) are energetic, generating radical ions, usually with extensive fragmentation. These ionisation techniques usually require the molecules to be already in the gaseous phase before the ionisation occurs, which is why they are limited to molecules that are sufficiently volatile. Soft ionisation techniques, on the other hand, allow the ionisation of intact molecules, with less fragmentation observed, due to the lower energy transferred to the analyte. Soft ionisation techniques are not limited by analyte volatility, as hard ionisation techniques are, and they are easily applicable for the analysis of heavier, more polar biomolecules^{75–77}.

One of the most famous soft ionisation techniques is Electrospray Ionisation (ESI) developed by John Fenn⁷⁸ in 1984. ESI was catalytic for the further development and hyphenation of mass spectrometry to LC systems, since ESI can generate intact gas-phase ions from analyte molecules present in a liquid mixture and thus can facilitate the gas-phase ion generation from chromatographic eluates.

The first step in ESI is the infusion of the solution containing the analyte into a metal capillary in which voltage of several kV is applied. Depending on the ionisation mode positive or negative potential energy is used. The electric field generated leads in distribution and separation between positive and negative charges near the meniscus of the liquid with the ions of the selected charge to move towards the tip of the capillary, while the ions of the opposite charge move towards the inside of the capillary. The repulsion of the charges overcomes the surface tension of the solution and the surface begins to expand, forming a Taylor cone at the tip of the capillary and a jet of small, highly charged droplets emerges from the cone when the excess charge overcomes the surface tension, while a coaxial gas flow (usually N₂) assists the spraying process⁷⁹⁻⁸².

After the formation of the charged droplets, the solvent starts to evaporate, while the charge remains the same. The thermal energy generated by the inert gas flow and the air at atmospheric pressure facilitates the solvent evaporation. With the decrease of the droplets size the repulsion between the charges present increases and eventually the repulsion forces overcome the surface tension. This leads to fission of the droplet – also known as Coulomb fission – releasing a jet of smaller, highly charged progeny droplets. The fission of the parent droplets is repeated until very small, charged droplets are formed. Two main mechanisms are believed to be involved in the gas-phase ion formation – the charged residue model and the ion evaporation model. In the charge residue model, some of the very small droplets occurred after the evaporation of the solvent and the repeated fissions would contain only one analyte molecule in addition to ionic charges present on the droplet's surface. Evaporation of the solvent in such droplet would take place, leaving the analyte molecule in the gas phase carrying the remaining charges, resulting in the formation of a gas-phase ion. The ion evaporation model assumes that after the evaporation of the solvent and the shrinkage of the originally generated ESI charged droplets, the charges repulsion

overcomes the molecules adhesion to the surface of the droplet. This results in gas-phase ions directly emitted (“evaporated”) from the surface of the droplet^{82–85}.

Positive ion in ESI (and for soft ionisation techniques generally) is often the ion polarity of choice for historic reasons. The polarity used depends primarily on the chemical structure and the functional groups expected to be present in the analyte. Molecules with acidic hydrogens, such as fatty acids, oligonucleotides, glycerophospholipids etc., could readily lose a proton (H^+) and become deprotonated negatively charged ions, while molecules containing nitrogen and amine groups could readily accept a positive charge through protonation. Adduct formation, with the addition of a cationic or anionic group in the analyte molecule, is another way of generating positive and negative ions respectively. Sodium (Na^+), Lithium (Li^+), and ammonium (NH_4^+) analyte adducts are often observed in the positive ion mode, while chlorine (Cl^-), formate ($HCOO^-$), and acetate (CH_3COO^-) are some of the adducts that can be formed in the negative ion mode. Despite the fact that positive ion mode is the preferred polarity used in mass spectrometric studies, negative ion mode presents certain advantages, such as lower background noise and improved ionisation efficiency (depending on the analyte), while it is less prone to ion suppression due to its increased selectivity towards acidic molecules^{25,86,87}.

The coupling of separation techniques like LC to mass spectrometry (LC-MS) revolutionised the field of bioanalysis, and the hyphenated technique is now used for a wide range of biological and chemical analysis, including proteomics and metabolomics, drug metabolism assays, and toxicological screening^{88–90}. Despite the fact that other ionisation sources have been previously or currently used as interfaces in LC-MS – such as thermospray ionisation and atmospheric pressure chemical ionisation (APCI) – ESI revolutionised the field and its applications on complex mixtures^{91–93}.

There is a wide variety of mass spectrometers that can be used for the analysis of complex mixtures, exhibiting different mass accuracy, mass resolution, resolving power. Mass accuracy describes the proximity between the accurate mass, i.e., the experimentally measured m/z value, to the exact mass (theoretical mass), while mass resolution describes how well two peaks can separate from each other, and it is a function of both peak width and the measured m/z value. There are two definitions

describing mass resolution. The first one – the 10 per cent valley definition – calculates resolution of two peaks of equal intensity at masses m and $m-\Delta m$, as $R = m/\Delta m$, where Δm the mass difference between the two peaks at 10% of either of the peak's height. The second definition – the peak width definition – describes the resolution of a single peak at mass m , as $R = m/\Delta m$, where Δm is the peak width, at 50% of the peak's height, also known as full width at half maximum (FWHM)^{94–97}. Resolving power according to IUPAC refers to the ability of a mass spectrometer to provide a specified value of mass resolution, and it is a term used to describe the ability the instrument to distinguish two adjacent ions with of equal intensity^{97,98}. It is often used interchangeably to mass resolution, however, resolution relates to the mass spectrometric data, while resolving power is a function of the mass spectrometer^{74,96,99}. The higher the resolving power of a mass spectrometer, the better the separation between two ions with close m/z values. Different mass spectrometers can be used depending on the type of studies and the requirements in terms of mass accuracy and resolution. Instruments of low resolving power, such triple quadrupoles (QqQ), can be used for targeted analysis, often utilising MS/MS for the analysis of specific ions with known m/z value. On the other hand, mass spectrometers with high resolving power, can provide accurate m/z values of several decimal places, often used for untargeted analysis, i.e., for the detection of as many ions as possible from a given mixture, which is often the preferred approach in metabolomics and lipidomics assays. Time-of-Flight (ToF), Fourier Transform-Ion Cyclotron Resonance (FT-ICR), and Fourier Transform Orbitrap (FT Orbitrap) mass spectrometers can be used for accurate mass measurements. Hybrid instruments, which combine two or more mass analysers, also exist to facilitate MS/MS analysis. One of the most common hybrid HRMS instruments is the Quadrupole Time-of-Flight (QToF), which is the main mass spectrometer used in this PhD thesis^{100–103}.

In QToF MS, the ions generated in the ionisation source are first introduced into the quadrupole mass analyser, which operates using an electric field generated by direct current (DC) and radiofrequency (RF) voltage. The quadrupole can operate either as a mass filter – where DC/RF ratios are adjusted to only allow transmission of ions of a certain m/z value – or it can operate in RF-only mode, so that all ions are transmitted. QToF instruments have a collision cell, used in MS/MS analysis, where the ion of choice collides with neutral gas molecules, often argon or nitrogen gas, which result

in the fragmentation of the ions. After their fragmentation, product ions are transmitted to the ToF analyser for their detection. In ToF MS, all the ions are accelerated and given the same kinetic energy. The main principle of the ion separation in a ToF mass analyser is that the ion's kinetic energy, $E_k = mv^2/2$ – where m is the ion's mass and v its velocity – is equal to the potential energy, $E_p = zeV$, where z is the ion's charge, e the charge of an electron, and V the accelerating potential of the electric field applied:

$$mv^2/2 = zeV$$

$$v = \left(\frac{2zeV}{m} \right)^{1/2}$$

From the equation above it is evident that the velocity of the ion is inversely proportional to its m/z value.

The time the ion needs to travel through the flight tube (time-of-flight, tof) can be calculated by dividing the flight tube distance (L) with the ion's velocity:

$$tof = \frac{L}{v} = \frac{L}{(2zeV)^{1/2}} \left(\frac{m}{z} \right)^{1/2}$$

Since all ions have the same kinetic energy, lighter ions have higher velocities, thus, needing less time to travel through the flight tube and reach the detector than heavier ions, allowing the separation of ions based on their mass-to-charge ratio^{101,104,105}.

Some ToF instruments employ a configuration named “reflectron” in order to increase the instrument's resolving power and compensate for velocity dispersions and uneven distribution of the initial kinetic energy between ions of the same m/z value. The reflectron is an electrostatic mirror, placed in the drift region of the ToF, in which ions penetrate until their kinetic energy is 0, at which point they get reaccelerated by the electrostatic field and move towards the opposite direction and towards the detector. The more energetic ions (the faster ions) penetrate deeper into the ion mirror than the slower ions. The additional distance the more energetic ions have to cover compensates for the spatial dispersion and kinetic energy spread among the ions of the

same m/z value, which allows them to reach the detector at the same time, thus improving the resolution¹⁰⁶.

Orthogonal acceleration (oa) is another feature often found in ToF instruments, which can improve the resolving power and allows the coupling with techniques that generate a continuous ion beam such as ESI. oaTOF instruments utilise an electrostatic field to accelerate the ion beam orthogonally, to the ion formation axis. One of the main advantages of orthogonal acceleration is that it provides distinct time events (pulsed ion beam), which is compatible with ToF analysis. Additionally, it minimises velocity and spatial dispersion between ions, thus improving the resolution^{75,107,108}.

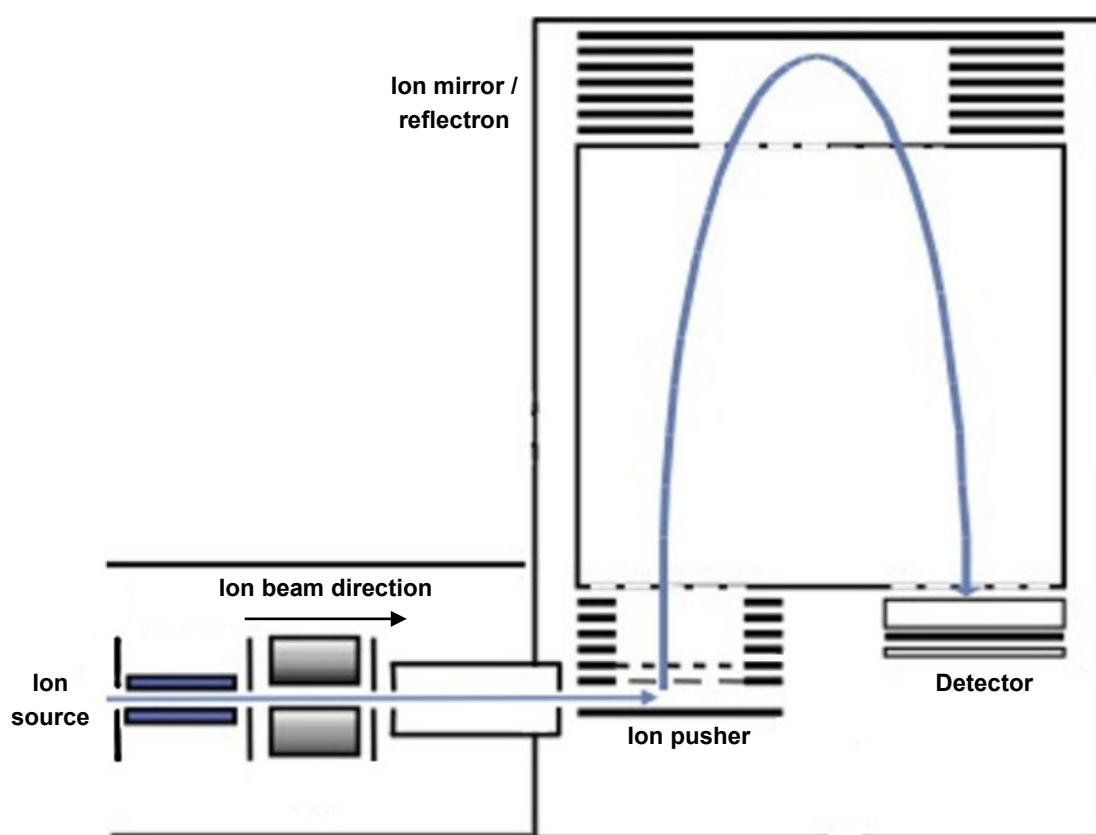


Figure 7. Illustration of a hybrid QToF mass analyser, using orthogonal acceleration (ion pusher diverts the ion beam an orthogonal direction) and the reflectron, which compensates for the spatial dispersion and kinetic energy spread among the ions of the same m/z value and improves the mass resolution. Figure adapted from Lucini et al. (2015), reproduced with permission from Elsevier.

1.3.1.2. Applications of LC-MS to cell analysis

Since its development and commercialisation, LC-MS has become the gold standard technique in bioanalysis, mainly due to the separation capabilities and complexity reduction LC provides, in combination with the high sensitivity of mass spectrometry¹⁰⁹.

Many studies have utilised LC-MS for the lipidomic and metabolomic analysis of cellular material. Rombouts et al.¹¹⁰ demonstrated the use of the hyphenated technique for the differentiation between human-transformed (T) and non-transformed (NT) immortalised colon cell lines using metabolomic and lipidomic LC-MS data for the investigation of colorectal cancer, while Yusufi et al., 2017¹¹¹, and Yeo et al., 2018¹¹², demonstrated the use of LC-MS for the metabolomic and lipidomic analysis of Chinese Hamster Ovary (CHO) cells used for the production of biopharmaceutical compounds.

Despite the applicability of LC-MS in cellular analysis its throughput capabilities are still limited, with the limiting factor being the requirement for sample preparation prior to the introduction of the sample in the LC-MS system – the analysis itself is also slow, requiring several minutes of chromatographic separation per sample¹¹³. While LC-MS can be used for the in-depth lipidomic and metabolomic characterisation of various cell lines, sample preparation of the cells is time-consuming and laborious, including potent organic solvents, multiple steps of centrifugation, and lipid/metabolite extraction^{110–112,114}. A widely used lipid extraction method is the Bligh & Dyer¹¹⁵ total lipid extraction and purification, using chloroform, methanol, and water to create a biphasic system, separating the sample molecules between an organic phase – chloroform layer, where lipids are expected to be – and an aqueous phase – methanol/water layer with the more hydrophilic, polar molecules. The extensive pre-treatment of the cells required for the LC-MS analysis does not allow dynamic, real-time analysis, thus failing to capture the rapid changes that occur in the cell over time and the use of potent organic solvents, like chloroform, could create a bias in the results^{31,32,116}.

1.3.2. Direct Infusion (DI) and Flow injection (FI) Mass Spectrometry

Direct infusion mass spectrometry (DI-MS) is an analytical technique in which a liquid sample is introduced to the ion source and the mass spectrometer in a continuous flow stream. DI-MS methods often use syringe pumps to deliver a continuous flow stream of the liquid sample. In flow injection mass spectrometry (FI-MS) an aliquot of the sample is injected into the stream of a liquid carrier and – without the chromatographic separation – is introduced in the mass spectrometer⁹⁷. FI-MS methods usually utilise LC systems in which the autosampler is used for the injection of the sample in the stream of the mobile phase and its subsequent introduction in the mass spectrometer – bypassing the chromatographic separation^{117,118}. DI-MS and FI-MS techniques have been proposed as faster alternatives to LC-MS for the analysis of cell cultures, as they have better throughput capabilities since the separation step is omitted, presenting a significant reduction in total analysis time (typically <1-2 min), allowing the analysis of hundreds of samples per day. Sample pre-treatment is still required, however, the preparation protocols used are simpler than the ones required for LC-MS, which also improves the throughput capabilities of these techniques^{113,119,120}. Metabolomic and lipidomic studies based on flow injection and direct injection techniques utilise high resolution instruments, such as Orbitrap and ToF, which enable the detection of thousands of m/z features present in biological samples¹¹³.

1.3.3. Matrix Assisted Laser Desorption Ionisation (MALDI)

Matrix Assisted Laser Desorption Ionisation (MALDI) is an ionisation technique developed in the 1980s, taking place at low pressure and utilising lasers for the desorption and ionisation of the surface molecules of condensed samples. The main feature of the technique is that, prior to analysis, samples are co-crystallised with an organic compound – the “matrix” – which usually exhibits strong absorbance in the UV range. During the ionisation process, the laser beam irradiates the matrix and the analyte, the matrix absorbs some of the laser energy and the material is desorbed from the surface of the sample. Several ionisation mechanisms have been proposed for MALDI, with the two prevalent ones being the “lucky survivor” and the gas phase protonation model. In the “lucky survivor” model the clusters of matrix and analyte, generated from the laser irradiation, contain pre-charged analyte molecules in clusters with counterions, resulting in zero net charge. When the clusters dissociate, the analyte can be either neutralised, or maintain the charge and become gas-phase ion. The gas phase protonation model indicates that gas phase collision of neutral analytes with protonated or deprotonated matrix molecules could result in proton transfer reaction, thus generating protonated or deprotonated gas-phase ions^{121,122}.

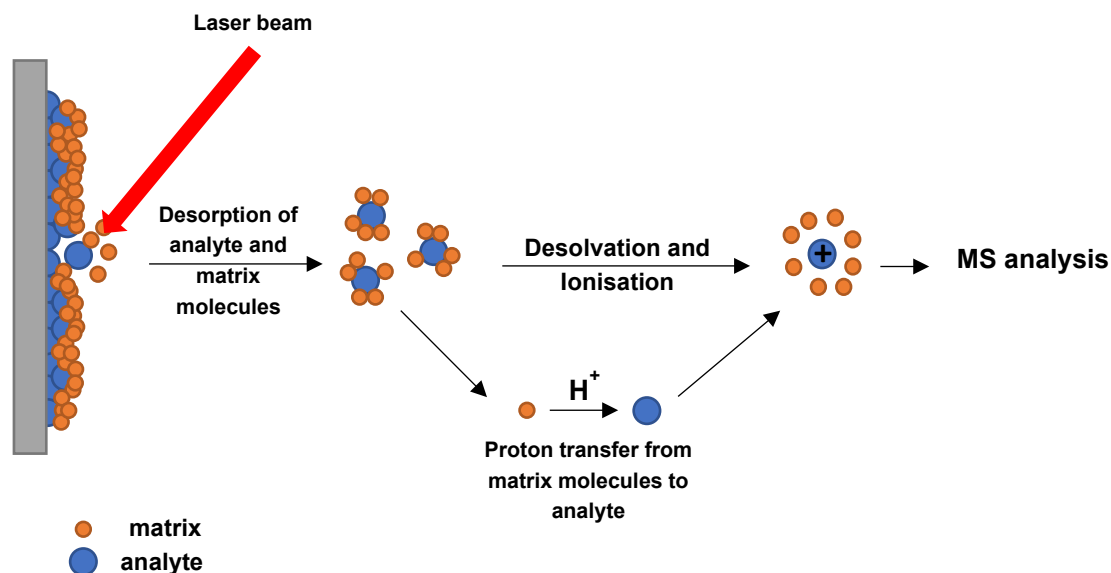


Figure 8. Ionisation of analytes in MALDI MS. Figure adapted from de Hoffmann et al. (1996), reproduced with permission from John Wiley and Sons Ltd. © 2003.

MALDI-MS is commonly used as a mass spectrometry imaging (MSI) technique, mainly on tissue sections, however it has also been used for high-throughput lipidomic and metabolomic analysis of cells^{123,124}.

In comparison to LC-MS, MALDI-MS analysis of cells requires simpler sample pre-treatment and has better throughput. However, depositing the cells on conductive target plates compatible with MALDI-MS and the application of the matrix still requires time, while the co-crystallisation of the matrix compound with the analyte is a delicate process and could be a potential source of contamination and error in the results.

1.3.4. Secondary Ion Mass Spectrometry (SIMS)

Secondary ion mass spectrometry (SIMS) is a desorption ionisation technique, operating under high vacuum, where the surface of a condensed phase sample is sputtered by a beam of high-energy ions or multiply charged gas clusters – referred to as the primary beam. During this process, energy is transferred to surface molecules, sputtering neutral molecules and newly formed ions – secondary ions – which are then analysed by the mass spectrometer. SIMS is often used for the analysis of cells, since its potential for high spatial resolution, both 2D and 3D, can give information on a subcellular level^{125–127}. The development of OrbiSIMS – a hybrid instrument that couples the high spatial resolution of SIMS with the high resolving power Orbitrap mass analyser – is one of the latest advancements in the field of secondary ion mass spectrometry as it combines subcellular imaging capabilities with high mass accuracy (< 1 ppm)¹²⁸. Despite the applicability of SIMS in high spatial resolution single cell MSI, the technique presents certain difficulties. The outcome of the analysis is highly dependent on the sample preparation of the cells, which need to be chemically fixed or cryofixed for analysis. In addition, fragmentation of ions occurs with SIMS due to the high energy of the primary ions, which increases difficulty in the interpretation of data and the identification of ions observed in the mass spectra^{125,129}.

1.3.5. Ambient ionisation mass spectrometry

Ambient ionisation mass spectrometry has been significantly developed in recent years due to its ability to ionise molecules from the surface of various types of samples in their native state, with minimal or no sample preparation. The main characteristic of these techniques is that the analysis takes place under ambient conditions – not just pressure, but also in an open environment, and therefore the techniques can be coupled to different mass analysers with minor modifications¹³⁰.

1.3.5.1. Desorption Electrospray Ionisation (DESI)

Desorption Electrospray Ionisation (DESI) was first described by Takats et al.¹³¹ in 2004, and was the first ambient ionisation technique reported. The mechanism of DESI combines the main features of desorption ionisation (DI) techniques and ESI, as nebulised, charged droplets of solvent – directed towards the surface of the analyte – are used to desorb the sample molecules on the surface, generating charged droplets containing the analyte molecules, which then generate ions by solvent evaporation and droplet fission, following the same ion formation mechanisms as ESI described in section 1.3.1.1^{131–133}.

DESI is most often used for tissue MSI analysis, however there have been studies using DESI for the analysis of cell cultures after the removal of growth medium and the cryofixation of cells on a DESI-compatible surface^{134,135}, demonstrating lipid-rich mass spectra of the cells.

1.3.5.2. Acoustic mist ionisation (AMI)

Another high-throughput ambient ionisation technique which has been reported is Acoustic Mist Ionisation mass spectrometry (AMI-MS), first described by Sinclair et al.¹³⁶, in 2016. AMI-MS utilises acoustic droplet ejection (ADE) that delivers ultrasonic pulses to low volumes of liquid samples, creating a “mist” of femtoliter-sized droplets, which become charged with the application of an electric field. The charged droplets are introduced to the mass spectrometer through a high-temperature

capillary, where the droplets desolvate, leaving charged, gas-phase analyte molecules, ready for mass spectrometric analysis. This technique can be used in a high-throughput manner when applied to micro titer plates, and the studies conducted until now have demonstrated the detection of a wide range of molecules, from small metabolites to multiply charged peptides and proteins^{136,137}.

Bachman et al¹³⁸. demonstrated the applicability of AMI-MS for the high-throughput analysis of cell lysates of adherent and suspension cells, acquiring mass spectra rich in small molecules and phospholipids. AMI-MS is potentially one of the very few technologies reported to analyse cells in such a high-throughput manner – reporting up to 3 samples per second – however, sample preparation, including the lysis of the cells, is still required for analysis.

1.3.5.3. Rapid Evaporative Ionisation Mass Spectrometry (REIMS)

Rapid Evaporative Ionisation Mass Spectrometry (REIMS) is an ambient ionisation technique where condensed phase biological samples are rapidly heated, generating a molecule-rich aerosol that is subsequently analysed with mass spectrometry. The samples can receive energy and are heated using various mechanisms, namely, contact heating, Joule heating – which subjects the biological material to RF alternating current –, or radiative heating – using laser irradiation. Radiative heating causes the localised heating of the biomass by the absorption of laser energy from the water molecules found within the sample. On the other hand, Joule heating occurs via the application of electric current to the conductive components of the sample. The localised heating, in both cases, leads in vaporisation of the endogenous water molecules followed by vapour expansion and subsequent disruption of the biomass, resulting in the generation of a molecule-rich evaporation plume¹³⁹. The aerosol is then aspirated from the sampling site, via polytetrafluoroethylene (PTFE) tubing, using the vacuum of the mass spectrometer, or a venturi air jet device. Before the aerosol enters the vacuum of the REIMS interface, it passes through a stainless-steel capillary, where it is mixed with a solvent matrix – usually propan-2-ol (IPA) – introduced orthogonally to the aerosol. Using a solvent matrix, the aerosol particles and clusters are diluted in the solvent's droplets and the solution is then introduced

into the vacuum region of the REIMS interface, with pressure at approximately 1 mbar, where the droplets get accelerated by adiabatic expansion due to the low pressure. The accelerated droplets collide in a heated surface (approximately 800°C), where impact events with the high-temperature surface results in solvent evaporation and gas-phase ion formation^{139,140}.

REIMS was first described in 2009 by Schafer et al.¹⁴¹ as a method to conduct mass spectrometric analysis *in vivo* during surgery. This technology was named “iKnife”, since the source of heating was the surgical diathermy knife, which utilised alternating current to rapidly heat the tissue and ablate surface molecules during surgery. The generated aerosol was then analysed by a mass spectrometer in the operating theatre, and the data generated featured predominantly glycerophospholipids, which in conditions such as cancer can be very informative. This technology is particularly useful in cancer surgery, since the iKnife can successfully detect cancer margins by the differences in the molecular profile of the cancer versus the healthy tissue. The initial study conducted by Schafer et al. showed good differentiation between histologically different areas of a porcine kidney, with the timescale of the REIMS analysis at 0.1-0.3 seconds, thus enabling real-time tissue identification – subsequent studies using the iKnife showed similar identification accuracy between different tissue types^{141–143}. After the initial use of REIMS as an *in vivo* mass spectrometric technique, REIMS was used for other applications, including *in situ* tissue analysis^{144,145}, bacteria cultures^{146–148}, and *in vitro* models, such as cell lines or organoid analysis^{142,149–151}.

LD-REIMS is a recently developed ambient ionisation technique that relies on the desorption of various biological samples, with minimal or no pre-treatment, under ambient conditions. The technique uses an IR laser for the rapid heating and evaporation of the sample, and the subsequent aerosol is then transferred into a mass analyser¹⁴⁶. The resulting mass spectra feature predominantly lipid species, i.e., fatty acids, glycerophospholipids, lipid dimers etc.

LD-REIMS and Laser Desorption Ionisation (LDI) methods share some similarities – mainly the use of lasers to desorb molecules from surfaces – however the ion formation mechanism of the two methods is different. The ion formation

in LD-REIMS, and REIMS in general, is believed to be a combination of the solvent matrix addition and the subsequent generation of solvent/analyte aerosol, with impact events in the heated collision surface, as demonstrated by Jones et al.¹³⁹. On the other hand, in LDI methods, the ion formation follows a different route, which is highly dependent on the presence of matrix molecules – in the case of MALDI – and/or the chemistry of the surface the samples are deposited on, as it happens in the case of surface assisted LDI (SALDI) and surface enhanced LDI (SELDI)^{97,152}.

REIMS cell analysis

The first study that utilised REIMS for the analysis of cells was conducted by Strittmatter et al.¹⁴⁹ in 2016, where the technique was evaluated for the analysis of the NCI60 cell line panel, and the results validated using LC-MS of the same cell lines¹⁵³. The sample preparation described was very minimal, consisting only of centrifugation of the harvested cells – REIMS analysis was performed directly

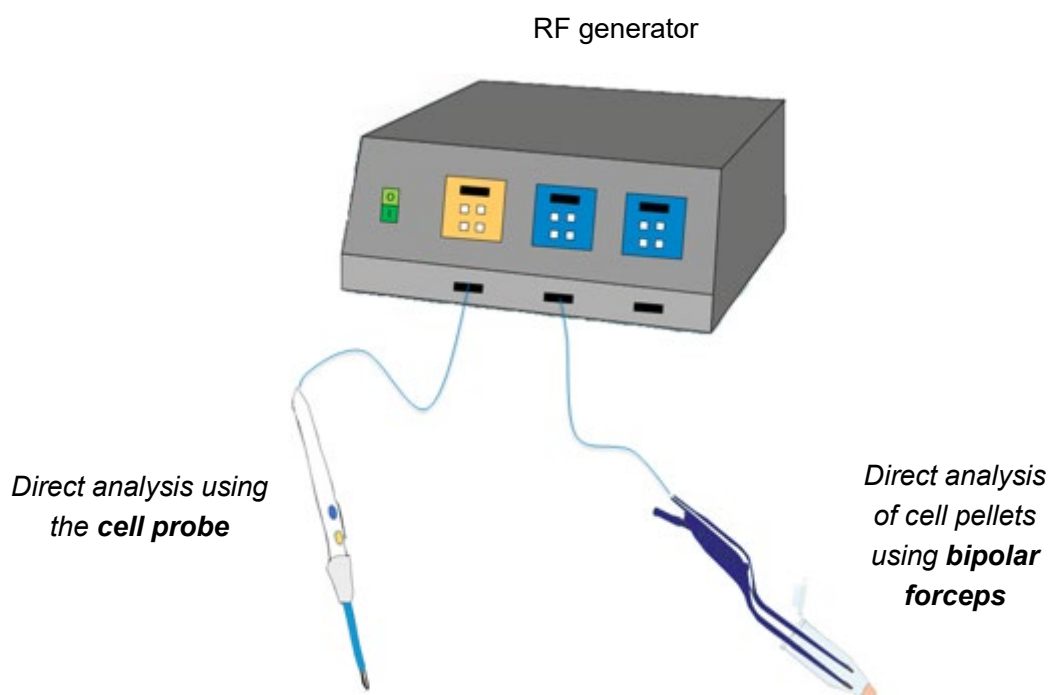


Figure 9. Schematic of cell probe and bipolar forceps REIMS sampling techniques used for the direct analysis of cell pellets. Both sampling methods coupled to a REIMS atmospheric interface have been used in the past for the direct mass spectrometric analysis of cells.

on cell pellets without any further pre-treatment. In this study a handheld bipolar sampling probe (also referred to as bipolar forceps) was used, connected to an RF power generator. The cellular material was enclosed between the tips of the two electrodes of the bipolar forceps, as shown in Figure 9, which rapidly heated and evaporated the cell pellet, generating an ion- and molecule-rich aerosol which was transferred to the mass spectrometer. REIMS was proven to be an effective tool for the characterisation and classification of biologically related cell lines, and the lipids identified were also detected with LC-MS¹⁵³.

A more recent study that utilised bipolar forceps REIMS for cell analysis was conducted by Koundouros et al.¹⁵⁰, in 2020. In this study, the REIMS technology was used again with RF powered bipolar probes for the analysis of cell lines in a condensed and pelleted form. The bipolar forceps REIMS, coupled with an Orbitrap mass analyser, gave important metabolic signatures on mutant PIK3CA cell lines¹⁵⁰.

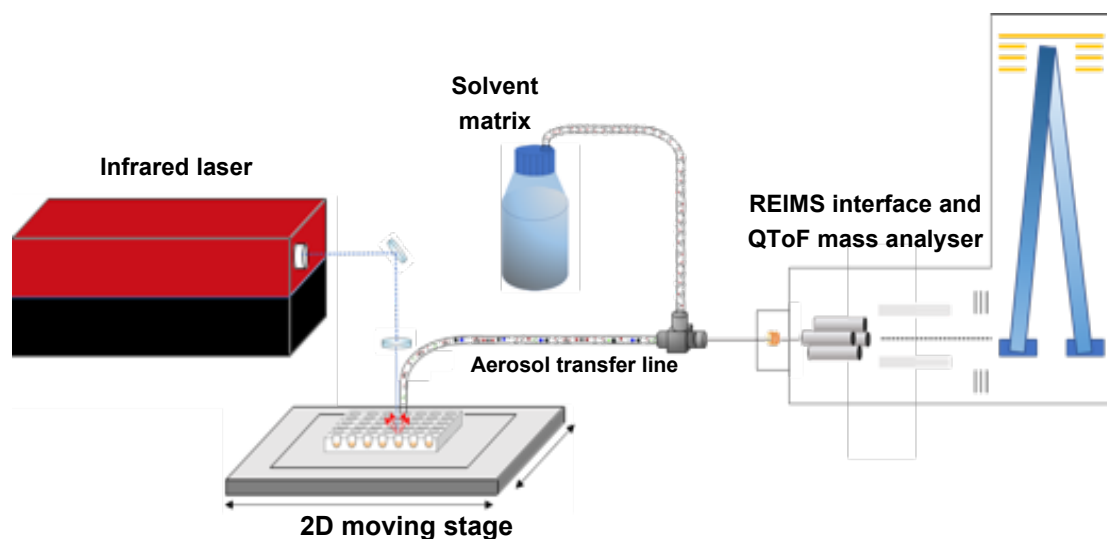


Figure 10. High-throughput LD-REIMS platform. An IR laser is used for desorption of cellular material and generation of the aerosol. The aerosol is aspirated through a PTFE transfer line using the vacuum of the source inlet of the mass spectrometer. Before introduction of the aerosol in the REIMS interface, it is desolved in solvent matrix, propan-2-ol (IPA), to enhance declustering. The mixture is then introduced into the REIMS atmospheric interface where it collides on a heated collision surface, resulting in the evaporation of the solvent. The ions are then detected by a QToF mass analyser.

IR LD-REIMS, using a CO₂ laser, was used by Paraskevaidi et al.¹⁵¹, for the rapid analysis of liquid-based cytology (LBC) samples of exfoliated cells – analysed in cell pellet format, after centrifugation – for the screening of cervical cancer, while it has also been used extensively for the analysis of bacterial cultures^{154–156}.

The main aim of this PhD is to develop and optimise protocols based on the use of an automated LD-REIMS autosampler (Figure 10) for the high-throughput lipidomic and metabolomic analysis of different types of cells with minimal or no pre-treatment, analysed either as cell pellets, or as cell monolayer directly from the cell culture well plate.

1.4. Statistical analysis and data interpretation of MS data

1.4.1. Raw MS spectra pre-processing

Raw mass spectra acquired from biological samples present some challenges in their subsequent manipulation and interpretation, due to their complexity and the large amount of molecular information they contain. Pre-processing of the data is usually necessary to deconvolute/enhance its quality by filtering out noise and background peaks – which can be chemical or instrumental, and do not contain biological information^{157,158}. Raw MS data usually contain several millions data points per spectrum, which can be reduced after pre-processing, producing a more manageable dataset, of MS peaks and ion intensities, for statistical analysis¹⁵⁹.

A crucial step that is shared in most MS data pre-processing workflows is the peak detection, where computational algorithms are used to reduce the raw spectrum to a shorter list of peaks, for example by using the relative signal-to-noise ratio (S/N) or continuous wavelet transform (CWT)¹⁵⁹. The detected peaks from all spectra within the dataset can be then nominally aligned using internal standards, e.g., reference peaks found within the measured data. These standards can also be utilised to verify the performance of peak picking algorithms, and to recalibrate the mass spectra and obtain more accurate m/z values¹⁵⁹. Signal smoothing and baseline correction are also important steps in the preprocessing of the MS data and aim to remove instrument-derived noise. Peak alignment and matching are also performed to correct and adjust

small drifts in m/z values. This step ensures that the molecules detected across are matched into a single m/z value, which will allow their comparison and further processing^{160,161}.

To enhance the interpretability of the data, normalisation is another important step in MS data pre-treatment, and it is used to generally remove systematic variation of the mass spectrometric data, caused by experimental factors that cannot be readily controlled or predicted, e.g., the amount of material analysed. One of the most common normalisation methods in metabolomics and lipidomics is total ion count (or total ion current) normalisation (TIC), where the intensity values are presented relative to the total signal intensity of the whole spectrum, which enables interpretation of relative concentration of species in terms of the total ions detected^{162,163}. Apart from the normalisation of the data, prior to the statistical analysis, metabolomic and lipidomic data often need to undergo further pre-treatment, namely transformation and scaling, to correct for unwanted variance that is not biologically relevant. Different methods can be used for the transformation and scaling of the data, including centering, autoscaling, pareto scaling, logarithmic transformation, and power transformation. Centering is one of the simplest data pre-treatment methods, and its main characteristic is that it removes the offset of the data, giving them values fluctuating around zero, rather than around the mean value. Centering is usually applied in combination to other methods, as it is not often enough to correct the unwanted variation and heteroskedasticity of the data. Scaling methods are also used, dividing each variable with a certain – depending on the method – factor, the scaling factor which is different for each variable. The most common scaling approaches used are the autoscaling and the pareto scaling, in which the scaling factor is the standard deviation and the square root of the standard deviation respectively. Finally, transformations are also used to apply non-linear conversion of the data, to correct for heteroskedasticity, and make skewed distributions more symmetrical. One of the most popular transformation methods is log transformation, where each variable is replaced with its logarithmic value. Power transformation is also used for the correction of the data, usually by converting the variables' values into their square root values^{164–166}.

1.4.2. Statistical analysis and biomarker discovery

After the pre-processing of the data, the output is usually organised into a matrix format for further statistical analysis. Univariate – focuses on each variable separately – and multivariate – considers multiple variables and the relationship between them simultaneously – statistical techniques are used widely in metabolomic and lipidomic studies, for biomarker discovery or predictive modelling for classification and stratification studies¹⁶³. These statistical techniques can be either unsupervised, i.e., statistical methods revealing naturally occurring patterns in the dataset, without the need of any prior information¹⁶⁷, or supervised, which use labelled data for classification and inference^{168,169}.

Two of the simplest statistical methods applied in mass spectrometric data are t-tests and ANalysis Of VAriance (ANOVA), which are used to analyse the variance between populations, and determine if the data of two (t-test) or more (ANOVA) groups are statistically different from each other. t-test and ANOVA measure the different groups' mean values and the spread of variance in the data, calculating the p-value, which describes the probability that all groups/populations are the same. A p-value of 1 indicates that there is 100% chance of the populations to be the same – no variance between the groups – while a p-value of 0 means that there is 0% chance of the populations to be the same. The lower the p-value the higher the importance in the differences observed between the populations, while p-values ≤ 0.05 are generally considered to indicate that the variance observed is statistically significant^{170–172}.

Metabolomic and lipidomic data are high-dimensional data, which usually requires multivariate statistical tools and dimensionality reduction methods. Principal Component Analysis (PCA) is a prime example of unsupervised, multivariate statistical analysis of mass spectrometric data, as it effectively performs dimensionality reduction by re-mapping it onto a basis of ranked variances in a dataset^{173,174}. The primary goal of PCA in metabolomic and lipidomic studies is to visualise differences and patterns between different classes in a multivariate dataset. As opposed to PCA, Partial least squares-discriminant analysis (PLS-DA) is a supervised method used to describe the direction of the maximum variance in the data, taking into consideration the class of the samples – thus, being a supervised method.

In addition, PLS-DA can identify the most important variables, providing the variable importance on projection (VIP) score, which describes the significance of each feature in the separation of the groups¹⁷⁵.

Predictive modelling algorithms are most commonly used for classification problems; well established methods include linear discriminant analysis (LDA), logistic regression (LR), and random forest (RF) algorithms, all commonly used in mass spectrometric data analysis. LDA is a linear classification method, used to find a linear combination of features separating two or more groups/classes; it is a supervised method used to separate different groups by maximising the distance between the mean values between the groups, while at the same time it tries to minimise the variance within each group^{176–178}. LR is also used for the construction of classification models. LR does not require a linear relationship between the variables, as it applies a nonlinear sigmoidal function in multivariate datasets and the output is the calculation of the probability for a given classification^{163,179–182}. Random forest classifier uses decision trees to split the features into partitions based on common observations and attributes. This is an iterative process that stops when the algorithm has successfully split data with the same characteristics into groups and conclude the output decision. Subsequently, the algorithm aggregates the decisions from the multiple decision trees and selects the majority decision^{183,184}.

Biomarker discovery, i.e., the discovery of molecular indicators of physiological or pathophysiological conditions, diseases, pharmacological responses etc., is one of the main objectives in metabolomic and lipidomic studies. Feature selection is one of the most crucial steps in biomarker discovery and can be performed using various chemometric approaches. Many of the statistical methods described above can perform feature selection, as in the case of PLS-DA, which can rank the most significant features based on their VIP score, while univariate methods, such as t-tests can also be used to identify statistically significant features based on their p-value¹⁸⁵. Feature selection algorithms, such as Recursive Feature Elimination with Cross-Validation (RFECV), has also been used in metabolomics and lipidomics for the selection of a subset of m/z values, exhibiting the best representation of the different classes defined in the dataset^{186,187}. RFECV refines the predictive models by iteratively removing features (m/z values), based on a chosen model performance metric, the

redundant features that have no impact on the classification are eliminated. This model refinement step is a standard practice in machine learning, as classification model rebuilt with only the selected features are generally more robust and less over-fitted¹⁸⁸.

1.4.3. Metabolite and Lipid tentative annotation and identification

After feature selection further analysis can be conducted for the molecular identification of the m/z values of interest. Establishing the elemental composition based on the accurate molecular mass obtained – when a high resolution mass spectrometer is used – is one of the first steps in the identification process. The deviation between the experimental m/z value and the proposed theoretical value is usually expressed in ppm based on the mass difference between the two values:

$$\Delta ppm = \frac{(\text{experimental } m/z - \text{theoretical } m/z)}{\text{theoretical } m/z} \times 10^6$$

During this stage, apart from the Δppm deviation, we should also consider which elements are expected to be in the molecular formula. Certain elements are not expected to be present in biological systems and they can be excluded from the assignment of the elemental composition to a given m/z value, while others such as H, C, N, and O are very abundant in biological systems. In addition, the isotopic distribution of certain elements, such as Cl and S can be very distinctive in the mass spectrum and can be used as an indicator of the presence of these elements in the molecule. Finally, the “nitrogen rule”, which indicates that a neutral molecule has an odd nominal mass when it contains an odd number of nitrogen atoms and an even nominal mass when it contains an even number of nitrogen atoms, can further refine the list of the candidate elemental composition¹⁸⁹. Algorithms can be used to generate the elemental composition based on restrictions, such as the number of elements, isotopic patterns, carbon/hydrogen ratios and nitrogen, oxygen, phosphor, and sulphur ratios relative to the carbon atoms¹⁹⁰.

Once the elemental composition(s) is established, a list of potential chemical structures is generated, and more experimental work is usually required for the definitive identification of the compound. This often comprises LC-MS/MS analysis for the identification of the molecule based on its retention time and fragmentation pattern

and its separation from other isomers, potentially also including the use of stable isotope-labelled standard compounds, to confirm the retention time of the molecule¹⁹¹.

In 2007, the Chemical Analysis Working Group (CAWG) of the Metabolomics Standards Initiatives (MSI) tried to implement a set of minimum reporting standards and set confidence levels for the metabolite identifications¹⁹². The most recent publications describe 5 levels of confidence, depending on the workflow followed and the experimental work undertaken for the metabolite annotation. Level 1 – validated identification – is the highest confidence level, where the structure of the metabolite is confirmed with a minimum of two independent and orthogonal data, validated with a reference standard, analysed under identical analytical conditions. Level 2 – putative identification – describes the identification of the metabolite structure, using predictive structure evidence, such as MS/MS data exhibiting diagnostic fragments of the candidate metabolite, but without confirmation with a reference standard. Level 3 – preliminary identifications – is used to express the level of confidence of the annotation obtained by accurate mass measurement and isotopic distribution patterns, used to produce a list of tentative metabolite structures from databases. Finally, in Level 4, candidate molecular formulas are generated based on the accurate mass and isotopic patterns (based on the rules described above to establish the elemental composition), while in Level 5 – the lowest confidence level – only the exact m/z value of interest is determined^{193,194}.

Chapter 2

Hypothesis and Aims

Chapter 2: Hypothesis and Aims

2.1. Hypothesis

LD-REIMS is capable of the high-throughput, direct analysis of cell cultures, in their native state, with minimal or no sample preparation.

2.2. Aims

1. To develop, optimise, and validate protocols for the analysis of adherent cancerous cells.
 - a. Comparison of bipolar forceps and cell probe REIMS with LD-REIMS for analysis of 5 breast and 5 colorectal cell lines in pelleted form.
 - b. Development and validation of LD-REIMS methodology for analysis of cancerous cells directly from the culture plate, as a cell monolayer, living or frozen.
2. To establish the applicability of LD-REIMS for molecular characterisation of genetically modified cells producing therapeutic biopharmaceutical compounds.
 - a. Classify genetically modified Chinese Hamster Ovaries (CHO) cell lines producing the therapeutic monoclonal antibody adalimumab as high or low producers, based on their metabolic and lipidomic profile.
 - b. Identify metabolites and molecules that are significantly different between high and low producing cells lines.
3. To assess the applicability of LD-REIMS for the detection and investigation of drug-induced phospholipidosis mechanisms in alveolar macrophages treated with cationic amphiphilic drugs (CADs).
 - a. Investigate metabolic and lipidomic differences between untreated (control) rat alveolar macrophages, and macrophages treated with amiodarone, chlorcyclizine, and tamoxifen.

- b. Compare *in-vitro* and *ex-vivo* models of drug induced phospholipidosis when analysed with a LD-REIMS autosampler, and DESI-MSI and REIMS imaging, respectively.

Chapter 3

Method development, Optimisation, and
Validation of LD-REIMS cell analysis

Chapter 3: Method development, Optimisation, and Validation of LD-REIMS cell analysis

3.1. Introduction

In chemistry, an analytical method is a method used for the chemical characterisation of matter, either qualitatively (detection and identification of molecules present) or quantitatively (the quantity or concentration of a specific molecule)¹⁹⁵. An analytical method should be developed and optimised to be “fit-for-purpose”, i.e., to exhibit the required performance, and demonstrate in a reproducible and accurate way the composition of the samples analysed^{196,197}.

As described by Zheng et al.¹⁹⁶, the basic steps for the development, optimisation, and validation of an analytical method are summarised in Figure 11. The first step is to establish the target – the intended analyte, and the desirable characteristics of the analytical method, (e.g., if the method needs to be qualitative or quantitative), including expected performance criteria.

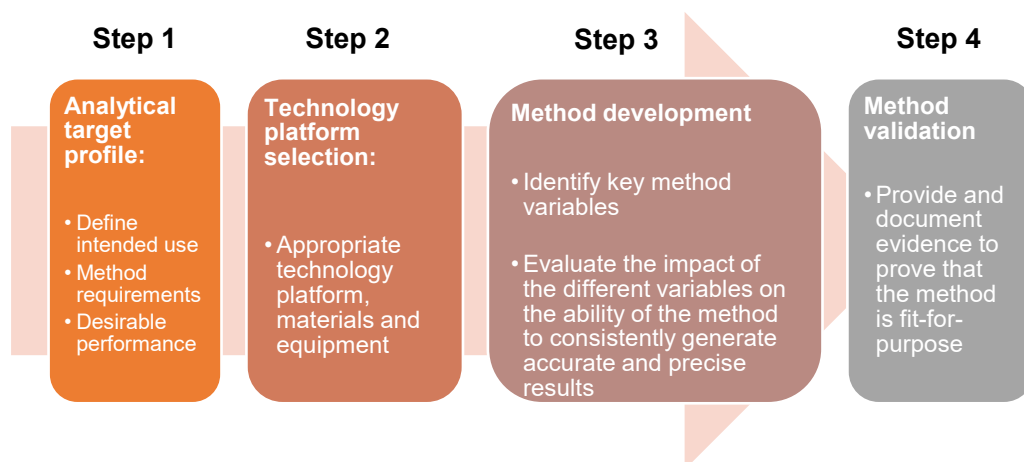


Figure 11. Basic steps for the development and validation of an analytical method.

The second step of this process is to establish the technological platform to be used, which requires information on the most suitable techniques or approaches which satisfy the desirable method attributes and characteristics established on Step 1. For example, if the method requires the quantification of antibodies then a suitable

technology platform might be Enzyme-Linked ImmunoSorbent Assay (ELISA), while if the intended use of the method is the detection of small polar metabolites, then a suitable technique might be Hydrophilic Interaction Liquid Chromatography coupled with a mass spectrometer (HILIC-MS).

After selecting the appropriate analytical platform, the key variables and characteristics of the method should be identified. The different variables of the selected method should be systematically assessed and their impact on the resulted measurements should be established. In this stage the method parameters should be optimised so that the method would ideally produce accurate and reproducible results.

After establishing the optimal parameters of the method in Step 3, method validation should be performed to prove that the method is “fit-for-purpose”. Accuracy, precision, repeatability, and reproducibility are often used to describe the fitness of an analytical method for its intended use. Accuracy describes the closeness of the measurement to the “true” or known value, while precision describes the closeness of the results among themselves, and it is often described as the deviation of each data point from the mean value. Precision is often expressed as repeatability, intermediate precision (or within-laboratory reproducibility) and reproducibility, depending on the level of variability included¹⁹⁸. Repeatability expresses the closeness of the measurements of the same sample/analyte over a short period of time, using the same analyst and equipment, while intermediate precision describes the agreement between the measurements of the same sample/analyte over a longer period of time, in the same laboratory, by the same analyst, using the same equipment. Reproducibility (also known as interlaboratory reproducibility) describes the agreement between the measurements of the same sample/analyte, using the same methodology but in different sites, with the measurements obtained by different analysts^{195–198}.

In this PhD thesis, the goal is the development and validation of a high-throughput analytical method for the analysis of cells in their native state, or as close to their native state as possible. Ambient ionisation mass spectrometry, and more precisely high-throughput LD-REIMS was identified as a suitable technological platform for this analysis. In this chapter, the optimisation of the sample preparation and LD-REIMS parameters for the measurement and characterisation of adherent cancerous cells are described. The precision – expressed as repeatability of the method and intermediate

precision – are assessed for the validation of the method. The results obtained by LD-REIMS were compared to bipolar forceps and cell probe REIMS methods that were used in the past for the analysis of cell pellets^{147,150,153,199}. For the method development, optimisation, and validation, 5 human breast cancer cell lines (BT549, MCF7, MDAMB231, MDAMB468, T47D) and 5 human colorectal cancer cell lines (C2BBE1, LS180, LS513, RKO, CL40) were used. All 10 cell lines were immortalised, adherent cell lines, often used in cancer research as in vitro models. Finally, LD-REIMS's accuracy in a biological hypothesis is demonstrated, using a method similar to that described in a study conducted by Koundouros et al.,¹⁵⁰ where it was found that oncogenic PIK3CA mutations enhance polyunsaturated fatty acid (PUFAs) production. Additionally, it was observed that cPLA2 inhibition suppresses PIK3CA-induced carcinogenesis. In the study, results were validated using a REIMS setup, with bipolar forceps REIMS, LC-MS, and biological assays on cells and tissue, so the aim here is to reproduce similar observations using the LD-REIMS platform for the analysis of PIK3CA mutant cells and the effect of cPLA2 inhibition in production of PUFAs.

3.2. Materials and Methods

3.2.1. Cell Culture

3.2.1.1. Human breast and colorectal cancer cell lines

Human breast cancer cell lines (BT549, MCF7, MDAMB231, MDAMB468, T47D) and human colorectal cancer cell lines (C2BBE1, LS180, LS513, RKO, CL40) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) media (Gibco). Human colorectal cancer cell line CL40 was cultured in DMEM/F-12 media (Gibco). Media for all cells was supplemented with 10% Foetal Bovine Serum (FBS) (Gibco). All cells were cultured at 37°C in a 5% CO₂ incubator. Cells were grown in 10 cm² plates and used for experiments when reaching 70-80% confluency. The cell lines were provided by the Institute of Cancer Research (ICR) and the cell culturing was performed by Aurelien Tripp, PhD candidate at ICR.

3.2.1.2. PIK3CA mutant and wild type MCF10A breast cancer cell lines

Human breast cancer cell lines, PIK3CA wild type (WT) MCF10A and PIK3CA mutant isogenic cell lines MCF10A E545K and MCF10A H1047R (referred to in this thesis as MUT1 and MUT2 respectively), were cultured in 10 cm² plates with DMEM/F-12 growth medium (Gibco) supplemented with 5% horse serum (Gibco), 20 ng/ml epidermal growth factor (EGF) (PeproTech EC, Ltd.), 100 ng/ml cholera toxin, 0.5 mg/ml hydrocortisone and 10 mg/ml insulin (all purchased by Sigma-Aldrich). For experimental conditions, where media free of fatty acid exogenous source was required, horse serum was replaced with 1% fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich). Half of the biological replicates of the MCF10A cell lines – wild type and mutants – were treated with a cPLA2 inhibitor, ASB14780. The inhibitor was supplemented at a concentration of 100 nM and DMSO was used as treatment control. All cell lines were maintained at 37°C and 5% CO₂. The cell lines were provided by ICR and the cell culturing was performed by Aurelien Tripp, PhD candidate at ICR.

3.2.2. Mass Spectrometry sample preparation

3.2.2.1. Cell pellets

Cell lines were cultured in 10 cm² plates until reaching 70-80% confluency. One hour prior to harvesting, media was changed. Plates were then washed twice with PBS, flash-frozen in liquid-nitrogen and scraped on ice with 1 mL PBS to recover the cell extract. Cell extracts were then transferred into a 1.5 ml microcentrifuge tubes (Eppendorf UK Limited), underwent centrifugation at 10,000 rpm, at 4°C, for 5 minutes. After removal of the supernatant, cell pellets were flash-frozen in liquid nitrogen and stored at -80°C until the day of the analysis. For LD-REIMS cell pellet analysis, the cell pellets were resuspended in the microcentrifuge tubes with 200 µL of 150 mM ammonium acetate (Sigma-Aldrich) (diluted in sterile water) in order to transfer the cellular material in a 96 well plate (Eppendorf™ 96-well V-Bottom PCR Microplates). The plate with the cell suspension underwent centrifugation at 5,000 rpm, at 4°C for 5 minutes to remove the ammonium acetate solution and generate the

pellets in the 96-well plates. The supernatant was then discarded, and the plate was kept in -80°C till the day of the analysis.

3.2.2.2. Cell monolayer

For the LD-REIMS cell monolayer analysis, cells were analysed directly from the cell culture wells (Greiner Bio-One, polystyrene, cell culture microplate, 96 well, u-bottom, clear) without need of extensive sample preparation. Cells were cultured according to conditions described in sections 3.2.1.1. and 3.2.1.2. and seeded at approximately 15,000 cells per well. Multiple biological replicates were generated for each cell line and condition in the 96 well plates and incubated at 37°C and 5% CO₂ until confluency of 70-80% was reached. Subsequently, the growth medium was removed, and the cells were washed twice with 150 mM of ammonium acetate (diluted in sterile water). Plates were cultured in duplicates. One of them was flash frozen in liquid nitrogen and kept in -80°C until analysed. The other was analysed with LD-REIMS straight after the washes with the ammonium acetate, to acquire the mass spectrometric profile of living cells and compare matching plates between the two approaches.

3.2.3. Mass Spectrometer Cleaning and Calibration

A Xevo G2-XS QToF mass spectrometer (Waters Corp.) was used for REIMS and LD-REIMS analysis. The day before the analysis propan-2-ol was introduced into the system to assess the noise levels and potential contamination and perform cleaning of the REIMS interface and the Stepwave ion guide if necessary. To clean the instrument, the REIMS interface was removed, and the inlet capillary and the Stepwave ion guide were removed and cleaned by immersion in a solution of 70:30 v/v propan-2-ol/Water (Optima™ LC/MS Grade, Fisher Chemical™, Fisher Scientific) and sonication for 15 minutes in an ultrasonic bath. The instrument pieces were then reassembled, and the instrument was pumped down. Calibration was conducted on the day of the analysis with a 0.1 mM sodium formate solution in propan-2-ol (Optima™ LC/MS Grade, Fisher Chemical™, Fisher Scientific), infused through a REIMS atmospheric interface (MediMass Ltd.) using a syringe pump (Remote Infuse/Withdraw Pump 11 Elite Nanomite Programmable Syringe Pump, Harvard Apparatus) and a 5 mL Hamilton

syringe at a rate of 100 $\mu\text{L}/\text{min}$. After the calibration, propan-2-ol was introduced into the system for approximately 30 minutes with 0.1 mL/min flow rate via a binary UPLC pump (ACQUITY, Waters Corp.), to remove any remaining contamination from the calibration solution.

3.2.4. Sampling techniques

Three different sampling methods used for comparison purposes in this study to generate the cellular aerosol for the REIMS analysis, i.e., bipolar forceps, cell probe, and laser desorption.

3.2.4.1. Bipolar forceps

The first approach utilised handheld forceps, consisted of two electrodes, also referred to as bipolar forceps (Erbe Elektromedizin). The bipolar forceps were connected to a Valleylab Force Triad RF generator (Covidien) operated at 4W power setting in bipolar mode. The RF power supply was controlled with a foot switch. The sampling of the cell pellets was done directly from the microcentrifuge tube (Eppendorf UK Limited), where the bipolar forceps were inserted. When the bipolar forceps were on the cell pellet, electric current was applied through the biological material, resulting in the generation of a molecule-rich aerosol. The aerosol was aspirated through an embedded fluid line in the forceps, connected through 1 m long 2.4 mm outer diameter, 1/16 in. inner diameter PTFE tubing (Fluidflon PTFE tubing; LIQUID-scan GmbH Co. KG) to the inlet capillary of the REIMS atmospheric interface. Multiple technical replicates were acquired for each cell pellet.

3.2.4.2. Cell probe

The second sampling modality used was the cell probe (Waters Research Centre) – a prototype RF based ablation device, specifically designed for the direct analysis of cell pellets from the microcentrifuge tube. The power used was 4W in mode. The RF supply here was controlled through a button switch present on the handheld probe. Similarly, as before, the sampling took place directly from the microcentrifuge tube (Eppendorf UK Limited). The probe was inserted into the tube, and when came in

contact with the cell pellet, electric current was applied, resulting in the generation of a molecule-rich aerosol. The generated aerosol was aspirated into the mass spectrometer as described above.

3.2.4.3. Laser Desorption

The third sampling technique used an IR laser, specifically an optical parametric oscillator (OPO) laser (Opolette 2731 / 3034, OPOTEK – beam width: 4 mm, pulse duration: 7 ns), incorporated as part of an automated prototype – in-house built – LD REIMS autosampler. The laser operated at 2940 nm wavelength and 20 Hz repetition rate. Different laser energies were tested for the optimisation of the laser parameters. For most of the experiments described in this chapter the laser power used was 65 mW. The 96 well plate with the samples was placed in the plate reader's two-dimensional (2D) stage. The well plate stage was moved automatically along X and Y axes. Each well of the sample plate was aligned with the laser beam and an aerosol transfer capillary. The 2D moving stage and the laser parameters were controlled through a LabVIEW-based software, and the stage was moved underneath the laser beam to the chosen well. PEEK tubing was used to transfer the generated aerosol to the mass spectrometer and the mass spectra from the individual wells were acquired. The LD-REIMS autosampler and the LABView based software were developed by Daniel Simon, PhD candidate at Imperial College London.

3.2.5. REIMS interface

The Waters Xevo G2-XS QToF instruments require a REIMS atmospheric interface equipped with a heated collision surface and the introduction of a solvent matrix. The solvent matrix, usually HPLC grade propan-2-ol, introduced orthogonally to the aerosol transfer line configuration. The heated collision surface was set at a temperature of approximately 800°C placed between the inlet capillary and the ion guide. Here, a prototype REIMS atmospheric interface developed by MediMass Ltd was used.

3.2.6. Mass spectrometry

A Xevo G2-XS QToF (Waters Corp.) mass spectrometer was used for acquisition of mass spectra. Negative ion mode was used, and the data acquisition was performed in Sensitivity scan mode. The detailed instrument settings are summarised on Table 1. The acquisition of the mass spectra was performed using MassLynx V4.1 (Waters Corp.).

WATERS XEVO G2-XS QTOF – SETTINGS

SCAN TIME	1000 ms
TOF ANALYSER MODE	Sensitivity
ION MODE	Negative
MASS RANGE	50 – 1500 Da
RESOLUTION	22,000

Table 1. Mass spectrometer settings used for the bipolar forceps REIMS, cell probe REIMS and LD-REIMS for the analysis of the 5 colorectal and 5 breast cancer cell lines, and the PIK3CA mutant and wild type cell lines.

3.2.7. Data processing and statistical analysis

The mass spectrometric data acquisition was performed using MassLynx[®] V4.1 (Waters Corp.). An in-house pipeline developed (by Alvaro Perdonés-Montero, Yuchen Xiang, and James McKenzie, research associates at Takats' group at Imperial College London), (python and R-based) was used for the pre-processing of the mass spectrometric data.

Concisely, the main pre-processing steps included in the pipeline were denoising, peak smoothing, peak detection, peak alignment, and recalibration.

Denoising was first carried out using FFT (Fast Fourier Transform), removing high frequency noises, after which spectral smoothing by means of Savitzky–Golay filtering²⁰⁰ was also performed, for each scan. The enhanced spectra underwent peak detection, alignment, and mass recalibration using functions available within the R package MALDIquant^{201,202}. For the recalibration, known internal m/z values were

used. Specifically, for the experiments in this PhD thesis, m/z 480.3090 or m/z 572.4809, LPE(18:0) (as $[M-H]^-$ adduct) and Cer(d34:1) (as $[M+Cl]^-$ adduct) respectively, were used as internal lock mass values. These peaks were found to be present in all the mass spectra acquired and offer an average representation of the spectral range covered (m/z 50-1500 Da or 50-1200 Da for the majority of the experiments in this PhD thesis). The resulting lists of detected peaks were lastly aligned between different classes and runs to be saved (in .csv format) and used for post-processing.

For statistical analysis, the .csv output from the preprocessing pipeline was used as input. Total ion current (TIC) Normalisation of the data was first performed and single ion intensity box plots were generated in MetaboAnalyst 5.0^{203*}. The same normalized data was then analysed with an in-house, python-based pipeline (developed by Yuchen Xiang, research associate at Takats' group at Imperial College London) for

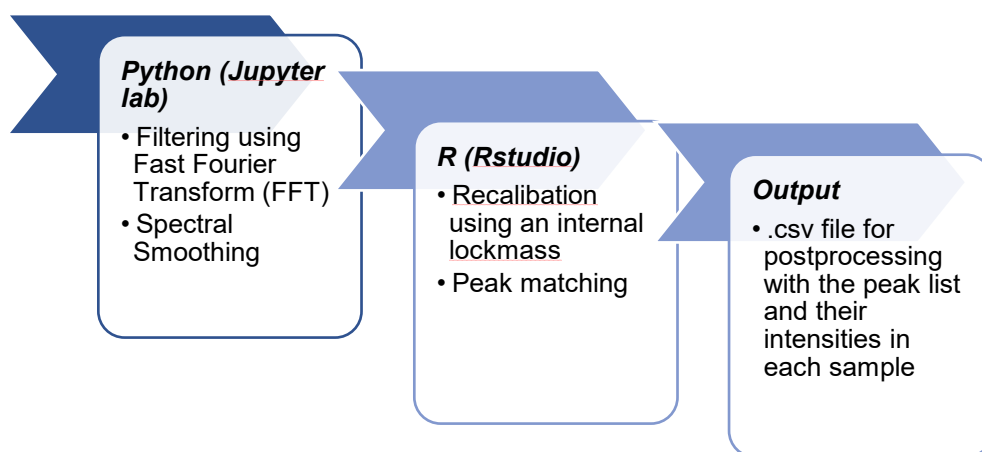


Figure 12. MS data preprocessing pipeline.

unsupervised & supervised multivariate analysis – here, principal component analysis (PCA) –, logistic regression (LR) analysis and recursive feature elimination and cross-

* MetaboAnalyst is an open access, online tool for streamlined metabolomics data analysis (<https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml>)

validation selection (RFECV) functions were utilised. A LR classification model was first trained on the input data and RFECV was then performed for model refinement, via the selection of the subset of most significant features in terms of their discriminative abilities between the different cell lines. To verify the performance of the models, leave-one-out cross validation (LOOCV) was used. Confusion matrices were subsequently generated, visualising:

- True positives, TP: cell lines predicted as their true label
- True negatives, TN: cell lines correctly not classified as a different cell line
- False positives, FP: cell lines incorrectly classified as a different cell line
- False negatives, FN: cell lines incorrectly misclassified as a different cell line

A schematic of the TP, TN, FP, and FN meaning in a confusion matrix is shown in Figure 13.

True label	TN	FP		TN	
	FN	TP	False negatives		
		False positives	20		
	TN			TN	
				1	19
Predicted label					

Figure 13. Example of confusion matrix, describing true positive (TP), true negative (TN), false positive (FP), and False Negative (FN) values.

The performance of each model can, then, be assessed based on its:

- **Accuracy:** $accuracy = \frac{TP+TN}{TP+TN+FP+FN}$
- **Precision:** $precision = \frac{TP}{TP+FP}$
- **Sensitivity:** $sensitivity = \frac{TP}{TP+FN}$
- **Specificity:** $specificity = \frac{TN}{TN+FP}$
- **F1 Score:** $F1\ Score = \frac{2 \times (sensitivity \times precision)}{sensitivity + precision}$

To cater for the potential imbalance between class sizes, the F1 score was chosen as the metric for the overall assessment of the classification models, as it combines both precision and sensitivity.

To test the repeatability between the measurements of each cell line analysed with different modalities, pairwise (Pearson) correlation between spectra of the same cell line and the same modality was computed, using a MATLAB based script written by Yuchen Xiang. The output of the statistical test was the mean Pearson's correlation coefficient (r) and its standard deviation (SD) for a given class (cell line/modality). The repeatability was assessed based the mean correlation coefficients, the higher the correlation coefficient, the more repeatable the data between the biological and technical replicates of the same cell line, analysed by the same modality (e.g., bipolar forceps REIMS) and hence the lower the standard deviation. To visualise the results, scatter plots of the SD (X axis) and the mean correlation coefficient (Y axis) were generated for easy comparison.

3.2.8. Lipid and metabolite identification

Lipid and metabolite annotation was performed using in-house and online databases (LIPIDMAPS, METLIN and HMDB) based on the accurate mass, elemental composition, isotopic ratio analysis and chemical/biological relevance. Lipid identification was performed by LC-MS/MS, based on the retention time and the manual MS/MS spectra interpretation. The Δppm error between the experimental accurate mass – the accurate mass as calculated after the recalibration step on the preprocessing pipeline – and the theoretical mass of the proposed metabolite/lipid was

calculated as described in the section 1.4.3., with a threshold of ± 10 ppm to accept the metabolite/lipid annotation.

3.2.9. LC-MS Lipidomics assay

For the lipid extraction of the cell pellets, 167 μ L of UPLC grade water (Optima™ LC/MS Grade, Fisher Chemical™, Fisher Scientific) was added to the pellet in the microcentrifuge tube (Eppendorf UK Limited) and vortexed for approximately 1 minute. To the cell lysate, 833 μ L of UPLC grade propan-2-ol (Optima™ LC/MS Grade, Fisher Chemical™, Fisher Scientific) was added, making a final volume of 1 mL and a ratio of 5:1 (v/v) IPA/Water. The mixture was vortexed again for 1 minute and then placed in a -20°C freezer for 2 hr for precipitation of proteins. Subsequently, the samples underwent centrifugation at 10,000 rpm for 10 minutes at 4°C, and the supernatant was transferred to HPLC vials for analysis. Before the analysis the internal standard lipid mixture was added to the lipid extracts. The lipid mixture used was the SPLASH® LIPIDOMIX® Mass Spec Standard (Avanti Polar Lipids). The composition and final concentrations of the lipid standards used are summarised in Table 2.

LIPID STANDARD	CONCENTRATION (μ G/ML)
PC(15:0/18:1(D7))	6.02
PE(15:0/18:1(D7))	0.21
PS(15:0/18:1(D7))	0.16
PG(15:0/18:1(D7))	1.07
PI(15:0/18:1(D7))	0.34
PA(15:0/18:1(D7))	0.28
LPC(18:1(D7))	0.95
LPE(18:1(D7))	0.20
CHOL ESTER(18:1(D7))	13.16
MAG(18:1(D7))	0.07
DAG(15:0/18:1(D7))	0.35
TAG(15:0/18:1(D7)/15:0)	2.11
SM(D18:1/18:1(D9))	1.18
CHOLESTEROL (D7)	3.94

Table 2. List of the lipids used as internal standards for the LC-MS lipidomic assay and their final concentrations.

LC-MS analysis was performed using an ACQUITY UPLC system (Waters Corp.) coupled to a Xevo G2-XS QToF mass analyser (Waters Corp.) using a LC-MS lipidomics assay developed and validated by the National Phenome Centre (NPC, Imperial College London)²⁰⁴. The column used for the chromatographic separation was a BEH C8 column, 1.7 μm , 2.1 x 100 mm (Waters Corp.) which was kept at 55°C during the analysis. Mobile phase A consisted of H₂O/IPA/ACN in a ratio of 2:1:1 (v/v/v), with a buffer system of 5 mM ammonium acetate, 0.05% acetic acid and 20 μM phosphoric acid (Sigma-Aldrich). Mobile B consisted of IPA:ACN in ratio of 1:1 (v/v), 5 mM ammonium acetate (Sigma-Aldrich) and 0.5% acetic acid (Sigma-Aldrich). The gradient profile used is described in Table 3 below:

MOBILE PHASE GRADIENT

TIME (MIN)	Flow (mL/min)	% Mobile phase A	% Mobile phase B
0.00	0.6	99.0	1.0
0.10	0.6	99.0	1.0
2.00	0.6	70.0	30.0
11.50	0.6	10.0	90.0
12.00	1.0	0.1	99.9
12.50	1.0	0.1	99.9
12.55	0.9	35.0	65.0
12.65	0.8	70.0	30.0
12.75	0.7	99.0	1.0
12.95	0.6	99.0	1.0
13.25	0.6	99.0	1.0

Table 3. LC-MS lipidomics, gradient profile

The MS spectra were acquired with a 50-2000 Da mass range (the mass range used here is different from the mass range used in REIMS as it is what the validated LC-MS method indicated), and a scan rate of 0.1 scan/sec in continuous mode, in negative ion mode. The mass spectrometer settings are summarised in Table 4.

MS PARAMETERS

SCAN TIME	0.1 sec
TOF ANALYSER MODE	Sensitivity
ION MODE	Negative
MASS RANGE	50 – 2000 Da
CONE GAS FLOW (L/HR)	150
DESOLVATION FLOW (L/HR)	1000
LOCKSPRAY FLOW (ML/MIN)	15
LOCKSPRAY CAPILLARY VOLTAGE (KV)	Negative ion mode: 2.0

Table 4. MS parameters used for the LC-MS lipidomic analysis

For the LC-MS/MS and DDA analysis the chromatographic and source settings remained the same. The quadrupole mass analyser was tuned before the analysis to have 1 Da resolution. For the MS/MS experiments the collision energy was tested and optimised for the specific precursor ions so that the parent ion peak/base fragment peak ratio was approximately 1:5. For the DDA acquisition, a collision energy ramp profile was used between 22-60 V for the 400-1500 Da mass range. The scan time used for the MS/MS and DDA analysis was 500 ms.

The LC-MS, LC-MS/MS and LC-DDA data were acquired and analysed with MassLynx V4.1 (Waters Corp.).

3.3. Results and Discussion

3.3.1. Comparison of the different REIMS modalities for the analysis of cell pellets

Multivariate analysis – namely PCA – was performed for each modality to assess data consistency. PCA scores plots of bipolar forceps and cell probe REIMS, and LD-REIMS analysis of 5 breast and 5 colorectal cancer cell pellets were produced. For the assessment of the classification capabilities of each modality, confusion matrices were generated based on the corresponding LR models, and the assessment was performed based on the average F1 score of each classification model.

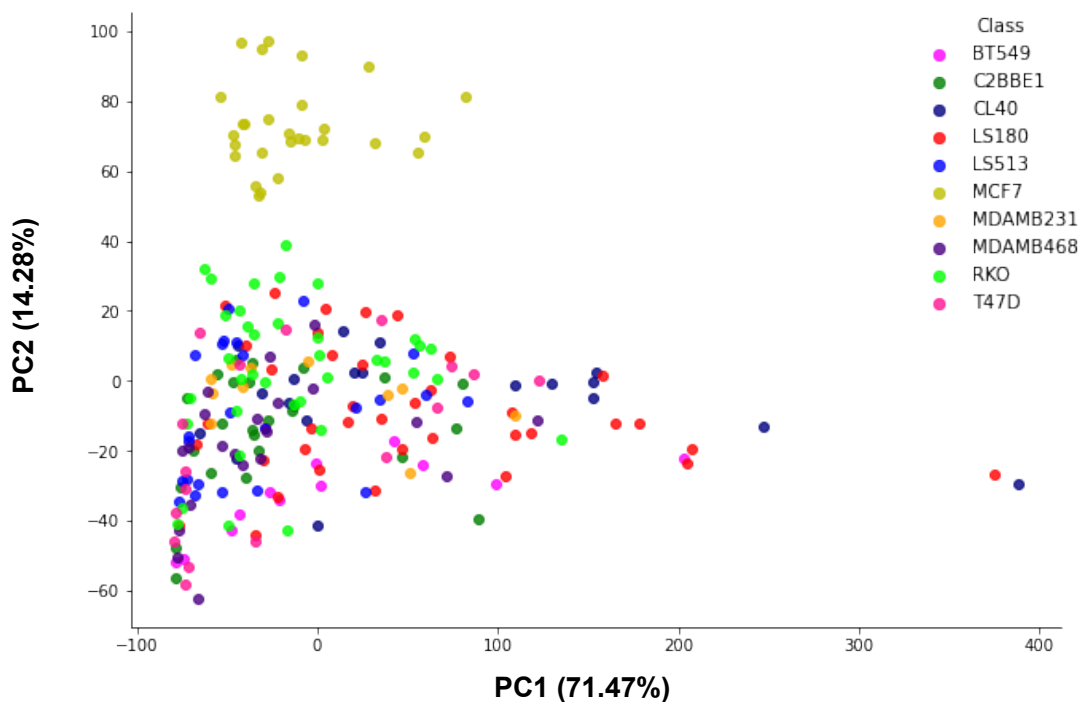


Figure 14. PCA scores plot of the 5 breast and 5 colorectal cancer cell lines analysed with bipolar forceps REIMS.

In the PCA scores plot of PC1/PC2 of the bipolar forceps data (Figure 14), MCF7 cell line seems to be forming a cluster separately from the other cell lines in the second largest variation in the data (PC2) according to PCA, indicating a radically different mass spectrometric profile. Upon closer inspection, the separation of the MCF7 cell line in the PCA seems to be due to increase in monounsaturated and saturated fatty acids, and a decrease in the intensity of complex lipids containing these fatty acyl chains, in the MCF7 cells, as demonstrated in Figure 15. This could be due to fragmentation of the complex lipids, during the evaporation of the cellular material and the ionisation process. Excess fragmentation of glycerophospholipids and the subsequent generation of the fatty acyl fragment ions could be due to various reasons, including the uneven distribution of the laser energy or the presence of more water content in the cell pellet which would again create changes to the laser energy distribution.

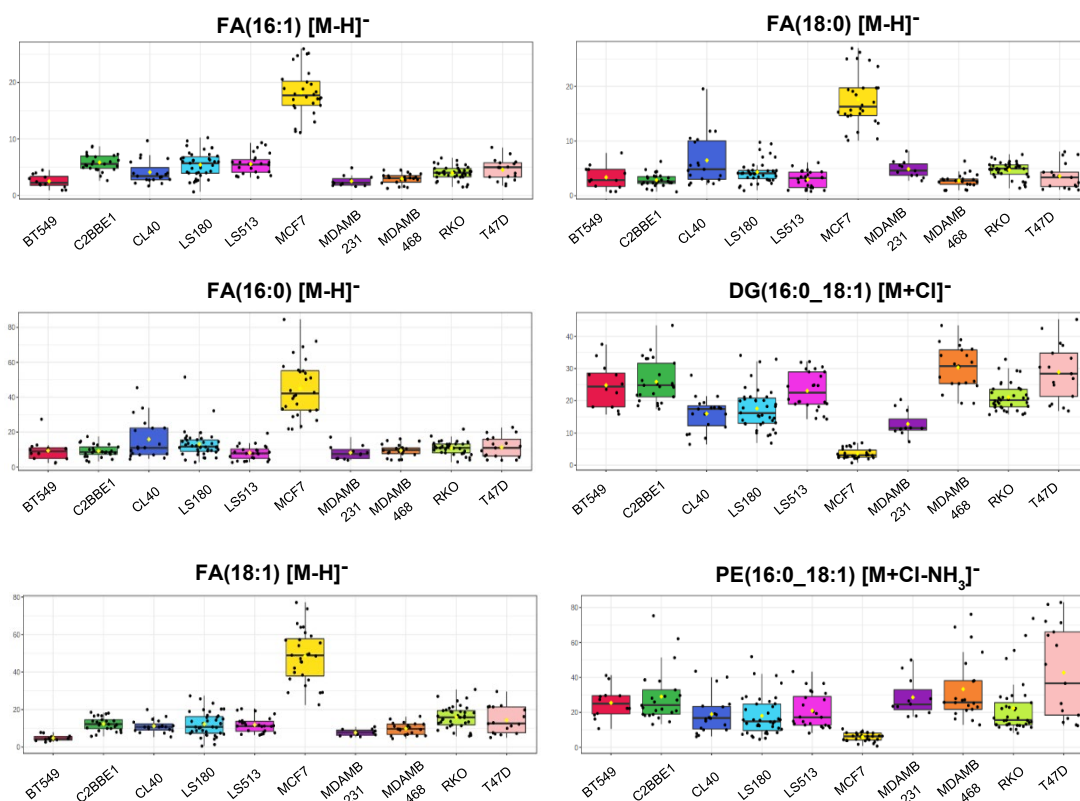
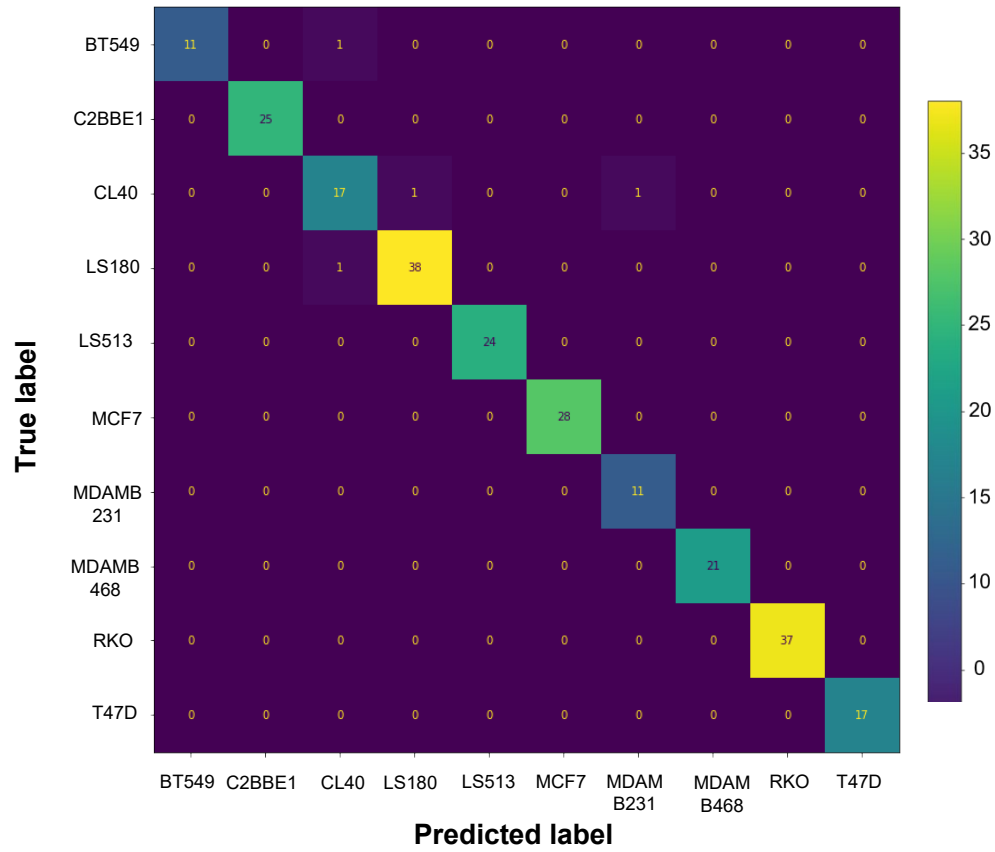


Figure 15. MCF7 cells seem to have increased monounsaturated and saturated fatty acids and decreased complex lipids containing these fatty acyl chains.

The classification performance, as shown by the confusion matrix and the F1 score (Figure 16) is near perfect for the bipolar forceps, with CL40 presenting the highest false prediction rate, as 2 out of the 19 replicates were misclassified. Most of the RFECV features used for the classification model training were lipids, including some fatty acids, but mainly phospholipids. The annotated m/z values are presented on Table 5. The majority of the detected lipids are chlorinated adducts $[M+Cl]^-$, thermal degradation products of chlorinated adducts with a loss of ammonia $[M+Cl-NH_3]^-$ and deprotonated ions with the loss of ammonia $[M-H-NH_3]^-$ for PC and PE lipids.



	Accuracy	Sensitivity	Specificity	Precision	F1
Average	0.988	0.979	0.998	0.979	0.978

Figure 16. Confusion matrix based on the LR classification model built using the RFECV features of the bipolar forceps REIMS.

Bipolar forceps REIMS RFECV features					
m/z	Annotation	Detected ion	Elemental formula	Theoretical m/z	Error (ppm)
253.2169	FA(16:1)	[M-H]-	C16H30O2	253.2168	0.4
283.2631	FA(18:0)	[M-H]-	C18H36O2	283.2637	-2.1
601.4644	DG(32:1)	[M+Cl]-	C35H66O5	601.4599	7.5
627.4807	DG(34:2)	[M+Cl]-	C37H68O5	627.4755	8.3
629.4944	DG(34:1)	[M+Cl]-	C37H70O5	629.4912	5.1
657.5224	DG(36:1)	[M+Cl]-	C39H74O5	657.5225	-0.2
678.4712	GlcCer(d30:1)	[M+Cl]-	C36H69NO8	678.4712	0.0
682.5944	Cer(d42:2)	[M+Cl]-	C42H81NO3	682.5905	5.7
684.6061	Cer(d42:1)	[M+Cl]-	C42H83NO3	684.6061	0.0
695.4689	PE(34:3)	[M-H-NH3]-	C39H72NO8P	695.4652	5.3
707.4481	PE(32:1)	[M+Cl-NH3]-	C37H72NO8P	707.4419	8.8
709.4565	PE(32:0)	[M+Cl-NH3]-	C37H74NO8P	709.4575	-1.4
716.5233	PE(34:1)	[M-H]-	C39H76NO8P	716.523	0.4
721.4791	PE(36:4)	[M-H-NH3]-	C41H74NO8P	721.4808	-2.4
722.4957	PC(O-30:2)	[M+Cl]-	C38H74NO7P	722.4891	9.1
723.4915	PE(36:3)	[M-H-NH3]-	C41H76NO8P	723.4965	-6.9
733.4578	PE(34:2)	[M+Cl-NH3]-	C39H74NO8P	733.4575	0.4
734.5271	GlcCer(d34:1)	[M+Cl]-	C40H77NO8	734.5338	-9.1
735.4726	PE(34:1)	[M+Cl-NH3]-	C39H76NO8P	735.4732	-0.8
742.5389	PE(36:2)	[M-H]-	C41H78NO8P	742.5387	0.3
744.5538	PE(36:1)	[M-H]-	C41H80NO8P	744.5543	-0.7
761.4888	PE(36:2)	[M+Cl-NH3]-	C41H78NO8P	761.4888	0.0
773.5276	PG(36:2)	[M-H]-	C42H79O10P	773.5333	-7.4
846.6557	HexCer(d42:1)	[M+Cl]-	C48H93NO8	846.659	-3.9

Table 5. List of the RFECV features and the annotations based on the accurate mass and isotopic pattern from the data acquired with bipolar forceps REIMS.

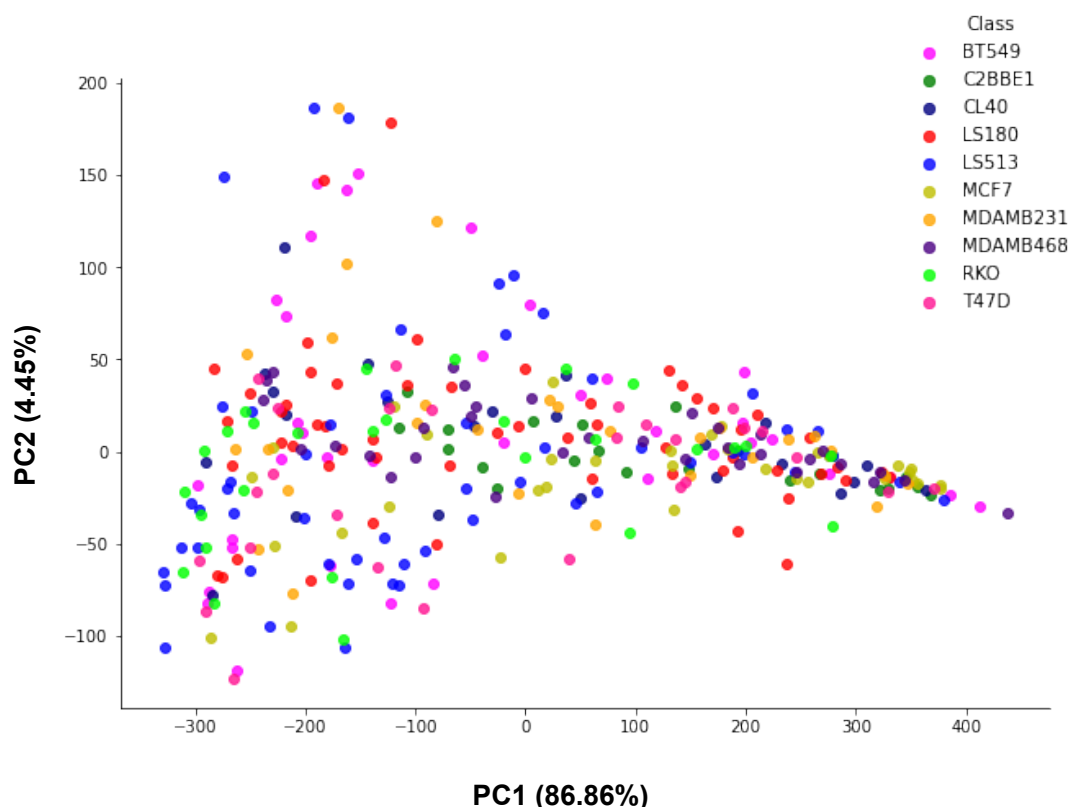
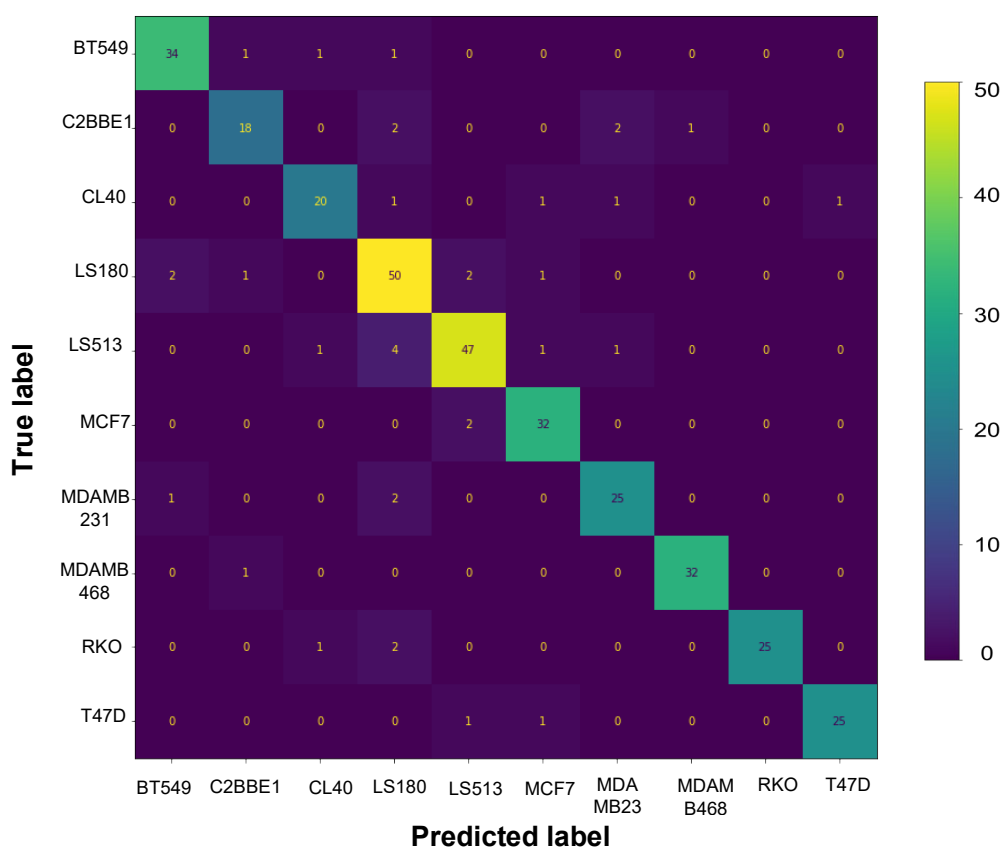


Figure 17. PCA scores plot of the 5 breast and 5 colorectal cancer cell lines analysed with cell probe REIMS.

The data acquired with the cell probe REIMS demonstrated poorer classification performance than the bipolar forceps, with the F1 score of the classification model found to be 0.897, (Figure 18). Here, as well, the CL40 cell line exhibits comparatively poorer predictive performance, with 4 out of 24 replicates misclassified as other cell lines. In addition, the largest PCA variations from the cell probe data (Figure 17) do not exhibit any visible separation between the different cell lines, meaning that these variations are unlikely to be biologically relevant.

The RFECV features obtained from the cell probe REIMS data are predominantly chlorinated adducts of diacylglycerides (DGs) and ceramides, while there is one thermal degradation product of PE(38:3), detected as $[M-H-NH_3]^+$ ion. The RFECV features and their annotations are summarised on Table 6.



	Accuracy	Sensitivity	Specificity	Precision	F1
Average	0.946	0.904	0.988	0.892	0.897

Figure 18. Confusion matrix based on the LR classification model built using the RFECV features for the cell probe REIMS

Cell probe REIMS RFECV features					
m/z	Annotation	Detected ion	Elemental formula	Theoretical m/z	Error (ppm)
417.2419	LPA(18:1)	[M-H ₂ O-H] ⁻	C ₂₁ H ₄₁ O ₇ P	417.2406	3.1
572.4815	Cer(d34:1)	[M+Cl] ⁻	C ₃₄ H ₆₇ NO ₃	572.4809	1.1
656.5761	Cer(d40:1)	[M+Cl] ⁻	C ₄₀ H ₇₉ NO ₃	656.5748	2.0
657.5210	DG(36:1)	[M+Cl] ⁻	C ₃₉ H ₇₄ O ₅	657.5225	-2.3
679.5098	DG(38:4)	[M+Cl] ⁻	C ₄₁ H ₇₂ O ₅	679.5068	4.4
682.5890	Cer(d42:2)	[M+Cl] ⁻	C ₄₂ H ₈₁ NO ₃	682.5905	-2.2
684.6023	Cer(d42:1)	[M+Cl] ⁻	C ₄₂ H ₈₃ NO ₃	684.6061	-5.6
685.5268	PE-Cer(d36:2)	[M-H] ⁻	C ₃₈ H ₇₅ N ₂ O ₆ P	685.5285	-2.5
734.5338	GlcCer(d34:1)	[M+Cl] ⁻	C ₄₀ H ₇₇ NO ₈	734.5338	0
751.5275	PE(38:3)	[M-H-NH ₃] ⁻	C ₄₃ H ₈₀ NO ₈ P	751.5278	-0.4

Table 6. List of the RFECV features of the cell probe REIMS and their lipid and metabolite annotations.

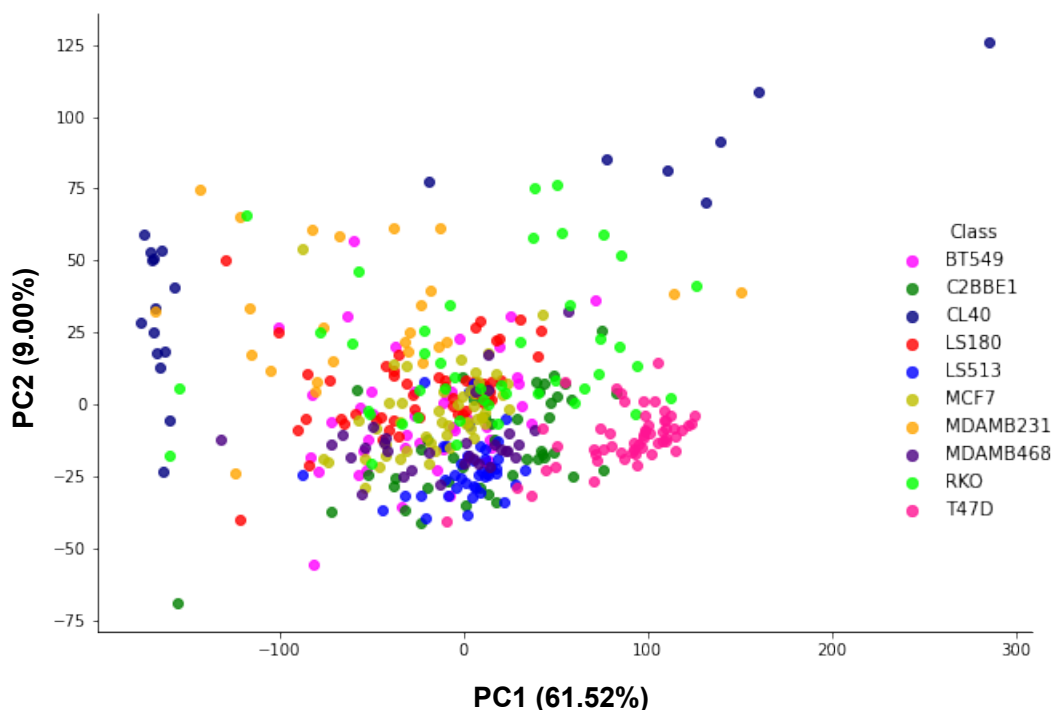
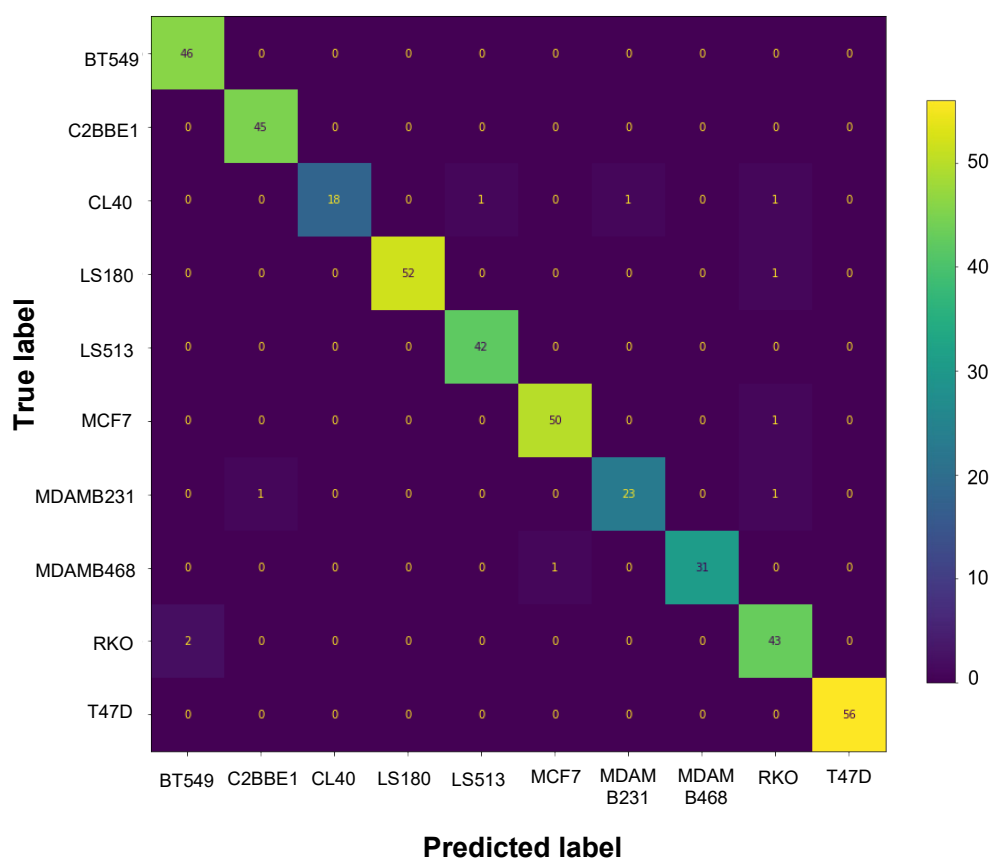


Figure 19. PCA scores plot of the 5 breast and 5 colorectal cancer cell pellets analysed with LD-REIMS.

LD-REIMS analysis of the 5 breast and 5 colorectal cancer cell lines in a pelleted form, generated a PCA scores plot with visible clustering between the scans of the same cell line, which account for a high percentage of variance in PC1 (61.52%) (Figure 19). The F1 score (Figure 20) of the classification model here is approximately the same as that of the bipolar forceps REIMS, i.e., 0.971. Similarly, the CL40 cell line analysed with LD-REIMS also exhibits poor classification performance with 3 out of 21 replicates misclassified. The consistent poor classification performance of the CL40 cell line in all three sampling techniques could be indicative of the cell line and its metabolic and lipidomic profile, potentially linked to the fact that this cell line requires different growth medium and different nutrients during the cell culturing.



	Accuracy	Sensitivity	Specificity	Precision	F1
Average	0.987	0.977	0.997	0.966	0.970

Figure 20. Confusion matrix based on the LR classification model built using the RFECV features for the LD-REIMS on cell pellets.

The LD-REIMS cell pellet RFECV features, and the annotations are summarised in Table 7. From the adducts and the lipids present here it is evident that the mass spectra obtained by bipolar forceps and cell probe REIMS are significantly different than the spectra acquired with LD-REIMS, which features predominantly deprotonated species, $[M-H]^-$, and a few chlorinated adducts, $[M+Cl]^-$, while thermal degradation products with the loss of ammonia for the PE and PC lipids are not observed. In addition, the range of different molecular classes detected with LD-REIMS is wider than that of the other two methods, with small metabolites, fatty acids, lysophospholipids, PEs, PE ether lipids, PSs and PIs also detected (observed as $[M-H]^-$ and $[M+Cl]^-$ ions). In the higher mass range of the LD-REIMS spectra gangliosides are also present.

LD-REIMS on cell pellets RFECV features					
m/z	Annotation	Detected ion	Elemental formula	Theoretical m/z	Error (ppm)
96.9593	Sulfate	[M-H]-	H ₂ SO ₄	96.9596	-3.1
196.0363	Glyceryl phosphorylethanolamine	[M-H ₂ O-H]-	C ₅ H ₁₄ NO ₆ P	196.0375	-6.1
214.0483	Glyceryl lphosphorylethanolamine	[M-H]-	C ₅ H ₁₄ NO ₆ P	214.0481	0.9
215.0316	Glucose	[M+Cl]-	C ₆ H ₁₂ O ₆	215.0322	-2.8
227.2012	FA(14:0)	[M-H]-	C ₁₄ H ₂₈ O ₂	227.2011	0.4
241.0121	Glucose 1-phosphate	[M-H ₂ O-H]-	C ₆ H ₁₃ O ₉ P	241.0113	3.3
277.215	FA(18:3)	[M-H]-	C ₁₈ H ₃₀ O ₂	277.2168	-6.5
279.2325	FA(18:2)	[M-H]-	C ₁₈ H ₃₂ O ₂	279.2324	0.4
303.2332	FA(20:4)	[M-H]-	C ₂₀ H ₃₂ O ₂	303.2324	2.6
305.2493	FA(20:3)	[M-H]-	C ₂₀ H ₃₄ O ₂	305.2481	3.9
307.2657	FA(20:2)	[M-H]-	C ₂₀ H ₃₆ O ₂	307.2637	6.5
309.2816	FA(20:1)	[M-H]-	C ₂₀ H ₃₈ O ₂	309.2794	7.1
327.2331	FA(22:6)	[M-H]-	C ₂₂ H ₃₂ O ₂	327.2324	2.1
329.2501	FA(22:5)	[M-H]-	C ₂₂ H ₃₄ O ₂	329.2481	6.1
331.2622	FA(22:4)	[M-H]-	C ₂₂ H ₃₆ O ₂	331.2637	-4.5
333.2768	FA(22:3)	[M-H]-	C ₂₂ H ₃₈ O ₂	333.2794	-7.8
391.2252	LPA(16:0)	[M-H ₂ O-H]-	C ₁₉ H ₃₉ O ₇ P	391.225	0.5
417.2396	LPA(18:1)	[M-H ₂ O-H]-	C ₂₁ H ₄₁ O ₇ P	417.2406	-2.4
419.257	LPA(18:0)	[M-H ₂ O-H]-	C ₂₁ H ₄₃ O ₇ P	419.2563	1.7
436.2808	LPE(O-16:1)	[M-H]-	C ₂₁ H ₄₄ NO ₆ P	436.2828	-4.6
452.2756	LPE(16:0)	[M-H]-	C ₂₁ H ₄₄ NO ₇ P	452.2777	-4.6
466.3284	LPE(O-18:0)	[M-H]-	C ₂₃ H ₅₀ NO ₆ P	466.3298	-3.0
478.2927	LPE(18:1)	[M-H]-	C ₂₃ H ₄₆ NO ₇ P	478.2933	-1.3
480.309	LPE(18:0)	[M-H]-	C ₂₃ H ₄₈ NO ₇ P	480.309	0.0
572.4844	Cer(d34:1)	[M+Cl]-	C ₃₄ H ₆₇ NO ₃	572.4809	6.1
581.3097	LPI(18:0)	[M-H ₂ O-H]-	C ₂₇ H ₅₃ O ₁₂ P	581.3091	1.0
687.543	PE-Cer(d36:1)	[M-H]-	C ₃₈ H ₇₇ N ₂ O ₆ P	687.5441	-1.6
689.5556	PE-Cer(d36:0)	[M-H]-	C ₃₈ H ₇₉ N ₂ O ₆ P	689.5598	-6.1
700.5226	PE(O-34:2)	[M-H]-	C ₃₉ H ₇₆ NO ₇ P	700.5281	-7.9
716.523	PE(34:1)	[M-H]-	C ₃₉ H ₇₆ NO ₈ P	716.523	0.0
722.512	PE(O-36:5)	[M-H]-	C ₄₁ H ₇₄ NO ₇ P	722.5124	-0.6
742.5385	PE(36:2)	[M-H]-	C ₄₁ H ₇₈ NO ₈ P	742.5387	-0.3
744.555	PE(36:1)	[M-H]-	C ₄₁ H ₈₀ NO ₈ P	744.5543	0.9
747.5134	PG(34:1)	[M-H]-	C ₄₀ H ₇₇ O ₁₀ P	747.5176	-5.6
750.5399	PE(O-36:5)	[M-H]-	C ₄₃ H ₇₈ NO ₇ P	750.5438	-5.2
752.5559	PE(O-36:4)	[M-H]-	C ₄₃ H ₈₀ NO ₇ P	752.5594	-4.7
764.5236	PE(38:5)	[M-H]-	C ₄₃ H ₇₆ NO ₈ P	764.523	0.8
766.5387	PE(38:4)	[M-H]-	C ₄₃ H ₇₈ NO ₈ P	766.5387	0.0
788.5405	PS(36:1)	[M-H]-	C ₄₂ H ₈₀ NO ₁₀ P	788.5442	-4.7
833.5169	PI(34:2)	[M-H]-	C ₄₃ H ₇₉ O ₁₃ P	833.518	-1.3
835.5333	PI(34:1)	[M-H]-	C ₄₃ H ₈₁ O ₁₃ P	835.5336	-0.4
857.5165	PI(36:4)	[M-H]-	C ₄₅ H ₇₉ O ₁₃ P	857.518	-1.7
859.533	PI(36:3)	[M-H]-	C ₄₅ H ₈₁ O ₁₃ P	859.5337	-0.8
863.5645	PI(36:1)	[M-H]-	C ₄₅ H ₈₅ O ₁₃ P	863.565	-0.6
883.5357	PI(38:5)	[M-H]-	C ₄₇ H ₈₁ O ₁₃ P	883.5337	2.3
885.5507	PI(38:4)	[M-H]-	C ₄₇ H ₈₃ O ₁₃ P	885.5493	1.6
887.5623	PI(38:3)	[M-H]-	C ₄₇ H ₈₅ O ₁₃ P	887.565	-3.0
1263.8422	Ganglioside GM3(d42:1)	[M-H]-	C ₆₅ H ₁₂₀ N ₂ O ₂₁	1263.8305	9.3

Table 7. List of the RFECV features of the LD-REIMS on cell pellets and their lipid and metabolite annotations.

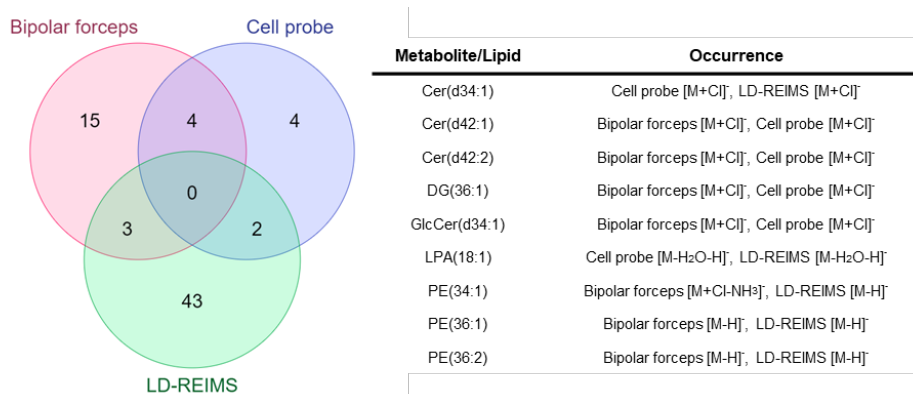


Figure 22. Venn diagram (left) showing the overlap of the significant lipids/metabolites detected in each modality. The occurrence of the overlapping features is shown in the table on the right with the respective adduct ions for each sampling method.

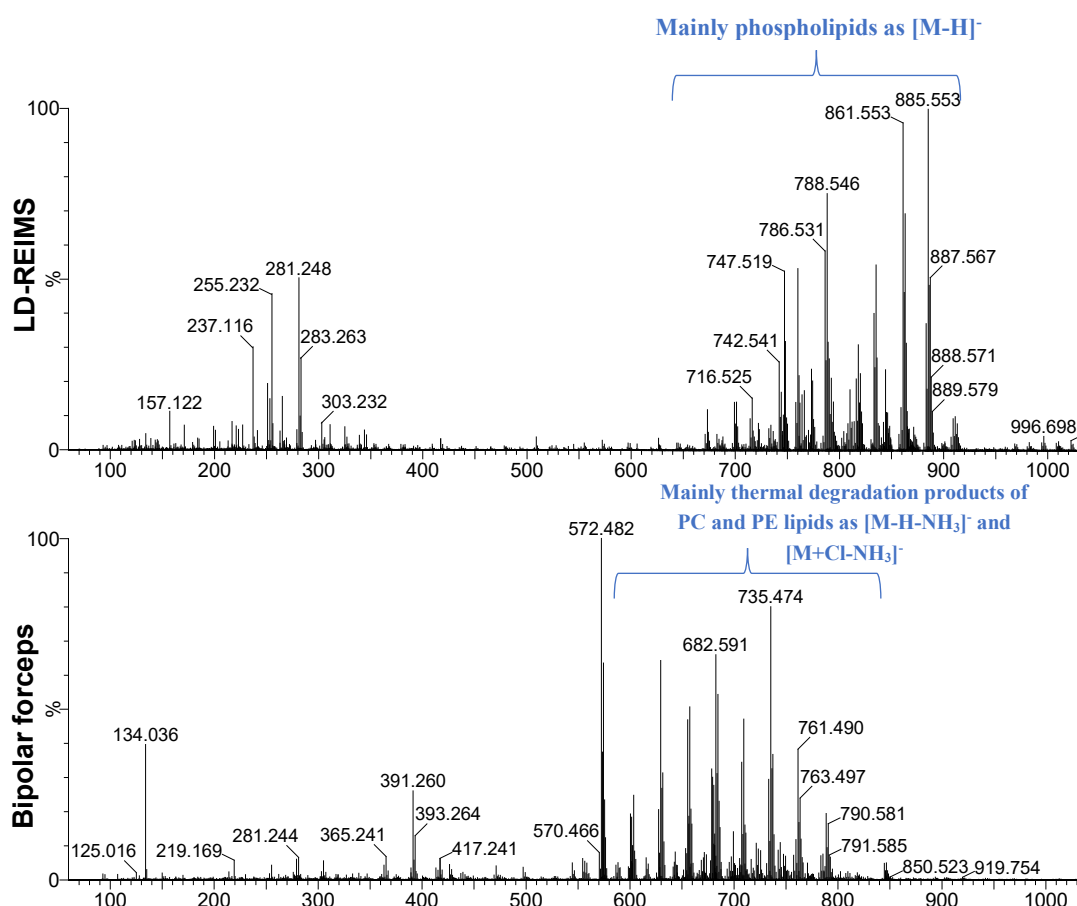


Figure 21. Representative mass spectra (1 scan) of the BT549 (breast cancer) cell line, analysed as cell pellets with LD-REIMS (top) and bipolar forceps (bottom). In the LD-REIMS spectrum we detect a large variety of phospholipid classes as [M-H]⁻ adducts, including PE, PS, and PI lipids. On the other hand, in the bipolar forceps REIMS spectrum, a big shift in the lipid masses occurs due to the thermal degradation products, i.e., deprotonated, and chlorinated PE and PC lipids after the loss of ammonia.

The mass spectra produced by the setups were significantly different, as demonstrated in Figure 22. These differences seem to reflect the sampling modality used, since the spectra from the LD-REIMS presented many differences when compared to the spectra produced by bipolar forceps and cell probe. The spectra produced by the bipolar forceps and the cell probe featured predominantly complex lipids and fatty acids, with thermal degradation products also present, mainly from PE lipids losing ammonia in the negative ion mode (as $[\text{PE-NH}_3\text{-H}]^-$ ions). On the other hand, in the mass spectra produced by LD-REIMS, we can see again the dominance of the phospholipids and fatty acids – however, the lipids were detected as deprotonated adducts, and no thermal degradation products were observed.

3.3.2. LD-REIMS on cell monolayer

In order to achieve the goals of the project (a simple, high-throughput method for native cell analysis), different sample preparation protocols were tested, and the LD-REIMS settings were optimised. Generation of cell pellets from a cell culture flask is still a fast process in comparison to the sample preparation required for LC-MS analyses – however, it is still time consuming and requires a large number of cells. In this section, a much simpler approach is suggested that includes the analysis of the cells as a cell monolayer, directly from a cell culture well plate. That allows the analysis of living or frozen cells, with the only sample preparation required being the removal of the growth medium and the washing of the cells with an appropriate buffer system, in this case 150 mM ammonium acetate diluted in sterile water.

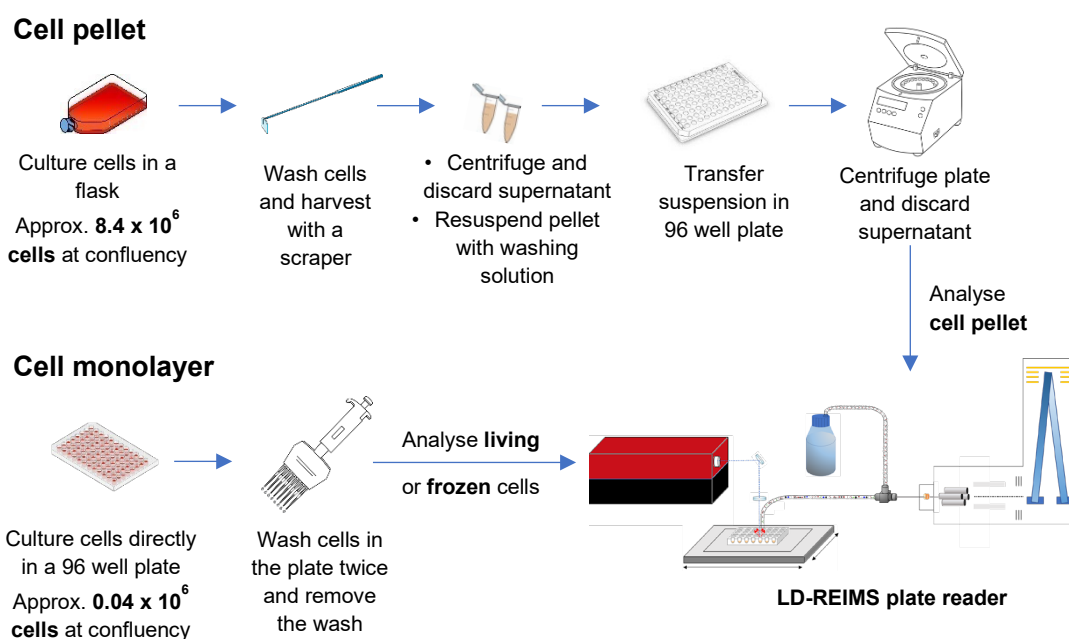


Figure 23. Workflow of cell pellet and cell monolayer preparation for the LD-REIMS analysis. Cell pellet generation requires a large number of cells, detachment of the cells from the culture flask and 2 steps of centrifugation. On the other hand, the LD-REIMS analysis of the cell monolayer can be performed directly on the cell culture well plate, by just removing the growth medium and washing the cells with the appropriate buffer solution.

3.3.2.1. Optimisation of the sample preparation process

Cell monolayer preparation for the LD-REIMS analysis

Sample preparation of the cell monolayer was one of the key variables for the development of the method, and the manipulation of the cells prior to the analysis was carefully considered and adjusted. The growth medium and supplements used for the cell culture have a high content of salts, which could negatively impact the signal of the mass spectrometer, and potentially cause ion suppression. Ammonium acetate has

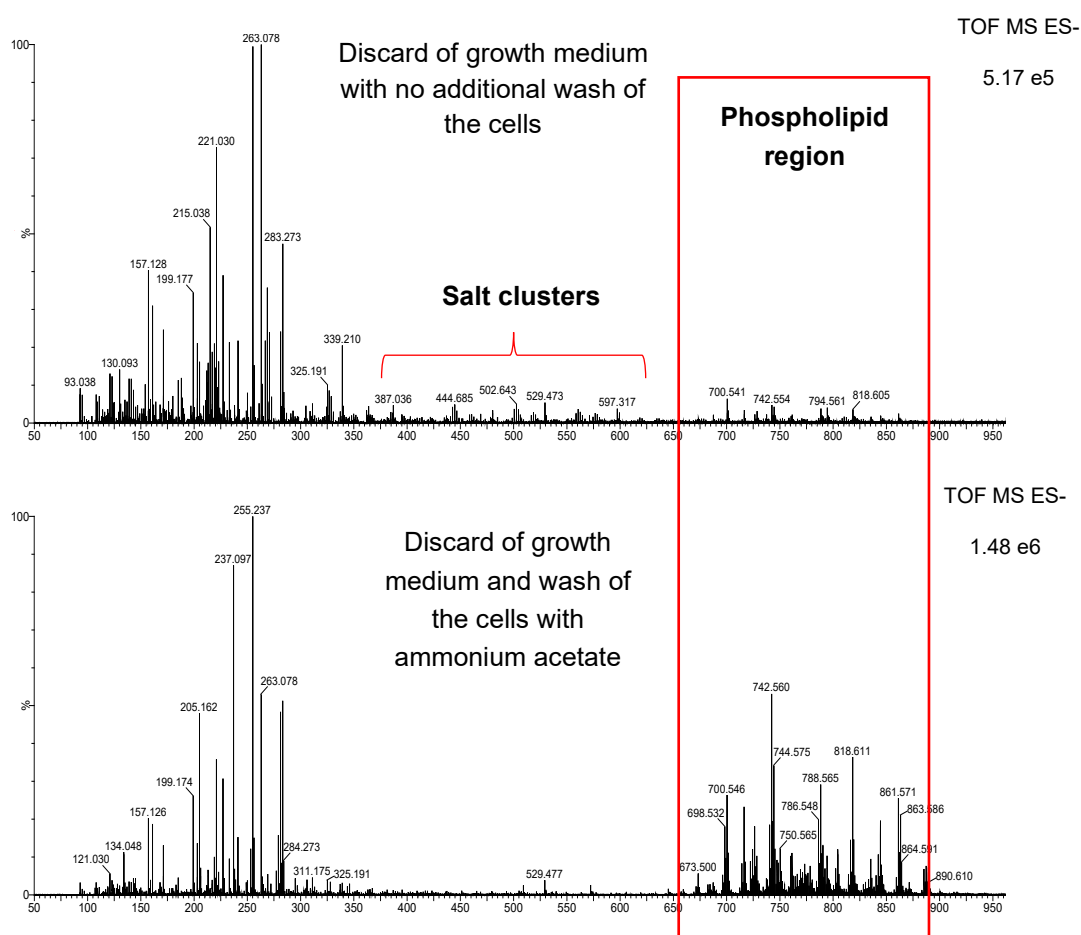


Figure 24. Representative spectra obtained from MCF7 breast cancer cell line. Top, LD-REIMS analysis was performed in the cell monolayer after the discard of the growth medium, without washing. Bottom, spectrum acquired on the cell monolayer after the growth medium was discarded and the cells washed with 150 mM ammonium acetate. In the “washed cells” spectrum, the signal, especially in the phospholipid region, is enhanced in comparison to the unwashed cells, while in the spectrum of the unwashed cells, the phospholipid signal is poor with mainly fatty acids and salt clusters observed in the area between 350 – 650 Da.

been used in the past for the washing of cells prior to the mass spectrometry analysis, and it seems to give better results than other washing systems, such as pure water or phosphate buffered saline (PBS), in terms of cell viability and removal of salt contaminants, which results in signal enhancement. The reason behind this could be the relative volatility of ammonium acetate, which helps in the evaporation of the remaining residues after washing. In addition, ammonium acetate provides similar osmotic pressure for the cells, which means it can preserve cellular contents and prevent the “burst” of the cellular membrane, as opposed to water, which creates an osmotic imbalance and cytolysis^{205,206}.

Figure 24 shows the benefits of the ammonium acetate washes. In the unwashed cell mass spectrum, the only pre-treatment the cells received was the removal of the growth medium, and no washing step was performed after. The overall intensity of the spectrum is significantly lower in comparison to the spectra produced after the washing of the cells with ammonium acetate (bottom spectrum in Figure 24). The phospholipid region seems to be enhanced in the ammonium acetate washed cells, while in the unwashed cells the phospholipid intensities are very low. Finally, the spectrum of the unwashed cells seems to feature salt clusters in the 350-650 Da range, which do not appear in the washed with ammonium acetate cells. The small metabolite and fatty acid region between 50 – 300 Da seem to be relatively comparable between the two spectra, with more species observed in unwashed cells, potentially due to the increase salt content from the growth medium, which could create more complex ion adducts, such as $[M-2H+Na]^+$ or $[M-2H+K]^+$, formed by the excess salt contents of the growth medium.

Laser power

The main settings of the LD-REIMS system tested and optimised were the laser power, the alignment of the 2D stage with the laser beam, and the aerosol transfer line inlet. Laser power was optimised based on the quality of the spectra produced, mainly in terms of ion intensities of the fatty acid and phospholipid region. Figure 25 demonstrates mass spectra of the MCF7 cell line, analysed as living cells, with

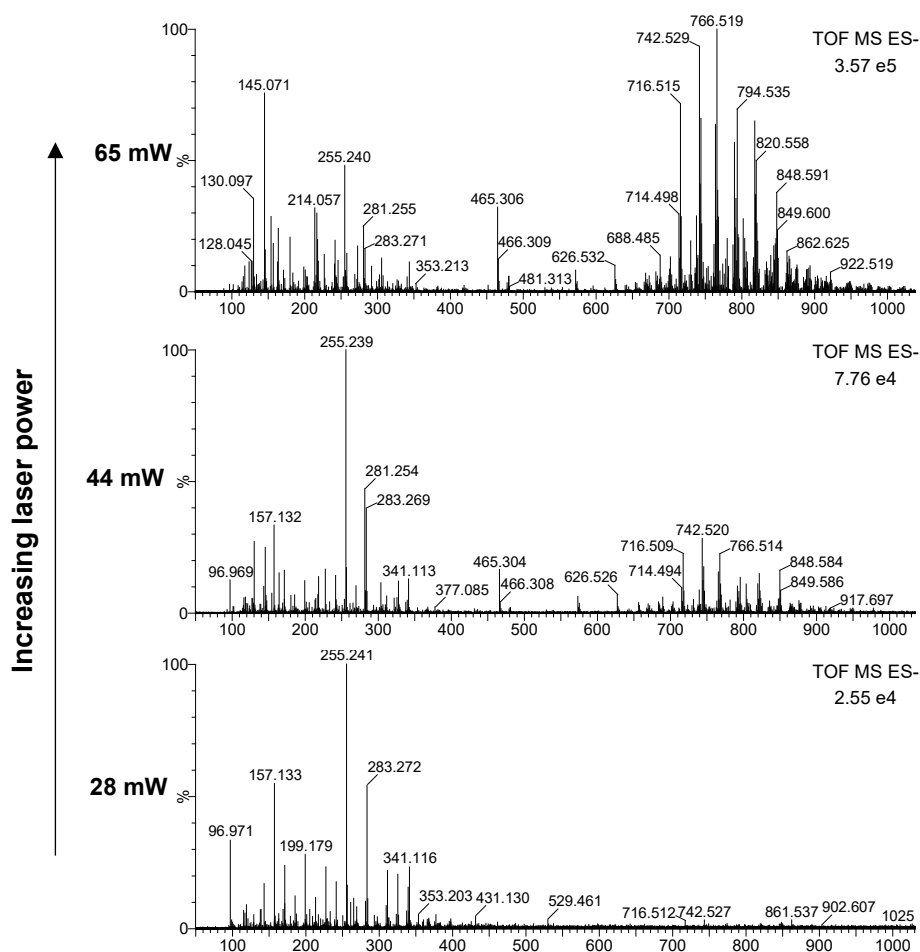


Figure 25. Mass spectra obtained to test the different laser energies. With increasing laser energy, signals in the phospholipid region between 600-900 Da increases in intensity. This is because higher laser energy causes more cellular material to be ablated. The laser energy used for the rest of the experiments in this chapter was 65 mW as it gives richer mass spectra and higher intensities, without producing thermal degradation products.

different power settings, and with the system aligned so that that the laser beam hits the centre of each well, where most of the cells are located. As expected, increasing laser power increases the ion intensity acquired, since higher laser power results in

more material ablated. In addition, the spectra shown in Figure 25 demonstrates predominantly deprotonated ions ($[M-H]^-$) – with increasing laser power there are no thermal degradation products, as in the case of bipolar forceps and cell probe REIMS.

Comparison of living and frozen cells

A benefit of being able to analyse the cell monolayer from the same well plate as they were cultured in is the option of either analysing the cells straight from the incubator after the washing step, which means that the cells are still living at the time of analysis or freezing the plate and analysing it on a different day. Flash freezing using liquid nitrogen has been used widely in biological research for cryopreservation of tissue and

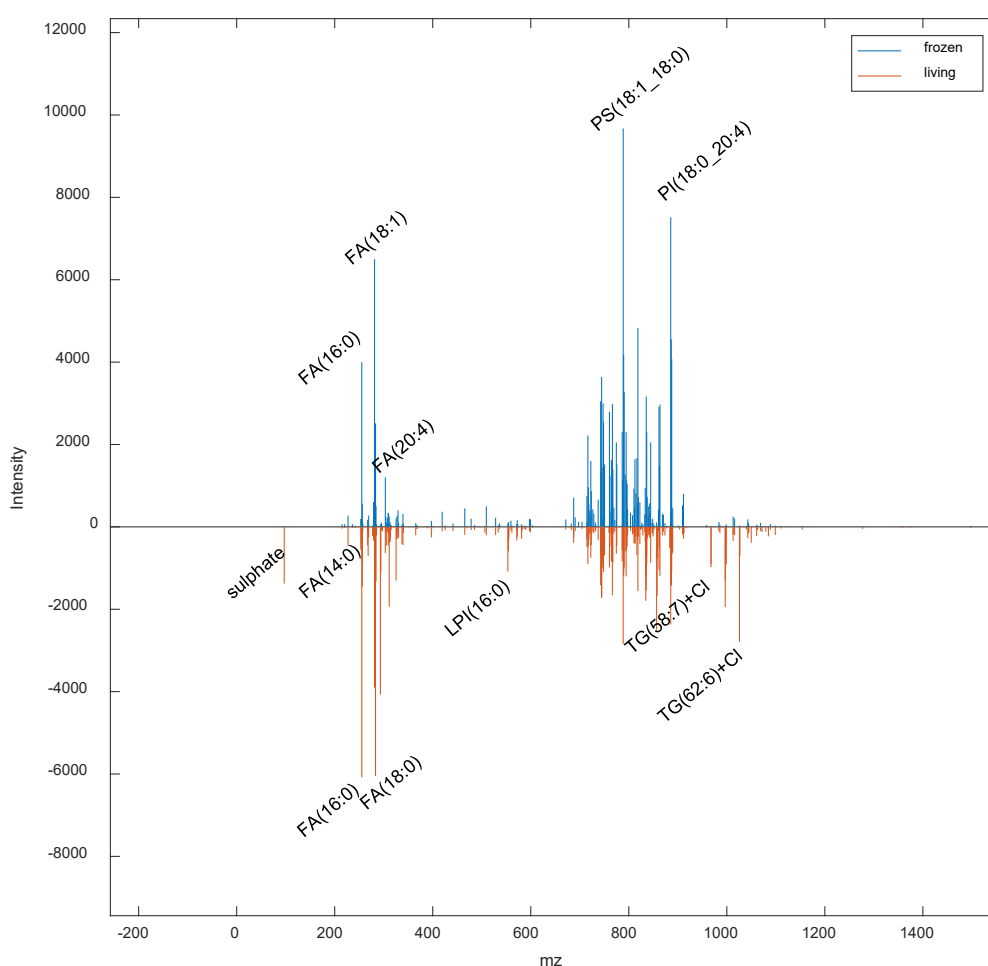


Figure 26. Average spectra of the 5 breast and 5 colorectal cancer cell lines produced by LD-REIMS. Top mass spectrum, cells analysed after they underwent flash freezing in liquid nitrogen and cryopreservation in -80°C (after washing the cells with ammonium acetate). Bottom mass spectrum, cell analysed directly after washing with ammonium acetate – living cells.

cells. Even though flash freezing generally helps to maintain the integrity of the biological samples, it has been found that freeze-thaw cycles can cause physical and chemical alterations in the cells and disintegration of the plasma membrane²⁰⁷.

In this section, the average mass spectrum of the 5 breast and 5 colorectal cell lines analysed after freezing with liquid nitrogen (prior to the freezing the cells were washed twice with 150 mM ammonium acetate) is compared to the average mass spectrum of the living cells analysed directly after the washes with ammonium acetate. The two spectra are visibly different, with the frozen cells having a richer phospholipid region than the living cells (Figure 26). On the other hand, the spectra of the living cells exhibit a richer small metabolite and fatty acid region, enhanced lysophospholipids, and triacylglycerides (TGs). Cell freezing has shown that can cause disintegration in the cellular membranes and decrease their fluidity²⁰⁷ – this could be the reason for the increased phospholipid signal in the frozen cells, as freezing can cause the disengagement of the membrane phospholipids from the bilayer, leading to easier detection by LD-REIMS. The sulphate, lysophospholipid and TG signals increased for the living cells in comparison to the frozen cells, which could be due to degradation of the frozen cells; however, it is also possible that some of these molecules were contained in the growth medium or the supplements added during the cell culturing, and that they are contaminants remaining in the wells after the cell washing in the living cells. Both methodologies give substantial biological information about the cell contents and can be used in a complementary way. Living cell analysis has the advantage of a dynamic, real-time snapshot of the cellular lipidome and metabolome, while analysis of frozen cells has the advantage of long-term cryopreservation of the cells.

Comparison between cell pellets and cell monolayer

The cell pellet and cell monolayer spectra acquired with LD-REIMS were compared to establish the differences between the two sample preparation methods. Generally, the ion intensity in the cell pellet spectra was found to be higher than in the cell monolayer, as demonstrated in Figure 27, which can be explained by the fact that there is more cellular material available in the cell pellet. However, the difference in the intensities of the two spectra seems to be due to the fatty acids, rather than the

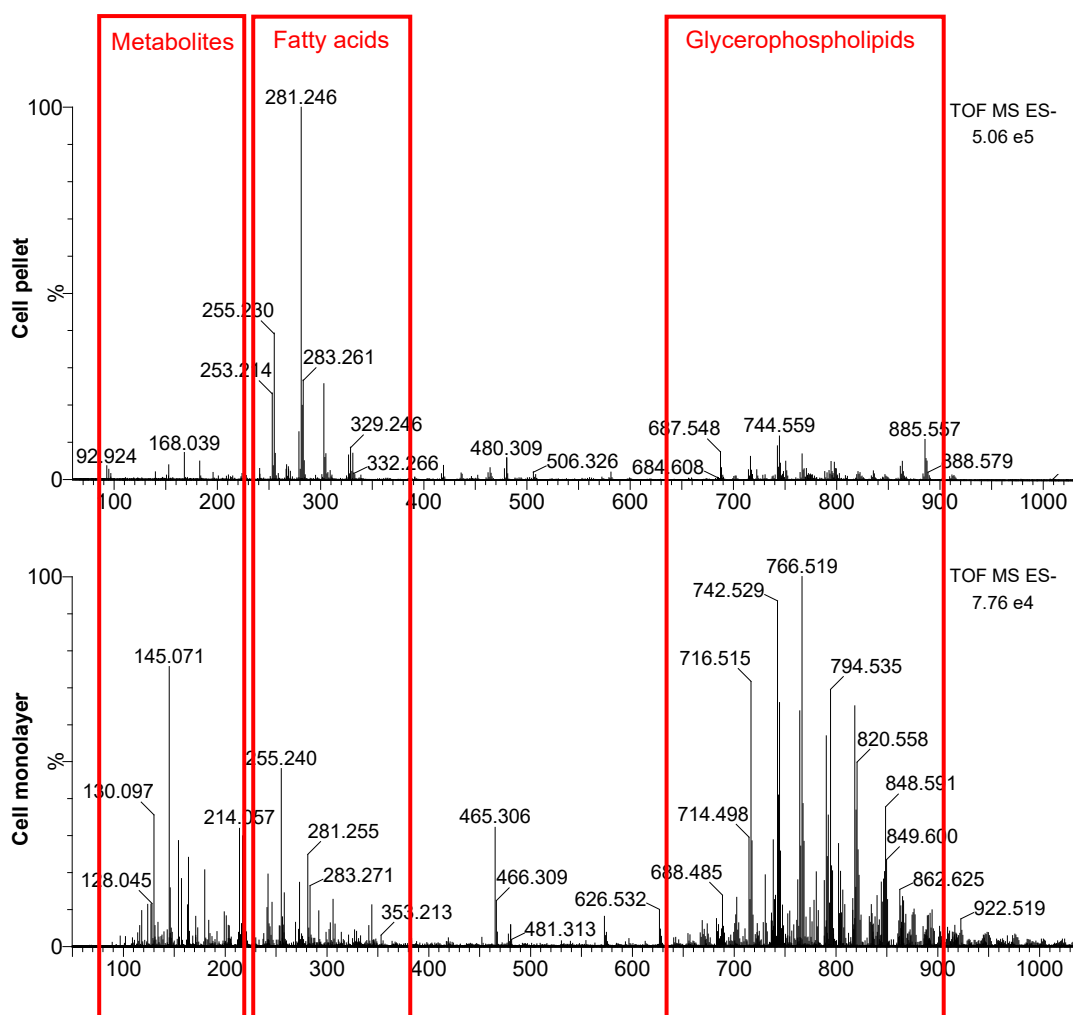


Figure 27. Representative spectra (1 scan) of the MCF7 cell line, analysed as cell pellet (top) and as cell monolayer (living cells in this case) (bottom) with LD-REIMS. The cell pellet spectrum appears to have higher ion intensity in comparison to the cell monolayer, potentially due to more cellular material available. The cell monolayer spectrum is enriched in small metabolites as opposed to the cell pellet spectrum.

phospholipids; FA(18:1) (m/z 281.246, $[M-H]^-$) is the base peak in the cell pellet spectrum, while in the cell monolayer the base peak is PC(16:1_16:0) (m/z 766.519, $[M+Cl]^-$). The cell monolayer mass spectrum is richer in small metabolites such as lactic acid, glutamate, glucose, and amino acids (Table 8), which is very advantageous for metabolic pathway analysis.

Metabolites detected in the cell monolayer					
m/z	Annotation	Detected ion	Elemental formula	Theoretical m/z	Error (ppm)
89.0245	Lactic acid	[M-H]-	C3H6O3	89.0239	6.7
96.9605	Sulphate	[M-H]-	H2SO4	96.9596	9.3
116.0704	Valine	[M-H]-	C5H11NO2	116.0712	-6.9
118.0503	Threonine	[M-H]-	C4H9NO3	118.0504	-0.8
124.0069	Taurine	[M-H]-	C2H7NO3S	124.0068	0.8
130.0861	Leucine	[M-H]-	C6H13NO2	130.0868	-5.4
145.0615	Glutamine	[M-H]-	C5H10N2O3	145.0613	1.4
146.0453	Glutamate	[M-H]-	C5H9NO4	146.0453	0
154.0615	Histidine	[M-H]-	C6H9N3O2	154.0617	-1.3
164.0709	Phenylalanine	[M-H]-	C9H11NO2	164.0712	-1.8
171.1391	Capric acid	[M-H]-	C10H20O2	171.1385	3.5
180.0668	Tyrosine	[M-H]-	C9H11NO3	180.0661	3.9
199.1701	Lauric acid	[M-H]-	C12H24O2	199.1698	1.5
214.0475	glycero-phosphoethanolamine	[M-H]-	C5H14NO6P	214.0481	-2.8
215.0324	Glucose	[M+Cl]-	C6H13O6Cl	215.0322	0.9
218.1018	Pantothenic Acid	[M-H]-	C9H17NO5	218.1028	-4.6
465.3042	cholesterol sulfate	[M-H]-	C27H46O4S	465.3039	0.6

Table 8. List of the metabolites detected in the cell monolayer, including glucose, glutamate, and amino acids, all taking part in important cellular metabolic pathways.

3.3.2.2. LD-REIMS cell monolayer – Classification performance and intermediate precision

After the optimisation of the different parameters for LD-REIMS analysis on cell monolayers, a series of experiments for the assessment and validation of the methodology were performed. As in the case of the LD-REIMS analysis of cell pellets and its comparison to cell probe and bipolar forceps REIMS modalities, the same 5 human breast cancer cell lines (BT549, MCF7, MDAMB231, MDAMB468, T47D) and 5 human colorectal cancer cell lines (C2BBE1, LS180, LS513, RKO, CL40) were used here for the assessment of the method. Here, only flash-frozen cell monolayers were used.

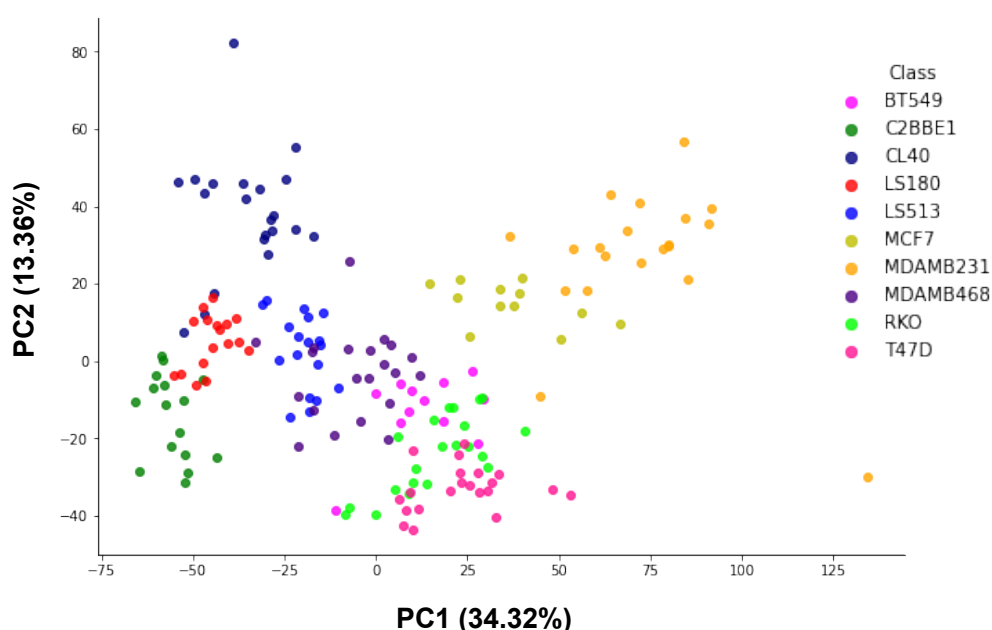


Figure 28. PCA scores plot of the 5 breast and 5 colorectal cancer cell lines analysed with LD-REIMS as cell monolayer.

The PCA scores plot (Figure 28) shows grouping between the scans of each cell lines and clearer separation than the LD-REIMS data obtained on cell pellet, and hence those from bipolar forceps, and cell probe REIMS methods (Figures 14, 17 and 19).

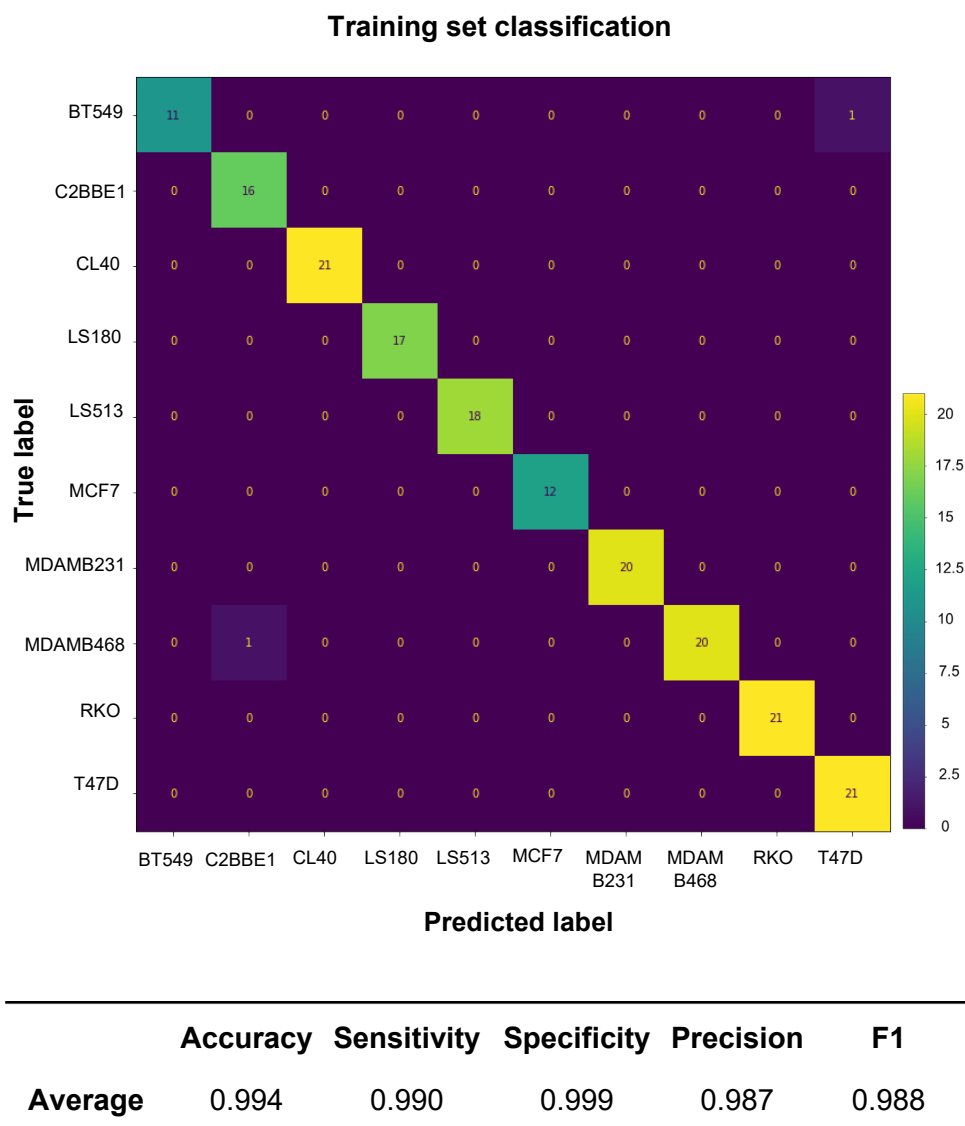


Figure 29. Confusion matrix based on the LR classification model built using the RFECV features for the LD-REIMS on cell monolayer.

A classification model based on LR was built here, in the same way as in the cell pellet analysis with REIMS modalities. The confusion matrix shown in Figure 29 demonstrates high classification accuracy of the cell lines analysed according to cross validation. Notably, the F1 score, at 0.988, is higher than both LD-REIMS and bipolar forceps REIMS on cell pellets, 0.971 (Figure 20) and 0.978 (Figure 16) respectively.

This classification model was then used for the prediction of an independent test dataset of the same cell lines acquired two months after the training dataset, using the same equipment and conditions in order to test the intermediate precision (also referred to as within-laboratory reproducibility) of the system and hence the machine

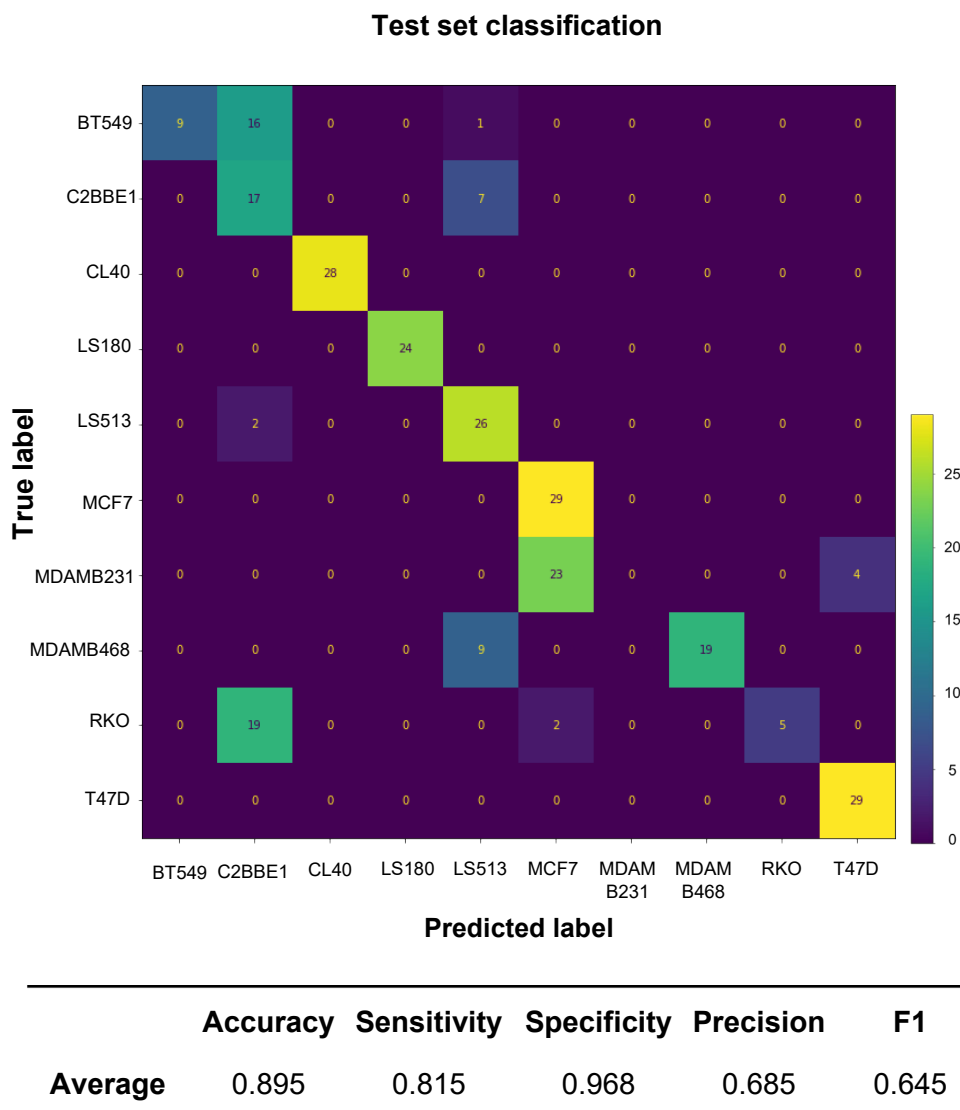


Figure 30. Confusion matrix based on the LR classification model built using the RFECV features for the LD-REIMS on cell monolayer.

learning model's robustness. As expected, the two datasets present some differences, and the classification model cannot successfully predict the true class of all the replicates. As demonstrated in the confusion matrix (Figure 30), however, some cell lines – CL40, LS180, MCF7 and T47D – have almost 100% successful classification prediction. Interestingly, CL40 cell line exhibited poor classification performance when analysed as a cell pellet with LD-REIMS, bipolar forceps, and cell probe REIMS

– but when analysing its cell monolayers with LD-REIMS reproducible results were observed, which consequently led to good classification performance even with the independent dataset. On the other hand, 23 out of the 27 replicates of MDAMB231 (breast cancer) were classified as MCF7 (breast cancer) and 4 out of 27 replicates of MDAMB231 were classified as T47D (breast cancer). The fact that the model is unable to distinguish breast cancer cell lines data from different time points indicates the similarity of the spectra between cell lines within the same cancer type. Similarly, the prediction of the RKO cell line, which is a colorectal cancer cell line, is low, with 19 out of 26 replicates misclassified as another colorectal cancer cell line, C2BBE1 (the exact values for the accuracy, sensitivity, specificity, precision, and F1 score for each cell line individually can be found in the Appendix). The inferior performance of the test set classification could be due to instability of the mass spectrometer from day-to-day (also known as a “batch” effect).

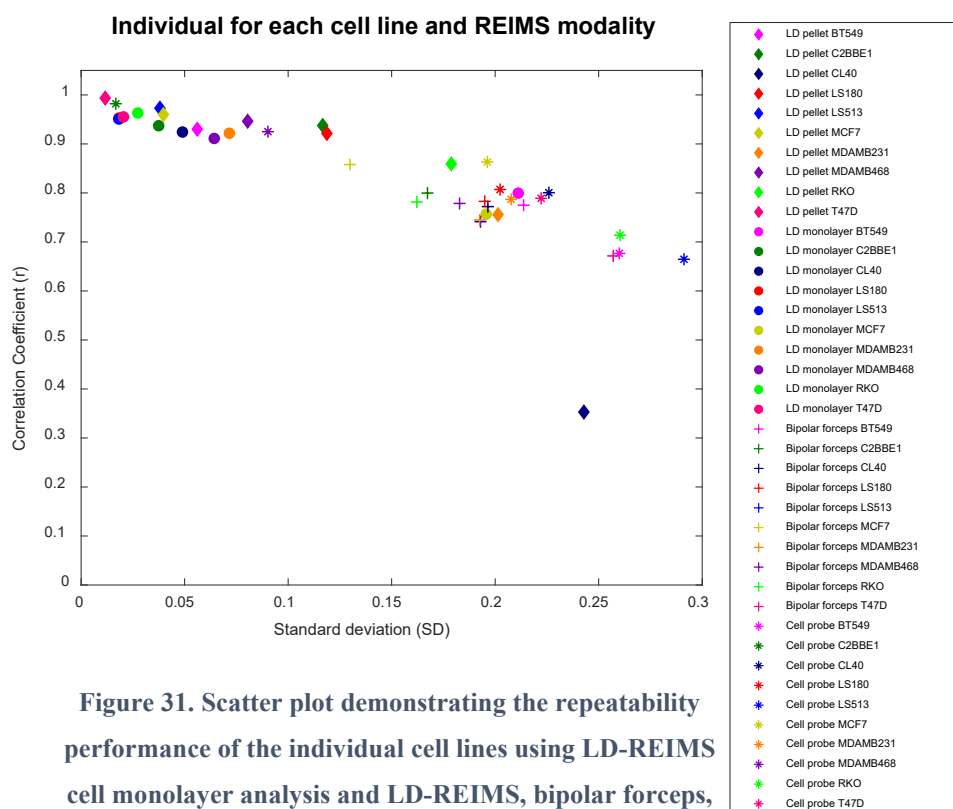
LD-REIMS on cell monolayer RFECV features					
m/z	Annotation	Detected ion	Elemental formula	Theoretical m/z	Error (ppm)
465.3050	cholesterol sulfate	[M-H]-	C27H46O4S	465.3039	2.4
722.5148	PE(O-36:5)	[M-H]-	C41H74NO7P	722.5125	3.2
742.5424	PE(36:2)	[M-H]-	C41H78NO8P	742.5387	5.0
744.5566	PE(36:1)	[M-H]-	C41H80NO8P	744.5543	3.1
747.5201	PG(34:1)	[M-H]-	C40H77O10P	747.5176	3.3
750.5419	PE(O-36:5)	[M-H]-	C43H78NO7P	750.5437	-2.4
762.5251	PS(34:0)	[M-H]-	C40H78NO10P	762.5285	-4.5
770.5722	PE(38:2)	[M-H]-	C43H82NO8P	770.5700	2.7
773.5384	PG(36:2)	[M-H]-	C42H79O10P	773.5333	6.6
774.5423	PE(O-40:7)	[M-H]-	C45H78NO7P	774.5438	-1.9
776.5567	PE(O-40:6)	[M-H]-	C45H80NO7P	776.5594	-3.5
810.5320	PS(38:4)	[M-H]-	C44H78NO10P	810.5285	4.3
812.5469	PS(38:3)	[M-H]-	C44H80NO10P	812.5442	3.3
819.5234	PG(40:7)	[M-H]-	C46H77O10P	819.5176	7.1
835.5378	PI(34:1)	[M-H]-	C43H81O13P	835.5337	4.9
844.6101	PS(40:1)	[M-H]-	C46H88NO10P	844.6068	3.9
861.5545	PI(36:2)	[M-H]-	C45H83O13P	861.5493	6.0
863.5687	PI(36:1)	[M-H]-	C45H85O13P	863.5650	4.3
883.5400	PI(38:5)	[M-H]-	C47H81O13P	883.5337	7.1
885.5549	PI(38:4)	[M-H]-	C47H83O13P	885.5493	6.3
887.5683	PI(38:3)	[M-H]-	C47H85O13P	887.5650	3.7

Table 9. List of the RFECV features of the LD-REIMS on cell monolayer and their lipid and metabolite annotations.

The RFECV features of the LR classification model are summarised in Table 9. As in the case of LD-REIMS cell pellet method, here as well the RFECV features are deprotonated ($[M-H]^-$) ions and are mainly composed of phospholipids. Further experiments are needed to establish the overlap of the feature coverage between LD-REIMS spectra and LC-MS.

3.3.3. Comparison of LD-REIMS cell monolayer analysis against the LD-REIMS, bipolar forceps, and cell probe REIMS on cell pellets

The precision assessment of the LD-REIMS cell monolayer analysis, and LD-REIMS, bipolar forceps, and cell probe REIMS analysis on the cell pellets of the 5 breast and the 5 colorectal cell lines was performed using pairwise correlation analysis between the measurements of the same cell line. The precision was assessed based on Pearson's



correlation coefficient (r) and the standard deviation (SD) of the data of each cell line. High correlation coefficient and low standard deviation means the datapoints are highly correlated with each other and the method is precise and repeatable. To

visualise this analysis, scatter plots of the mean correlation coefficient (r) – Y axis – and the standard deviation (SD) – X axis – were plotted for each cell line and each modality (Figure 31), and the average r and SD of the cell lines for each REIMS modality (Figure 32).

LD-REIMS cell monolayer and LD-REIMS cell pellet analysis seem to outperform the other two methods, as the correlation between the data points is $r > 0.9$ for most of the cell lines, and the standard deviation, $SD < 0.1$. On the other hand, bipolar forceps and cell probe REIMS correlation coefficients are between 0.7 and 0.85 for most of the cell lines, while their standard deviations are between 0.15 and 0.25, indicating significant variability between the spectra produced with these two sampling methods for each cell line.

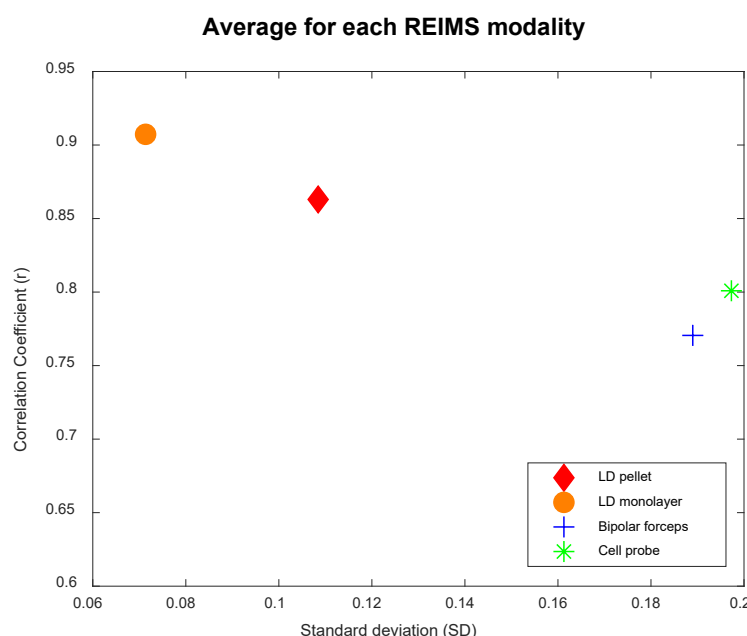


Figure 32. Scatter plot demonstrating the average repeatability performance of the cell lines using LD-REIMS cell monolayer analysis and LD-REIMS, bipolar forceps, and “cell” probe REIMS analysis on cell pellets.

When comparing the average correlation coefficient and standard deviation of all the cell lines for each REIMS modality (Figure 32), we can see that LD-REIMS on a cell monolayer outperforms all the other methods, including LD-REIMS on cell pellets, with the average correlation coefficient at 0.907 and the standard deviation at 0.071. LD-REIMS analysis on cell pellets is the second highest with correlation coefficient of 0.863 and standard deviation of 0.108. These differences between the cell pellet and the cell monolayer could be due to potential heterogeneity of the cell pellet – cellular debris tends to accumulate on the surface of the cell pellet, as it is lighter than the cells. Cell monolayers do not present this issue since the cells form a relatively uniform layer at the bottom of the well, which could improve the reproducibility of the analysis.

3.3.4. LD-REIMS on cell monolayer treated with cPLA2 inhibitors

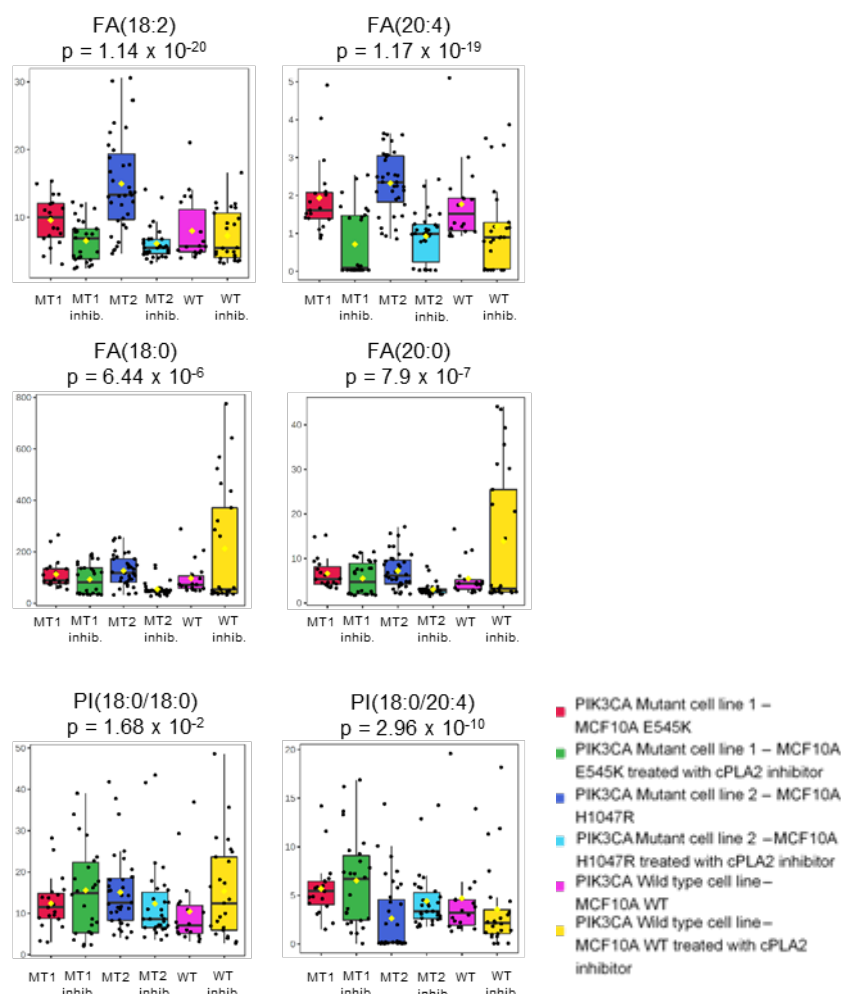


Figure 33. Box plots of the normalised ion intensity PUFAs, saturated fatty acids and PI lipids containing both saturated and polyunsaturated fatty acyl chains. In the box plots, the intensities of the 2 isogenic PIK3CA mutant (MT1 and MT2) cell lines are compared to the wild type (WT) cell lines, untreated and treated with the cPLA2 inhibitor ASB14780.

Cytosolic PLA2 (cPLA2) is an enzyme that releases PUFAs from the glycerophospholipids containing them, especially arachidonic acid (FA(20:4)). PUFAs are precursors of oxylipins, which are signalling molecules in cellular inflammatory processes²⁰⁸, holding important biological roles. Koundouros et al.¹⁵⁰, have observed that oncogenic PIK3CA mutations increases cPLA2 activity, which in turn increases PUFAs production, and that cPLA2 inhibition suppresses mutation driven PIK3CA-induced carcinogenesis. In their study, they validated these results

using a bipolar forceps REIMS system, LC-MS, and biological assays in cells and tissue.

This section aims to demonstrate that LD-REIMS analysis on cell monolayer can reproduce these results and conclude to similar observations on the cPLA2 activity in PIK3CA mutant cells, and thus validating the method against an already proven biological hypothesis. The analysis was performed in living and frozen cells, prepared as described in sections 3.2.1.2 and 3.2.2.2., and the results presented here were obtained from living cells, while in the frozen cells the same observations were made regarding the PUFAs and the enhanced arachidonic and cPLA2 metabolism.

As shown in Figure 33, inhibition of the cPLA2 enzyme using cPLA2 inhibitors such as ASB14780 can significantly alter the levels of arachidonic acid and other PUFAs, as Koundouros et al. demonstrated previously¹⁵⁰. Saturated fatty acids, such as FA(18:0) and FA(20:0) (Figure 33), seem to remain unchanged in all the cell lines regardless of whether they are untreated or treated with cPLA2 inhibitor, which is expected as cPLA2 releases mainly unsaturated fatty acids from the phospholipids containing them. Interestingly, PI lipids containing polyunsaturated fatty acyl chains (in this case, arachidonate fatty acyl chain) exhibit lower intensity in the untreated mutant cell lines, indicating their lysis by cPLA2 for the release of arachidonic acid, while with cPLA2 inhibition, their intensities increase. This could indicate that PI lipids are substrates for cPLA2 activity, and further investigation on the lipid classes that act as the enzyme's substrates could be useful in the future. In addition, PI(18:0/18:0) – containing saturated fatty acyl chains – does not exhibit any differences across the mutant and wild type cell lines, treated or untreated, confirming again the activity of cPLA2 in glycerophospholipids containing polyunsaturated fatty acyl chains.

3.4. Conclusion

In this chapter I aimed to demonstrate the main steps taken for the optimisation of the LD-REIMS cell analysis, either on cell pellet or cell monolayer, which may be living or frozen. The methods were compared with previously used approaches on cells using REIMS modalities, namely bipolar forceps, and cell probe REIMS. LD-REIMS

analysis on cell pellets exhibited better classification performance than the other two REIMS modalities used for the analysis of cell pellets, better repeatability, and broader molecular coverage. Potential reasons could be the automated nature of the technique, as opposed to the user dependent nature of the bipolar forceps and the cell probe REIMS, as well as the difference in the heating process, i.e., radiative (LD-REIMS) and Joule heating (bipolar forceps and cell probe).

Validation was also performed, comparing accuracy, precision and - in the case of the LD-REIMS cell monolayer analysis – intermediate precision (or within laboratory reproducibility). LD-REIMS on cell monolayers seems to outperform the other three modalities in terms of classification performance, molecular coverage, and closeness between the measurements. Finally, the optimised method was successfully tested in an already proven biological hypothesis, showing the increase in PUFAs in PIK3CA mutant cells and their decrease after the treatment with the cPLA2 inhibitor.

Chapter 4

Molecular characterisation of genetically modified
CHO cells producing therapeutic monoclonal
antibodies using LD-REIMS

Chapter 4: LD-REIMS on genetically modified CHO cells producing therapeutic monoclonal antibodies

4.1. Introduction

Small molecules and biopharmaceuticals are currently the two main classes of compounds used as therapeutic agents. Biopharmaceuticals – biological macromolecules used as pharmaceuticals, also known as biologics – are medicines derived from living cells or through biological processes. They are usually complex molecules, such as proteins, carbohydrates, nucleic acids etc., and some examples of biopharmaceuticals are vaccines, recombinant proteins, and monoclonal antibodies (mAbs)²⁰⁹.

In the early 1980s, monoclonal antibodies (mAbs) emerged as the new and innovative field in the pharmaceutical world. Since then, the number of therapeutic mAbs approved has increased every year. The reason behind the increased popularity of mAbs is their high specificity and affinity for both secreted and cell-surface targets. In addition to this, mAbs carry lower risks (specific binding and better side effect profile) in comparison to small molecules, and they are twice as likely to be successful in clinical trials in comparison to small therapeutic molecules^{210–213}.

The production of the therapeutic mAbs starts with recombinant DNA transfection into a host organism, which could be bacteria, yeast or mammalian cells, followed by the expression of the product²¹⁴. Mammalian systems are the main expression systems of recombinant, therapeutic mAbs. Of recent mAbs approvals, about 80% used mammalian cells as the expression system, and about 10% used *E. Coli* and yeast. The reason for that is the need for post-translational modifications, which can only occur in a mammalian expression system. The mammalian cells mainly used as host cells to produce therapeutic mAbs are the Chinese Hamster Ovary (CHO) cells, used in approximately 80% of the mAbs approved, while other mammalian cells that have been used are the Sp2/0 and NS0 cells, which are mouse myeloma cell lines^{210,211}. CHO cells are preferred over other cell lines

because they are robust and fast-growing cells, while the fact that they are suspension cells means they can easily grow in bioreactors, exploiting in full the space and the nutrients provided.

As mentioned above, to produce the recombinant mAbs, a plasmid of choice is transfected into the genome of the host CHO cell, where it gets randomly integrated in the host DNA. The random integration of the plasmid into the CHO cell genome results in the generation of multiple clones of the genetically modified (GM) CHO cell line. After the generation of multiple GM CHO cell line clones, single cell isolation systems are utilised to separate individual clones and grow them into single cell colonies. Subsequently the isolated single cells replicate and create larger colonies, various biological assays are performed to establish the health of the cells, and later the amount of the recombinant mAb produced²¹⁵.

The challenge of this process is the categorisation of the various cell clones produced – after the genetic modification – based on the yield of the recombinant mAb they have produced, i.e., their characterisation as low or high producing cell lines. The identification of the high and low producing cells in the early stages of biopharmaceutical development and manufacturing is crucial since the low producers must be disqualified early in the developmental process to avoid spending resources on cells which do not have the potential to produce adequate amount of product. Thus, financial and manufacturing resources should be focused on high-yielding GM CHO cells.

The fast screening and classification of the low and high producing cell lines, as well as a better understanding of the biological processes and the metabolic pathways activated in these cells, could give us insight into the main differences between the two groups and reduce the number of low producing clones moving towards further development. It has been proposed before that the composition of the lipid bilayer of the cell membranes could affect the protein yield and the protein secretion from the cells in the culture medium^{216,217}. In-depth understanding of the GM cells' biology and the cell membrane's role in the recombinant mAbs secretion is crucial for control of their growth, and ultimately control of production. Metabolomics and lipidomics could be useful in understanding the metabolic pathways of high and low producing cell lines, and

their combination with a high-throughput analytical platform like LD-REIMS could provide give us a biological ‘snapshot’ of these cells. Currently, LC-MS is the gold standard for the metabolomic and lipidomic analysis of CHO cells, which is however a time-consuming and laborious technique and cannot be used for high-throughput analysis^{112,216}.

Adalimumab is a therapeutic mAb that works by inactivating tumour necrosis factor-alpha (TNF α), and it is used as a treatment for rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, ulcerative colitis, psoriasis, hidradenitis suppurativa, uveitis, and juvenile idiopathic arthritis. Here, the ability of LD-REIMS to classify GM CHO cell lines as low or high producers, based on their metabolomic and lipidomic profiles is demonstrated, while the lipid species and the metabolites present in the high and low producers are used to understand the biological reasons behind their mAb yields.

4.2. Methods

4.2.1. GM CHO cell lines producing adalimumab

Five genetically modified CHO-K1 cell lines producing different quantities of the monoclonal antibody adalimumab (Humira®) were developed and provided by GSK (Biopharma group, R&D GSK, Stevenage, UK). The amount of adalimumab produced by each cell line was established at GSK’s facilities and is summarised in Table 10. The cell lines were classified as low or high-producing cell lines based on the amount of adalimumab produced. Cell lines which produced less than 25 mg/L were classified as “low producers”, in this case WT1, WT3, and WT4, while the cell lines which produced more than 25 mg/L were classified as “high producers”.

Cell line ID	Amount of adalimumab produced (mg/L)	Classification
WT1	21.2	Low producer
WT2	38.2	High producer
WT3	16.3	Low producer
WT4	<10	Low producer
WT5	30.5	High producer

Table 10. Cell lines producing < 25 mg/L classified as low producers and cell lines producing > 25 mg/L classified as high producers

4.2.2. CHO cell culturing protocol

Two different culturing protocols used for the CHO cell development. The first included the subculturing of the CHO cells in fresh growth medium and removal of the unwanted waste products, once the cell culture reached confluency, i.e., when the cell density reached approximately 2-3 million cells/mL of growth medium – measured with an automated cell counter (Bio-Rad). For simplicity, in this PhD thesis, the CHO cells cultured using this protocol will be referred to as “non-production” CHO cells.

The second protocol was based in “fed-batch” cell culturing which is used by the biopharma industry to push and stress the cells into producing more product. However, due to the high rate of flux and glycolysis, unwanted waste products such as lactate and ammonia are accumulated over time. This can slow down cell development and compromise quality of the produced mAb. The lactate accumulation has been mainly attributed to uncontrolled glucose metabolism and can result in acidified growth medium and osmolality changes²¹⁵. During the “fed-batch” production protocol, the cells are cultured in a shake flask with the appropriate growth medium containing the required nutrients. In contrast to the first cell culturing protocol, here the cells are not subcultured in fresh growth medium, they instead remain in the initial shake flask. The “harvesting” of the cells, i.e., the collection and isolation of the cells from the growth medium, takes

place when cell viability – measured with an automated cell counter (Bio-Rad) – drops below 50%. For simplicity, in this PhD thesis, the CHO cells cultured using this protocol will be referred to as “fed-batch” CHO cells. Figure 34 demonstrates the overall cell culturing process followed for the adalimumab producing cell lines.

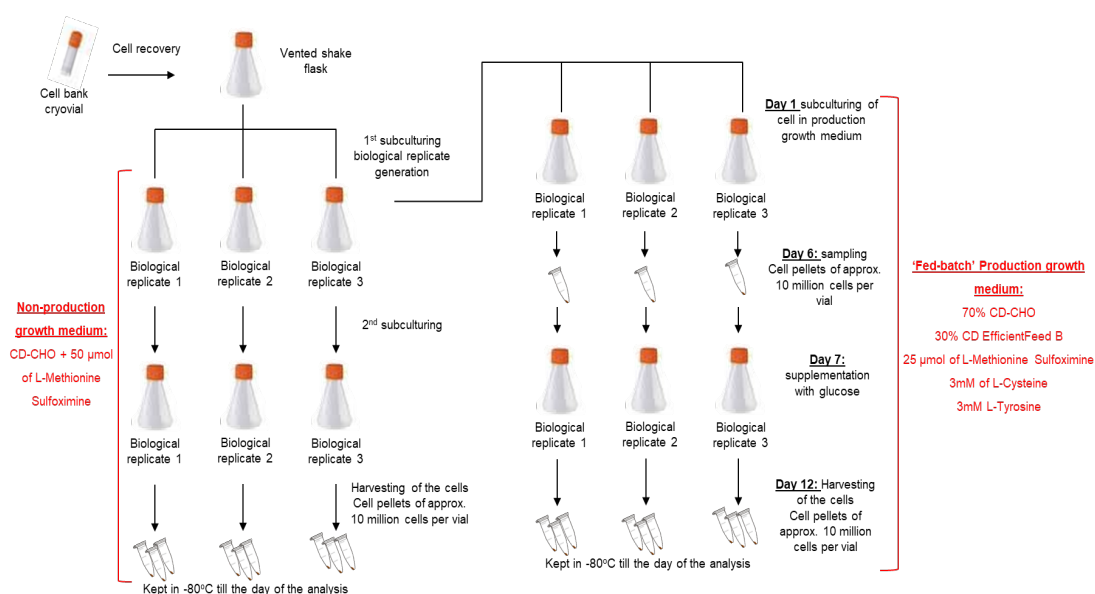


Figure 34. Schematic to describe the two culturing protocols used. Left – CHO cell culturing under “non-production” conditions and the generation of 3 biological replicates from the original cell bank cryovial. Right – CHO cell culturing using ‘fed-batch’ production protocol, where the cells are stressed to produce more product.

4.2.2.1. Cell recovery

The five cell lines, received in cell bank cryovials, were recovered and cultured at Imperial College London. Three biological replicates of each cell line were generated in line with GSK’s protocols. After thawing at 37°C the contents of each cryovial (which was approximately 1 mL), were added with a stripette dropwise into 13 mL of prewarmed complete growth medium, i.e., CD CHO medium (Gibco) supplemented with 50 µmol of L-Methionine Sulfoximine (Merck Millipore), in a 15 mL Falcon tube. Subsequently, the cell suspension underwent centrifugation at 1,000 rpm, at 4°C for 5 minutes. The supernatant was then discarded, and the cell pellets resuspended with 5 mL of complete growth medium. Each of the resulting suspensions was then

added to 15 mL of prewarmed complete growth medium in a 125 mL Polycarbonate Erlenmeyer Flask with Vent Cap (Corning) and incubated at 37°C and 5% CO₂, on a shaking platform.

4.2.2.2. Subculturing and generation of biological replicates

On Day 3, the five cell cultures reached approximately 2×10^6 cells/mL cell density and were transferred in fresh growth medium. At this point three biological replicates were generated by splitting the original cell cultures into three new cultures with seeding density of 0.3×10^6 cells/mL each. The three biological replicates of each of the 5 cell lines were incubated under the same conditions described above until Day 7, when they were transferred again into fresh medium and a cell density of 0.3×10^6 cells/mL.

4.2.2.3. ‘Fed-batch’ CHO cell culturing

The growth medium used for “fed-batch” production of the CHO cell lines contains more nutrient additives than the “non-production” cell culturing protocol. The stock solutions of the supplements added in the growth medium were prepared in cell culture grade, sterile (Gibco). L-Cysteine (Sigma-Aldrich) and L-Tyrosine (Sigma-Aldrich) stock solutions were prepared to a concentration of 300 mM, while D-Glucose stock solution was made to a final concentration of 1.6 M. All amino acid and glucose solutions were 0.2 µm filtered, sterilised before use, and stored at 4°C. After the stock solutions were prepared, the complete growth medium was prepared and consisted of 70% CD-CHO (Gibco) and 30% CD EfficientFeed B (Gibco), supplemented with 25 µM of L-Methionine Sulfoximine (Merck Millipore), and 3mM of L-Cysteine (Sigma-Aldrich) and L-Tyrosine (Sigma-Aldrich).

The cells were subcultured in the “fed-batch” growth medium from the three biological replicates described in section 4.2.2.2 at a cell density of 0.3×10^6 cells/mL. For the culturing of the production cell lines, 250 mL shake flasks with vented caps (Corning) were used and the final working volume of the cell suspension was 60 mL. The cell suspension of each cell line was then incubated at 37°C and 5% CO₂ on a shaking platform. Cell counting was performed on days 3, 6, 10, and 12 to confirm

that the percentage of living cells was not below 50%. On Day 6, the cell viability was between 95-100% and the cell density was between $5.8-10 \times 10^6$. A sample of 1 mL was taken from each flask, centrifuged at 4°C, 10,000 rpm for 5 minutes. After the centrifugation the supernatant was discarded, and the cell pellet was kept at -80°C for LD-REIMS analysis (these cells will be referred to as “fed-batch” Day 6 CHO cells later in the chapter). On Day 7, the cells were supplemented with 1% v/v of the 1.6 M stock solution of D-glucose (Sigma-Aldrich), i.e., 0.6 mL of the stock solution was added to each flask. On Day 10, cell counting was performed again; the cell viability was between 60-79%, while the cell density was between $5.3-6 \times 10^6$ cells/mL.

4.2.2.4. Cell harvesting and sample preparation for the LD-REIMS analysis

On Day 10, cell counting was performed on the cells cultured under “non-production” culturing conditions; all the cell cultures were found to have an average cell density of 2×10^6 cells/mL. The harvesting of the “fed-batch” cells was performed on Day 12, when the viability percentage was between 35-55% for each cell culture. Since CHO cells are suspension cells, LD-REIMS on cell pellets was deemed to be the appropriate method for their analysis. Therefore, the cell suspension of each flask, for both “non-production” and “fed-batch” culturing, was centrifuged at 1000 rpm, at 4°C for 5 minutes. The supernatant was discarded, and the appropriate volume of growth medium was calculated for the resulting suspension to have a cell density of 10×10^6 cells/mL. The cell suspension was then aliquoted into 1 mL volumes in microcentrifuge tubes (Eppendorf UK Limited), which underwent centrifugation at 10,000 rpm, at 4°C, for 5 minutes. The cell pellets were resuspended in the microcentrifuge tubes with 200 µL of 150 mM ammonium acetate (Sigma-Aldrich) (diluted in sterile water) and transferred in a 96 well plate (Eppendorf™ 96-well V-Bottom PCR Microplates) in a randomised way. The plate with the cell suspension underwent centrifugation at 5,000 rpm, at 4°C for 5 minutes. The supernatant was then discarded, and the plate was kept in -80°C till the day of the analysis.

4.2.2.5. LD-REIMS analysis

The LD-REIMS analysis was performed using the same parameters and conditions as described in the sections 3.2.4.3, 3.2.5 and 3.2.6 in Chapter 3. The samples analysed

included 3 biological replicates from each cell line in each condition, as summarised in Table 11.

Cell culture methods	Cell line ID	Biological replicates ID
“non-production” cell culturing	WT1 (low producer)	WT1_1, WT1_2 and WT1_3
	WT2 (high producer)	WT2_1, WT2_2 and WT2_3
	WT3 (low producer)	WT3_1, WT3_2 and WT3_3
	WT4 (low producer)	WT4_1, WT4_2 and WT4_3
	WT5 (high producer)	WT5_1, WT5_2 and WT5_3
“Fed-batch” cell culture Day 6	WT1 Day 6 (low producer)	WT1_1_D6, WT1_2_D6 and WT1_3_D6
	WT2 Day 6 (high producer)	WT2_1_D6, WT2_2_D6 and WT2_3_D6
	WT3 Day 6 (low producer)	WT3_1_D6, WT3_2_D6 and WT3_3_D6
	WT4 Day 6 (low producer)	WT4_1_D6, WT4_2_D6 and WT4_3_D6
	WT5 Day 6 (high producer)	WT5_1_D6, WT5_2_D6 and WT5_3_D6
“Fed-batch” cell culture Day 12	WT1 Day 12 (low producer)	WT1_1_D12, WT1_2_D12 and WT1_3_D12
	WT2 Day 12 (high producer)	WT2_1_D12, WT2_2_D12 and WT2_3_D12
	WT3 Day 12 (low producer)	WT3_1_D12, WT3_2_D12 and WT3_3_D12
	WT4 Day 12 (low producer)	WT4_1_D12, WT4_2_D12 and WT4_3_D12
	WT5 Day 12 (high producer)	WT5_1_D12, WT5_2_D12 and WT5_3_D12

Table 11. Cell pellets analysed by LD-REIMS, including cell line ID and biological replicates ID used.

4.2.3. LC-MS Lipidomics assay

LC-MS was used to analyse the CHO cells, to facilitate the lipid identification of the features detected with LD-REIMS. For the LC-MS lipidomics assay, the samples were prepared following the same protocol as described in the section 3.2.9 and analysed with the National Phenome’s Centre (NPC) lipidomic assay, again as described in section 3.2.9, including the addition of the internal standard lipid mix (SPLASH® LIPIDOMIX® Mass Spec Standard, Avanti Polar Lipids). The LC-MS, LC-MS/MS and LC-DDA data were acquired and analysed with MassLynx V4.1 (Waters Corp.).

4.2.4. Data analysis

The data acquired was pre-processed using the same process as described in section 3.2.7. The .csv output from the preprocessing pipeline with the list of the detected m/z values of the different samples and their subgroups, i.e., “non-production”, “fed-batch” Day 6 and “fed-batch” Day 12 cultured high and low producing cells, was then used for statistical analysis. Multivariate (PCA) analysis was performed using MetaboAnalyst²¹⁸.

For statistical analysis, the same methods described in section 3.2.7 were used and confusion matrices of the LR classification models predicting the true label of high and low producing CHO cells – with leave-one-out cross validation (LOOCV) – were used. RFECV was also performed here, as described in section 3.2.7., for model refinement, via the selection of the subset of most significant features in terms of their discriminative abilities between high producers and low producers. Here, for the training of the classification models for each condition (“non-production”, “fed-batch” Day 6, and “fed-batch” Day 12) the mass spectrometric data of the cell lines WT2 (high producer) and WT4 (low producer) was used. To test the model’s performance with an independent test dataset, the mass spectrometric data of the cell lines WT5 (high producer) and WT3 (low producer) was used.

Metabolite and lipid annotation and identification was carried out for the RFECV features as described in section 3.2.8.

4.3. Results and Discussion

4.3.1. Lipidomic differences between “non-production” and “fed-batch” CHO cells

Statistical analysis showed clear differentiation between high producing and low producing cell lines in all three culturing conditions, i.e., “non-production” and “fed-batch” Day 6 and Day 12. As the PCA plot in Figure 35 demonstrates, the main source of variance in PC 1 comes from the different culturing methods. However, high producers and low producers of each condition seem to also be differentiated from each other.

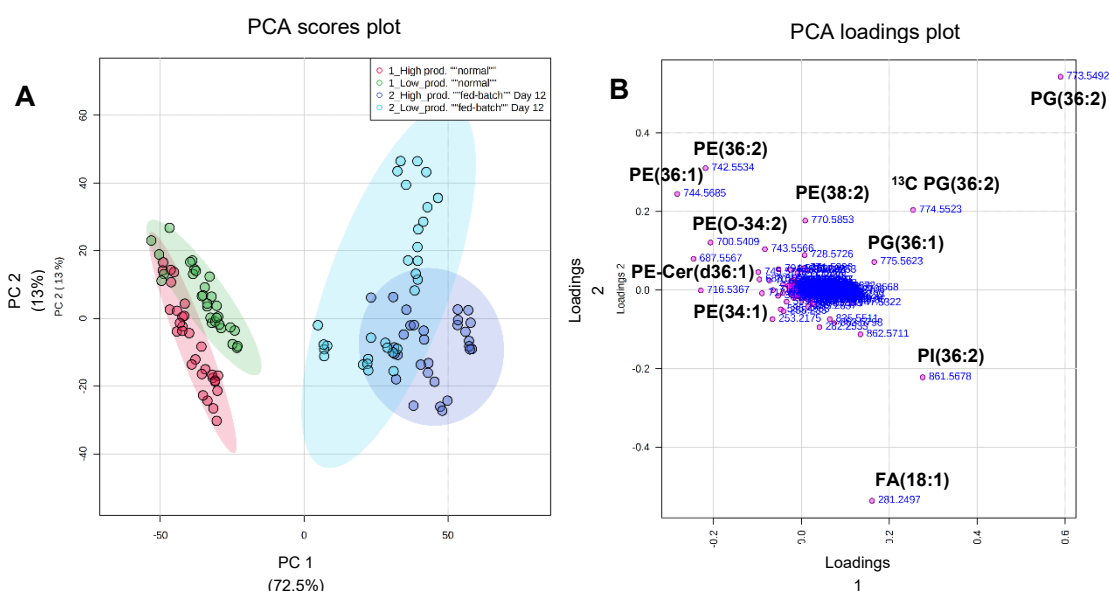


Figure 35. PCA scores plot showing the differentiation between the “non-production” and “fed-batch” Day 12 cell culturing and the respective groups of the high and the low producing cell lines, based on their mass spectrometric profile of the biological and technical replicates of each condition acquired with LD-REIMS. B) PCA loadings plot showing the features mainly contributing in the PCA.

Figure 36 shows representative spectra of the high-producing cell line WT2. Under “non-production” culturing conditions, m/z 744.5543, which based to accurate mass and MS/MS is PE(18:1_18:0), is the base peak in the complex lipid region, a trend we can also see in the “fed-batch” Day 6 culturing cells. In the case of the “fed-batch” Day 12 cultured cells, the lipidomic profile changes quite drastically, with the complex lipid region being dominated by the PG(18:1/18:1) and PI(18:1/18:1) lipids (m/z

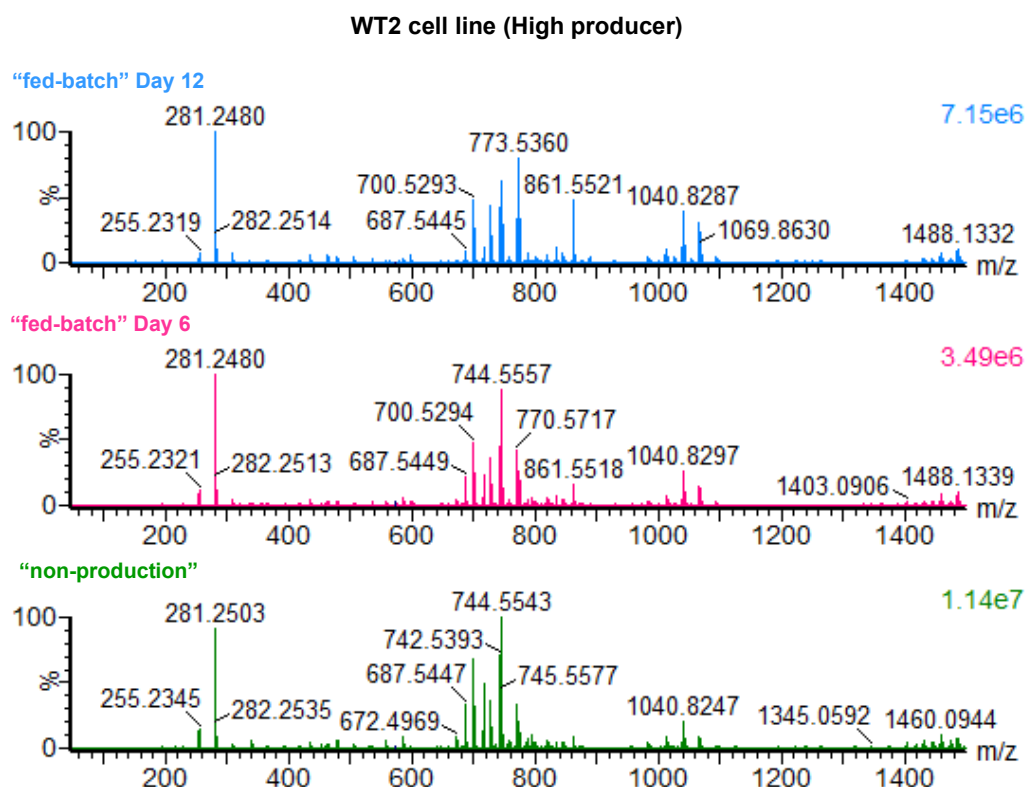


Figure 36. Representative mass spectra of the WT2 cell line, which is a high-producing cell line for each culturing condition.

773.536 and m/z 861.552) respectively. Figure 37 demonstrates representative spectra of the low producing cell line WT4 in each culturing condition. Similarly to WT2, m/z 744.554 (PE(18:1_18:0)) is again the most abundant peak in the complex lipid region for the “non-production” and “fed-batch” Day 6 condition. For the “fed-batch” Day 12 condition, we can see the peak with m/z 773.538 (identified as PG(18:1/18:1)) to be the most abundant peak in the complex lipid region, however the m/z 861.5523 (annotated PI(18:1/18:1)) we observed as one of the most abundant peak in the respective spectrum of WT2 cell line of very low intensity here. The drastic lipid changes observed in the “fed-batch” Day 12 CHO cell spectra could be an indication of their health state. As stated in sections 4.2.2.3 and 4.2.2.4, in “non-production” and “fed-batch” Day 6 cultured cells, the viability is between 95-100% - while in “fed-batch” Day 12 cultured cells, the viability drops to 35-55%, which could significantly alter the cells’ metabolic and lipidomic profile. The fact that the PE lipids are decreased could indicate eruption in the cell membrane, since the PE and PC lipids are the main components of the lipid bilayer in cellular membranes. By day 12 of the “fed-

batch” cell culturing, the cells are heavily stressed, producing both mAbs and waste, causing their viability to drop.

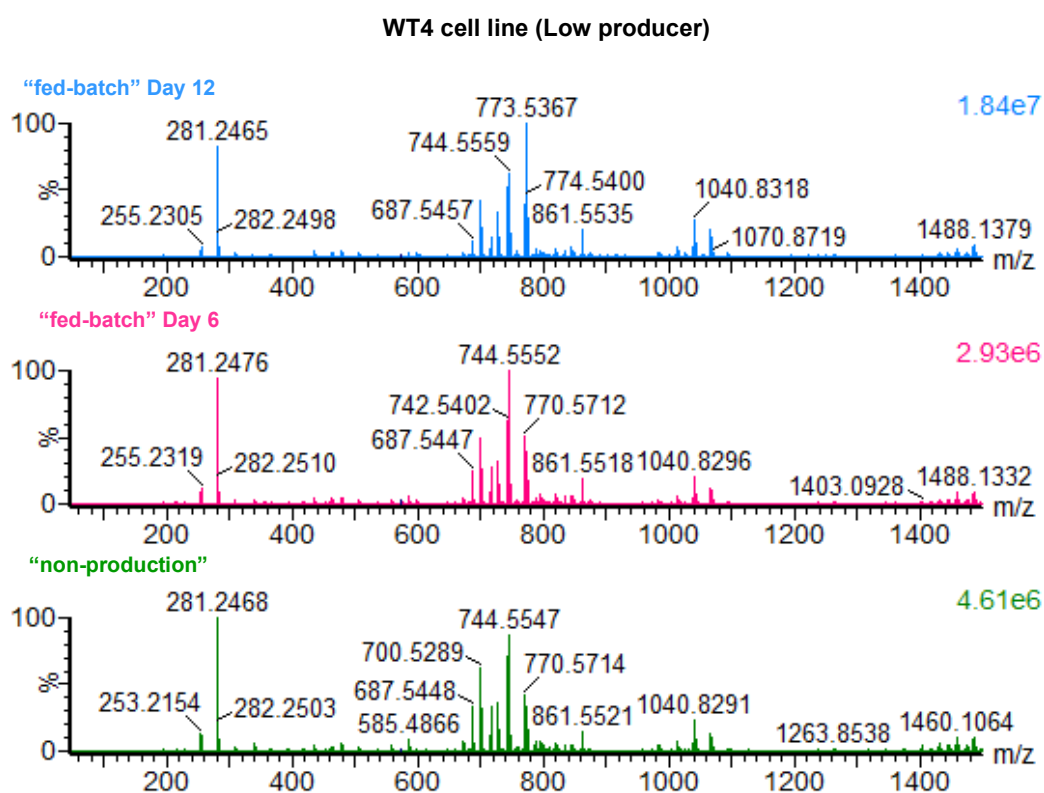


Figure 37. Representative mass spectra of the WT4 cell line, which is a low-producing cell line for each culturing condition.

4.3.2. Classification of high producers and low producers in the different cell culture conditions

As explained in the introduction, classification between high and low producing cell lines is very important for the biopharma industry, as it is vital to distinguish GM CHO cell lines capable of producing high protein yield as early in development as possible. That is the reason why in this section the capabilities of LD-REIMS high-throughput platform for the classification of the high producers and the low producers for both “non-production” and “fed-batch” cultured cells are investigated.

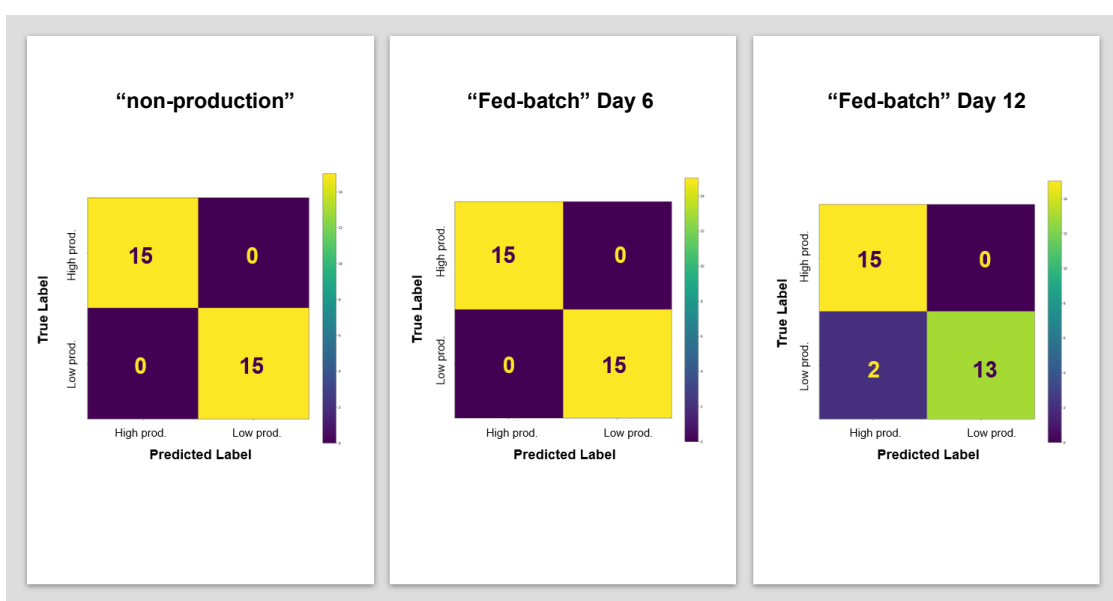


Figure 38. Confusion matrices of the test set cell lines used to test the classification performance of the models and the ability of the models to predict high and low producing cells

Classification models of the WT2 (high producer) and WT4 (low producer) cell lines were built based on LR, using the RFECV feature subset. WT5 (high producer) and WT3 (low producer) cell lines were used as the test dataset to assess the classification capabilities of the models built and establish if the models can predict the true class of the cell lines in each condition. The confusion matrices shown in Figure 38 show the prediction results of this process. As demonstrated, for the “non-production” and the “fed-batch” Day 6 cultured CHO cells the accuracy of the predictions is 100%, with both models classifying correctly the label of each technical and biological replicate of the WT3 and WT5 analysed. For the “fed-batch” Day 12 CHO cells the model is not as accurate, as it misclassifies 2 out of 15 replicates of the low producer, WT3,

however it predicts successfully all the datapoints of the high producing cell line. The fact that the “fed-batch” Day 12 classification model is not 100% accurate as the other two culturing conditions are, could be due to the health state of the cells at this point – 35-50% viability – regardless of their mAb yield.

4.3.2.1. PE ether lipids in high producing “non-production” CHO cells

In Figure 39, a Venn diagram shows the overlapping between the RFECV features used for the classification models of each condition and the main molecular classes of

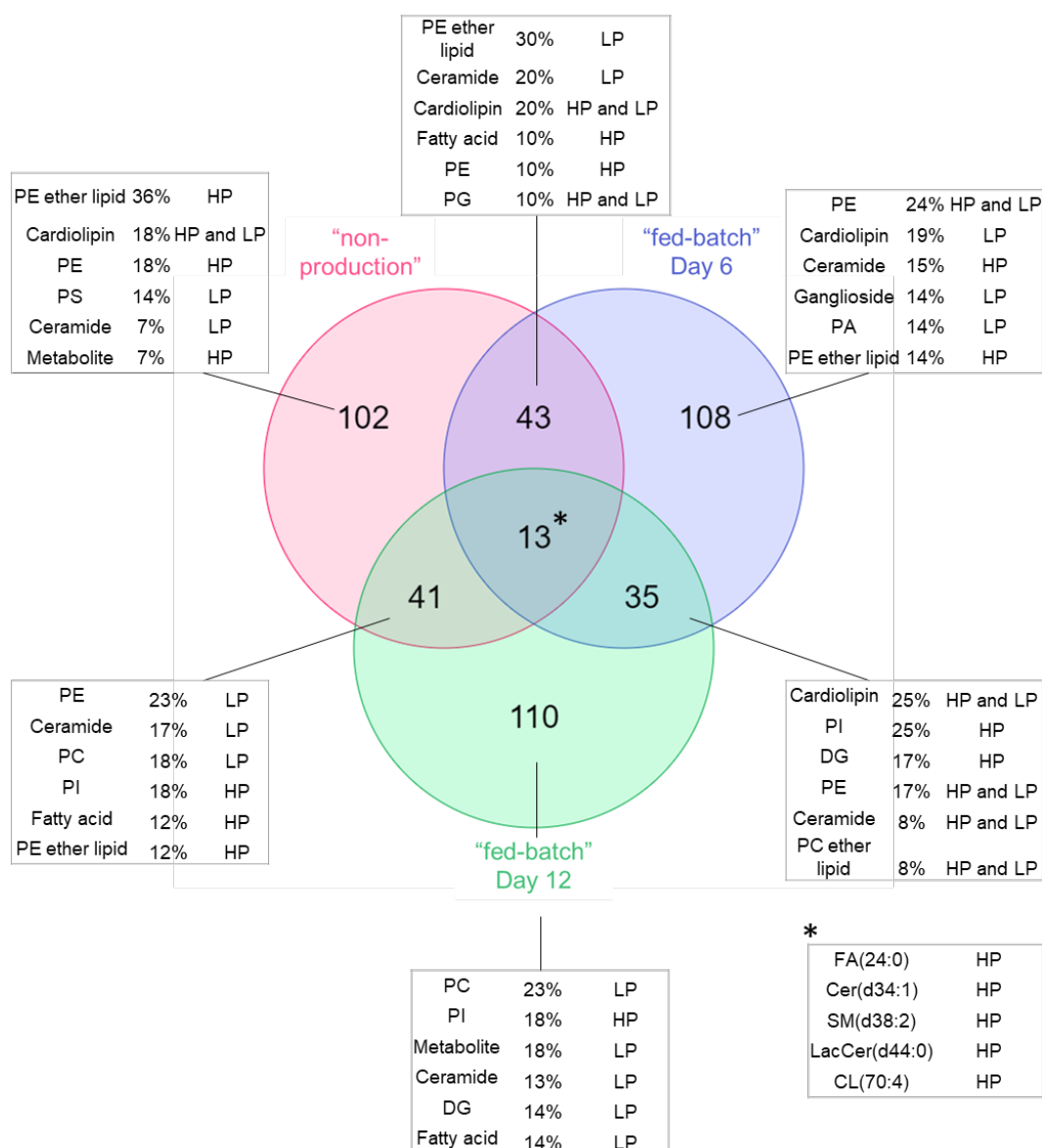


Figure 39. Venn diagram of the RFECV features used for generation of the classification models used. The tables are showing the main molecular classes observed in each group and if they were abundant in the high or low producing cell line of each condition. (HP: high producer and LP: low producer)

each group. As the diagram demonstrates there is not significant overlap between the groups, meaning that the classification models built for each condition are based on different ions. In the “non-production” culturing cells the RFECV features are dominated by PE ether lipids that are more abundant in the high producing CHO cell lines, mainly containing 18:1 fatty acyl chain (Table 15 in Appendix contains the detailed identifications of the RFECV features and their occurrence in each culturing condition). Ether lipids of PC and PE species can be quite abundant in certain cell types and tissues, up to 20% of the total lipidome. They offer a unique stereochemistry in the cellular membranes, and they are believed to influence the fluidity and membrane fusion. The absence of carbonyl group at the sn-1 position of the ether lipids, facilitates stronger intermolecular hydrogen bonds between the headgroups in the membrane⁶¹. As mentioned in Chapter 1, the acyl chain is linked to the glycerol backbone, either by an ether or a vinyl ether group. The latter is the most common subcategory of ether lipids, the plasmalogens, however, with mass spectrometry we cannot confirm the identification of a plasmalogen, as we cannot know exactly where the double bond in the fatty acyl chain is. Studies have shown that plasmalogens can protect the unsaturated membrane phospholipids from reactive oxygen species. By being the targets of these attacks, they potentially protect other phospholipids from oxidation^{219–223}. The fact that PE ether lipids seem to be the more abundant species in the high producer cell lines could mean that they have increased protection against oxidation.

One of the common PE ether lipids is PE(O-18:0_22:6) (Table 15, Appendix), containing the polyunsaturated fatty acyl chain 22:6. Heavier and more unsaturated fatty acyl chains make the cellular membrane more fluid and more vulnerable against peroxidation by ROS²²⁴.

4.3.2.2. PC and PI lipids in “fed-batch” CHO cells

“Fed-batch” Day 12 cultured CHO cells are dominated by PC and PI lipids. As shown in Figure 40, The PI lipids are very abundant in the high producers, while the PC lipids present the opposite trend, found increased in the low producing CHO cells. PIs are negatively charged structural blocks of cellular membranes, but they are also holding an important signalling function as precursors of phosphoinositides, bioactive lipids,

interacting with proteins in the cell membrane. The PI deficiency in the low producing cells could indicate dysregulation in the production of phosphoinositides^{225,226}.

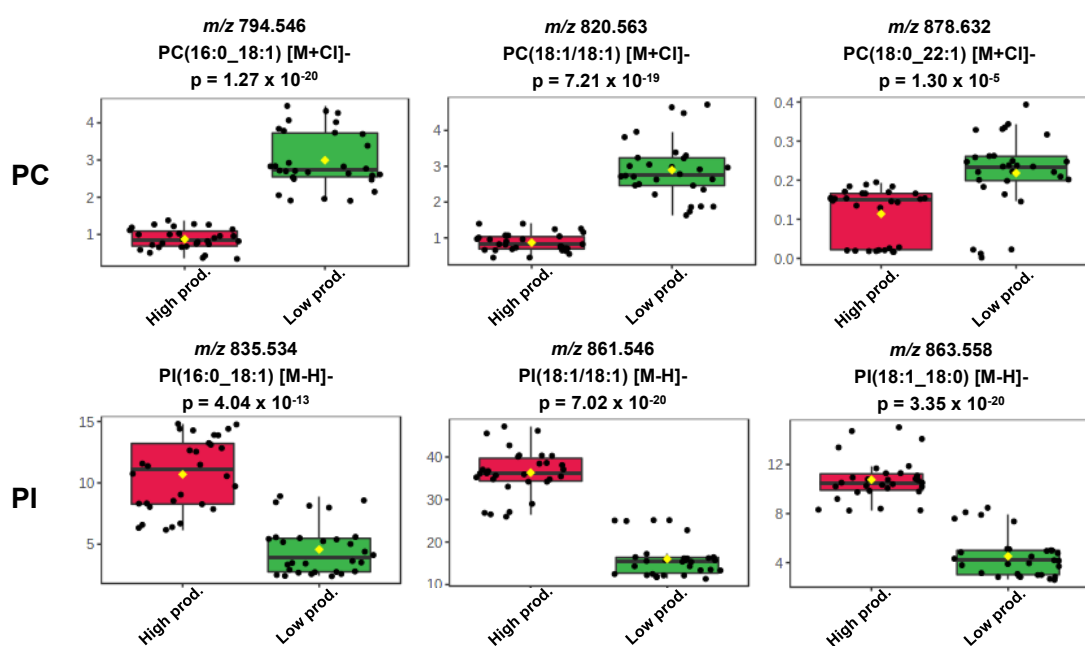


Figure 40. PC and PI lipids relative intensities in high and low producing CHO cells, cultures under “fed batch” conditions (harvested at Day 12)

Differences between PI/PC lipid ratios could have an effect in the cell membrane structure. A study performed by Stanishneva-Konovalova et al. suggested that the amount of PI lipids and their derivatives in the plasma membrane can affect the ordering of the PC lipids and affect the lipid bilayer thickness^{227,228}. Different ratios of these lipids between the high and low producing CHO cells, cultured under “fed-batch” conditions could indicate disruptions in the plasma membrane.

4.3.2.3. Incorporation of polyunsaturated fatty acids in the phospholipids detected in the low producing CHO cells

In the common features between the “non-production” and the “fed batch” Day 12 cultured cells, PEs are the dominated lipid class, including PE(18:1_20:1) and PE(18:0_20:1), which are more abundant in the low producing CHO cells, in both culturing conditions. That could indicate vulnerability of the cellular membrane of the low producers against peroxidation from ROS due to the heavy fatty acyl chain 20:1 incorporated in the PE lipids.

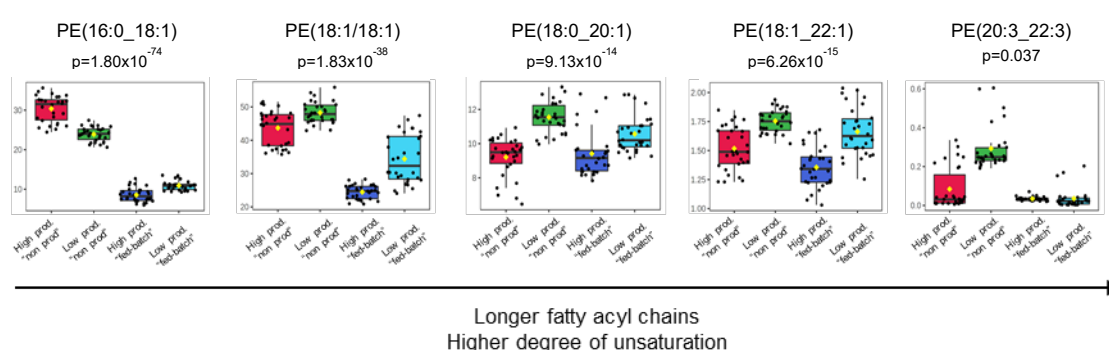


Figure 41. PEs are main components of the cellular membrane, heavier unsaturated fatty acyl chains could alter the permeability of the plasma membrane, creating a more fluid membrane, with low protection against peroxidation ROS.

The trend between the fatty acyl composition of the lipid membranes and their abundance in either low or high producers is demonstrated in Figure 41. PEs with heavier and more unsaturated fatty acyl chains tend to be more abundant in low producers CHO cells in both culturing conditions.

4.3.3. Amino acids and small metabolites in the low producing cell lines

Another interesting trend noticed when compared low and high producing CHO cells involves amino acids and small metabolites. As shown in Figure 42 the basic amino acids and small metabolites, such as glucose, tend to be higher in abundance in the low producing cell lines in both “non-production” and “fed-batch” culture cells.

The amino acids detected with LD-REIMS are taking part in the aminoacyl tRNA biosynthesis and in protein production. The high abundance of these amino acids in the low producers could mean that the aminoacyl tRNA biosynthesis pathway is disrupted in these cells and that the cells are failing to exploit these nutrients and incorporate them in their metabolism. It could also explain the low yield of the recombinant mAb from these cells as the amino acids are the building blocks of the proteins.

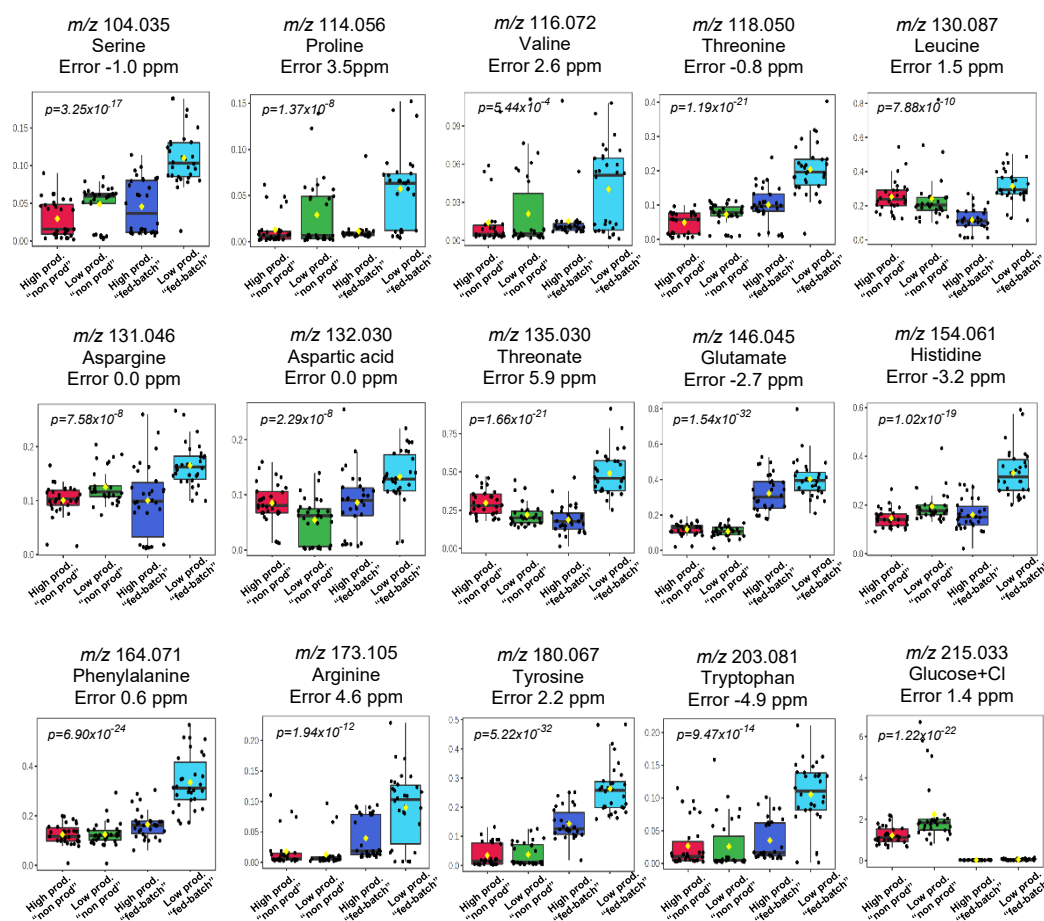


Figure 42. Relative abundance of amino acids and small metabolites in high and low producing CHO cell lines cultured under “non-production” and “fed-batch” conditions.

Similarly, high abundance of glucose in the low producers means that the cells are not exploiting the glucose available to them from the culture medium. However, this is only true for the “non-production” cultured cells, as in the “fed batch” cultured cells glucose is not present, indicating that the cells, both high and low producers are under stress.

4.4. Conclusion

LD-REIMS successfully differentiated the low and the high producing cell lines and provided valuable biological information in a high-throughput manner and with minimal sample preparation. Low producers CHO cells seem to have cell membranes with heavier, more unsaturated phospholipids incorporated, which could compromise their functions and membrane architecture. The upregulation of small metabolites, such as glucose and amino acids, in the low producing cells could mean that the low producers do not metabolise successfully the nutrients from the growth medium, which could have an important effect on the protein yield.

In addition, the low producing cell lines seem to be enriched in phospholipids with heavier chains and higher degree of unsaturation, while the high producing cell lines are mainly consisted of phospholipids with 16 and 18 carbon fatty acyl chains, saturated or monounsaturated. This trend is followed in the three most abundant lipid classes detected in CHO cells, i.e., phosphoethanolamines (PE), phosphocholines (PC) and phosphoethanolamine ether lipids (PE ether lipids). This could be an indication about the fatty acyl composition of the membrane lipids and overall the membrane architecture, with the high producers to have a more rigid, saturated membrane, while the low producing cell lines a desaturated, more fluid membrane, with low protection against peroxidation by reactive oxygen lipids (ROS)²²⁴. This could be the basis for future work, involving more experiments for the elucidation of these phenomena and the confirmation of hypotheses made based on the results in this PhD thesis.

Chapter 5

Investigation of Drug Induced Phospholipidosis
in alveolar macrophages using LD-REIMS

Chapter 5: Investigation of Drug Induced Phospholipidosis in alveolar macrophages using LD-REIMS

5.1. Introduction

Cationic amphiphilic drugs (CADs) are therapeutic molecules used to treat a wide range of conditions, from allergies and infections to heart disease and psychiatric disorders. CADs share common structural features, mainly their amphiphilic nature being both hydrophobic – containing aromatic rings – and hydrophilic – containing a hydrophilic side chain with an amine group²²⁹. At physiological pH (between 7.35 and

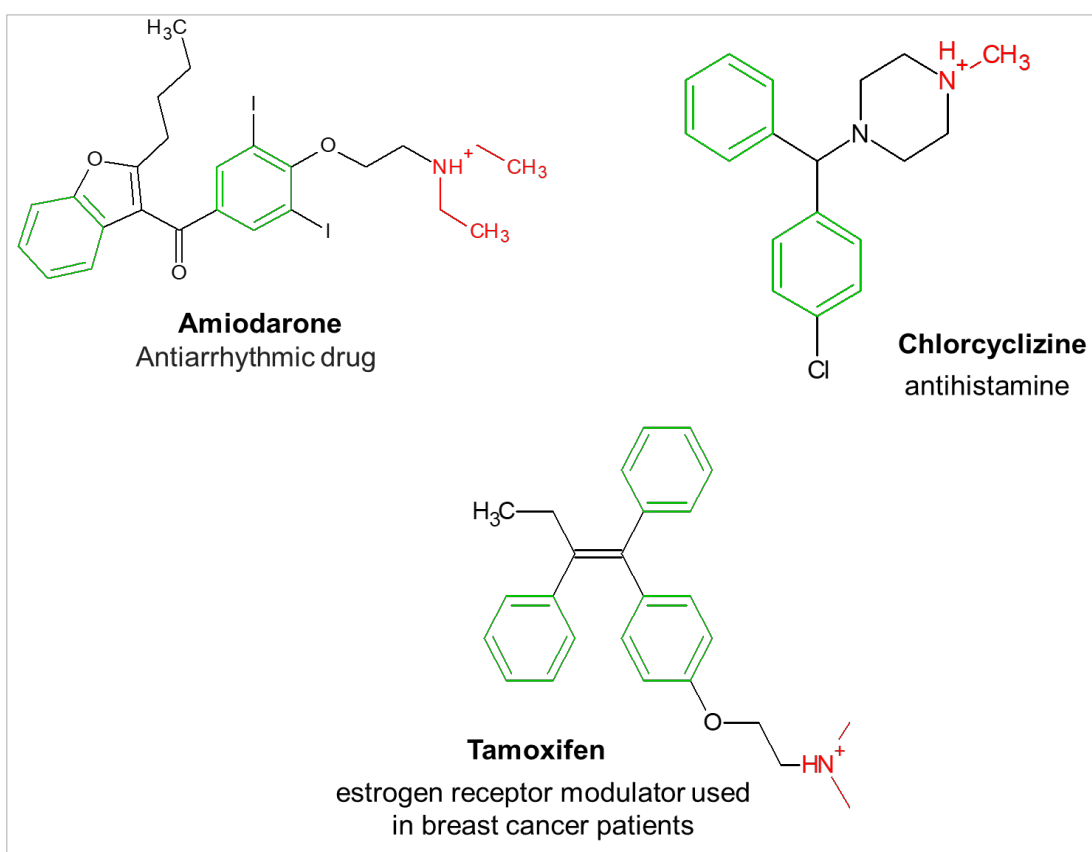


Figure 43. Three commonly prescribed CADs – amiodarone, chlorcyclizine, and tamoxifen.

CADs share common structural features, the hydrophobic aromatic rings (green) and the hydrophilic cationic amine groups (red). The term ‘amphiphilic’ describes the dual character of their chemical structures, i.e., both hydrophobic and hydrophilic.

7.45 with average of 7.40²³⁰) CADs are not protonated, which allows them to move across lipid membranes and cellular organelles. Once they enter the acidic environment of the lysosomes, however, the amine group becomes protonated, less able to diffuse through the lysosomal membrane, resulting in the accumulation of CADs in the lysosomes and degradation of the inner lysosomal membrane²³¹.

Even though CADs are widely used, and dozens have been approved for clinical treatment, they are known to cause phospholipidosis as a side effect, i.e., the excessive accumulation of phospholipids and the creation of intracellular lamellar bodies in cells and tissue. Drug-induced phospholipidosis is a lysosomal storage disorder, i.e. a metabolic disorder that results in lysosomal malfunction^{232,233}, however its mechanism is not yet fully elucidated. Phospholipidosis can occur in almost any tissue type, but is usually more prominent in the lungs, especially in cells depending greatly on lysosomal activity, such as alveolar macrophages. After exposure to CADs alveolar macrophages become “foamy”, as they grow heterogeneous in size, becoming enlarged with accumulation of lamellar bodies. Lamellar bodies are intracellular organelles storing lipids in alveolar macrophages and type II pneumocytes and they are the secretory organelles of the surfactant lipids in the lungs^{234–236}. In pulmonary phospholipidosis, accumulation of phospholipids are observed, as well as increased numbers of alveolar macrophages in the alveoli^{237–239}.

The gold standard for the detection of phospholipidosis in cells and tissue is electron microscopy (EM). However, the technique is time consuming, and the sample preparation is laborious, and thus not fit for the fast detection of drug induced phospholipidosis. Detecting phospholipidosis in the early stages of drug development is essential for the pharmaceutical industry, as phospholipidosis is considered an adverse effect and could result in regulatory rejection, or delays in the approval of a drug candidate from the regulatory authorities^{240–242}.

Apart from EM, in the recent years, LC-MS has been used for the detection of phospholipidosis, specifically for the detection of di-22:6-bis(monoacylglycerol)phosphate (BMP(22:6/22:6)), which has been suggested as a potential biomarker for drug induced phospholipidosis²⁴³. However, LC-MS is as well a time-consuming technique with extensive and laborious sample preparation, and detection and identification of BMP lipids is challenging since

they are structural isomers of PG lipids, with two monoacylglycerols bound to a phosphate group. BMPs can only be found in the inner membranes of late endosomes and lysosomes – which could be the reason for the presence of BMP(22:6/22:6) in cells dependent in lysosomal activity, such as the alveolar macrophages. Their presence in mammalian tissues is uncommon - however, they have been found elevated in certain cell types, such as macrophages, which depend greatly on lysosomal activity. As mentioned above, analytically, BMPs are difficult to identify since they are structural isomers to PGs and hence share the same m/z and fragmentation patterns in negative ion mode (Figure 44). Their differentiation can be achieved with chromatographic separation (where possible) and/or fragmentation (CID) in positive ion mode, which generates different spectra to their PG counterparts^{239,244} (Figure 45).

**Negative ion mode – identical MS/MS
spectra of PG and BMP lipids**

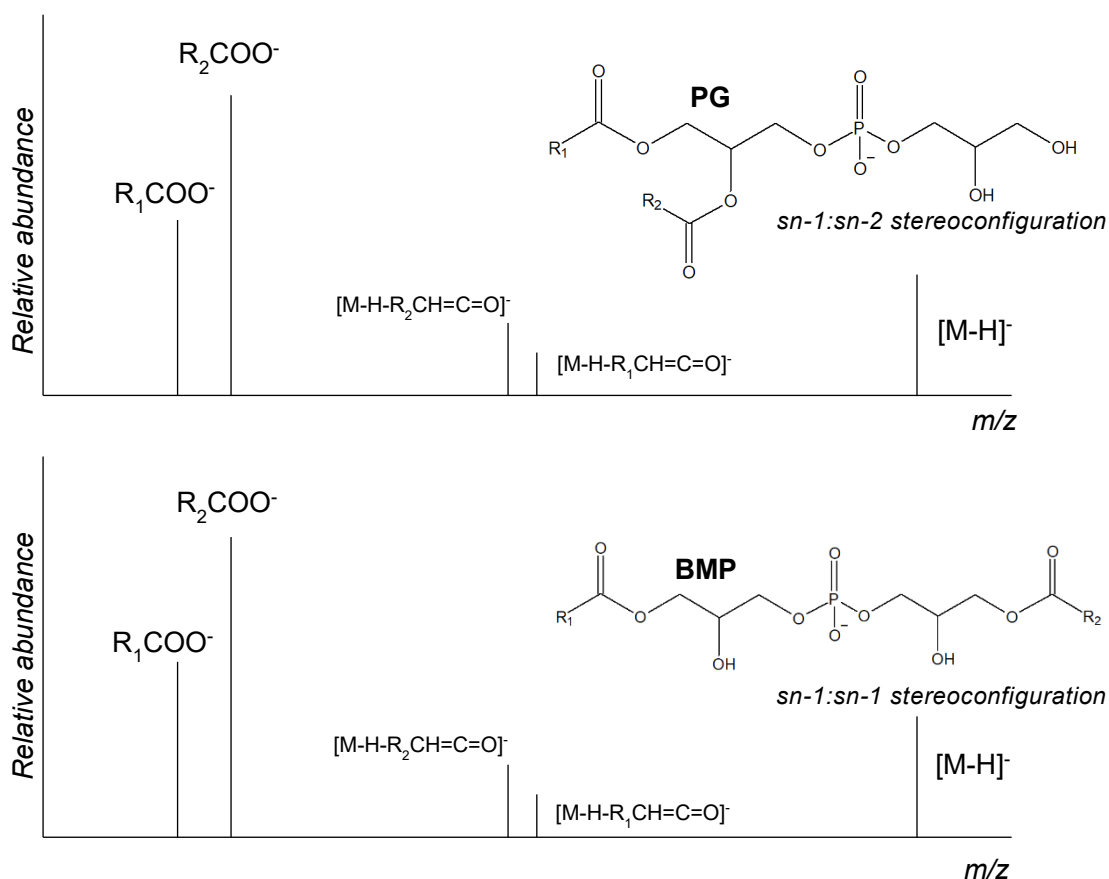


Figure 44. Illustration of the CID MS/MS fragmentation pattern of PG (top) and BMP (bottom) lipids in negative ion mode. The MS/MS spectra produced by these lipid isomers are identical, with the base peaks being the respective fatty acyl ions.

In this chapter, the aim is to investigate differences between alveolar macrophages treated with three CADs that are known to cause phospholipidosis – amiodarone, chlorcyclizine, and tamoxifen – to untreated/control alveolar macrophages, using a high-throughput LD-REIMS autosampler. In addition, ambient ionisation mass spectrometry imaging, and specifically DESI MSI and the recently developed LD-REIMS imaging is used for the comparison of *in vitro* and *in vivo* models for the effect of amiodarone in cellular metabolism. Comparability of *in vitro* and *in vivo* models is

Positive ion mode – different MS/MS fingerprint of PG and BMP lipids

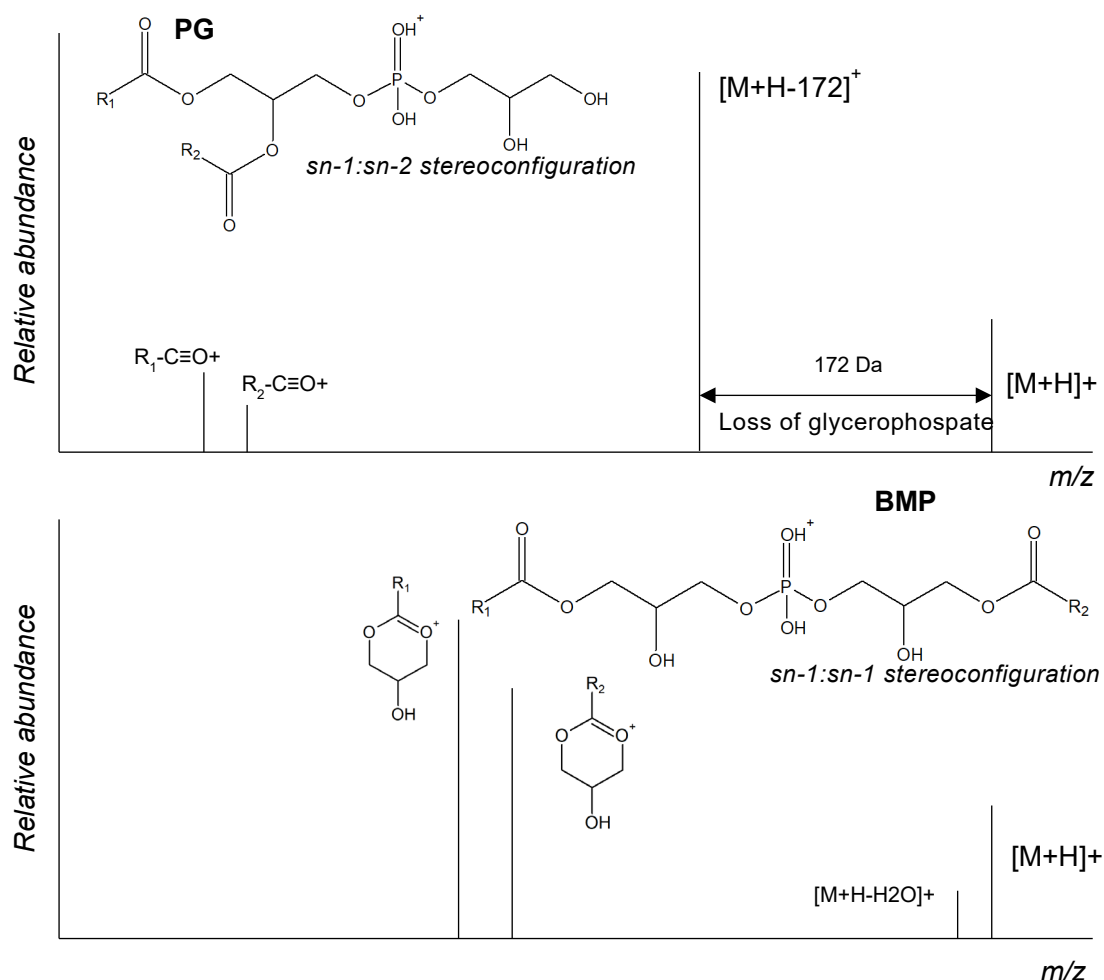


Figure 45. Illustration of the CID MS/MS fragmentation pattern of PG (top) and BMP (bottom) lipids in positive ion mode. The MS/MS patterns are visibly different. The PG (top) product $[M+H-172]^+$ reflects the loss of the glycerophosphate group, and less intense peaks are annotated as the acylium cations of the fatty acyl chains. By comparison, BMP's main peaks are annotated as the cyclic monoglyceride cations attached to the fatty acyl chain.

still challenging, mainly due to the complexity of an entire living organism compared to cell culture models. For that reason, it will be interesting to see the correlation of the lipidome and metabolome between the drug induced phospholipidosis tissue and cells, using the same ionisation technique, i.e., LD-REIMS with different setups – imaging for the tissue analysis, and high-throughput autosampler for the rapid screening of the cells. DESI MSI, as an established ambient MSI technique, is used for the validation of the LD-REIMS observations.

5.2. Materials and Methods

5.2.1. Sample preparation – rat alveolar macrophages

Rat lung alveolar macrophages – cell line NR8383 (ATCC CRL-2192) – were used as the model cell line in this study. The cells were cultured in T75 culture flasks (Corning) in Ham's F-12K (Kaighn's) Medium with 2 mM L-glutamine, adjusted to contain 1.5 g/L sodium bicarbonate, 85% (ATCC), and 15% heat inactivated fetal bovine serum (Gibco). A stock solution of 5 mg/mL amiodarone hydrochloride (Sigma-Aldrich, CAS 19774-82-4, purity $\geq 98\%$) was prepared in DMSO (Sigma-Aldrich) and further diluted in the complete growth medium to a final concentration of 2.5 and 5 $\mu\text{g/mL}$ ²⁴⁵. Stock solutions of 5 mg/mL of chlorcyclizine hydrochloride (Stratech Scientific, CAS 14362-31-3, purity $> 98\%$) and of Tamoxifen Citrate (Stratech Scientific, CAS 54965-24-1) were prepared in DMSO (Sigma-Aldrich) and further diluted in the complete growth medium to a final concentration of 5 $\mu\text{g/mL}$. The cells were incubated (37°C and 5% CO₂) in the growth medium treated with amiodarone, chlorcyclizine, and tamoxifen for 72 hr. Control (untreated) cells were incubated under the same conditions. Rat alveolar macrophages are semi-adherent, i.e., a percentage (approximately 50%) of the cells attach to the bottom of the T75 flask, while the remaining cells growing in suspension. After the incubation, the growth medium with the suspension cells was removed, underwent centrifugation for 5 minutes at 1000 rpm and 4°C, and the supernatant discarded in order to collect the non-attached cells in a cell pellet. In the T75 flask with the adherent cells, 1 mL of 150 mM ammonium acetate solution was added, and the cells were scraped; the cell lysates were then moved into 1.5 ml microcentrifuge tubes (Eppendorf UK Ltd.) and

underwent centrifugation at 1000 rpm and 4°C for 5 minutes, the supernatant was discarded, and the cell pellet of the adherent cells was collected. Both cell pellets, one from the suspension cells and one from the adherent cells, were resuspended in 100 µL of 150 mM ammonium acetate solution; they were merged into the same microcentrifuge tube and transferred into a 96 well plate (Eppendorf™ 96-well V-Bottom PCR Microplates). The plate then underwent centrifugation for 5 minutes at 5,000 rpm and 4°C in order to generate the combined cell pellet from both adherent and suspension rat alveolar macrophages. The supernatant was then discarded, and the plate kept at -80°C until the day of analysis.

5.2.2. Sample preparation – rat lung tissue imaging

Rat lung tissue dosed with amiodarone (7 day repeat oral administration of Amiodarone – 100 mg/kg – in male Wistar Han rat) and untreated control rat lung tissue were sectioned into 10 µm slices using a Cryotome FSE (ThermoFisher Scientific), and thaw mounted onto glass microscope slides (Superfrost Plus). The glass slides with the tissue sections were kept at -80°C until the day of analysis.

5.2.3. High-throughput LD-REIMS cell pellet analysis

LD-REIMS analysis of the cell pellets was performed using the same parameters and conditions described in sections 3.2.4.3, 3.2.5 and 3.2.6 of Chapter 3. The acquisition of the mass spectra was performed using MassLynx V4.1 (Waters Corp.).

5.2.4. LD-REIMS Imaging

The thawed tissue sections on glass slides were placed on a two-axis moving stage (Prosolia), underneath the laser lens. The laser used was an IR Opotek Opolette HE 2731 Tuneable OPO Laser, operated at 2940 nm wavelength, 20 Hz repetition rate and approximately 20 mW power. The image acquisition was performed at 70 x 70 µm spatial resolution, with 100-1500 Da mass range and 0.14 sec scan time. The laser beam desorbed the tissue surface producing an aerosol, which was introduced into a Xevo G2- XS QToF mass spectrometer (Waters Corp.). Propan-2-ol was used as a

matrix solvent during the sampling as described by Jones et al.¹³⁹. The imaging data acquired using HDImaging v.1.4 (Waters Corp.) and MassLynx v4.1 (Waters Corp.).

5.2.5. DESI MS Imaging

DESI MS Imaging analysis was carried out in triplicate on lung tissue sections treated with amiodarone and control lung tissue sections. A two-axis moving stage (Prosolia) mounted on a G2-XS QToF mass analyzer (Waters Corp.) was used. The DESI sprayer (Waters Corp.) was also mounted onto the DESI stage and aligned with a heated transfer capillary for the introduction of the desorbed ions into the mass spectrometer. The solvent, 9:1 (v/v) Water/Methanol (Sigma-Aldrich), was introduced to the DESI sprayer using a NanoLC pump (Waters Corp., U.S.A.) with a steady flow rate of 1.5 $\mu\text{L}/\text{min}$. All images were acquired at 100 x 100 μm spatial resolution, 50-2000 Da mass range and 1 sec scan time. The imaging data was acquired using HDImaging v.1.4 (Waters Corp.) and MassLynx v4.1 (Waters Corp.).

5.2.6. Oxylipin quantification

Rat lung tissue dosed with amiodarone and control rat lung tissue were mixed with 0.5 mL of 1:1 (v/v) Methanol/Water (Sigma-Aldrich) and homogenised using a bead beater. The homogenates were transferred into microcentrifuge tubes (Eppendorf UK Ltd.) and underwent centrifugation for 30 min at 13,000 rpm and 4°C. The supernatant was then put under a flow of N₂ gas until dry. The dried extracts were kept at -80°C until the day of analysis. On the day of analysis, the dried residues were resuspended in 120 μL of methanol/water 50:50 and analysed with LC-MS/MS based on the conditions described by Wolfer et al.²⁴⁶ at the National Phenome centre.

5.2.7. Untargeted Lipidomics LC-MS analysis

The extraction of the cells was performed as described in section 3.2.9. Rat lung tissue treated with amiodarone and control rat lung tissue were mixed with 1 mL of 5:1 IPA/Water (both UPLC grade, Optima™ LC/MS Grade, Fisher Chemical™, Fisher Scientific) and homogenised using a bead beater. The mixture was vortexed again for 1 minute and then placed in a -20°C freezer for 2 hr for precipitation of proteins.

Subsequently, the samples underwent centrifugation at 10,000 rpm for 10 minutes, at 4°C, and the supernatant was transferred to HPLC vials for analysis. Before analysis, an internal standard lipid mixture was added to the lipid extracts, volumes and concentrations described in section 3.2.9. The LC-MS analysis was performed following the National Phenome's Centre (NPC) lipidomic assay, using the same conditions described in section 3.2.9. LC-MS/MS analysis was performed for the ions of interest, using the same conditions described in section 3.2.9.

5.2.8. Western blot of rat alveolar macrophages

Western blot analysis was used for the quantification of PLA2 enzymes in amiodarone treated and control alveolar macrophages. In T75 flasks, the cells were washed with cold PBS while on ice, and 0.4 mL of cell lysis buffer solution (consisting of 10% concentrated cell lysis buffer (10x) (Cell Signalling Technology), 0.2% phenylmethylsulfonyl fluoride (PMSF) (Cell Signalling Technology) and 89.8% sterile water) was subsequently added. The flask with the cells and the cell lysis buffer remained on ice for 30 minutes. The lysates were then transferred to microcentrifuge tubes (Eppendorf UK Ltd.) and underwent centrifugation for 30 minutes at 12,000 rpm, and 4°C. Total protein concentrations were determined using PierceTM BCA protein assay kit (ThermoFisher Scientific). Based on the total protein concentration, the cell lysates were diluted with sterile water and Laemmli buffer (BioRad) to a final protein concentration of 1 µg/mL. The cell lysate mixture was then boiled at 90°C for 5 minutes before subjection to SDS-PAGE electrophoresis using precast gels (BioRad). The membrane was incubated in primary antibody overnight, and subsequently in secondary antibodies (cPLA2, iPLA2, and GADPH) for 45 minutes. The images of the membranes were developed using Azure 600 Western Blot Imaging System (Azure Biosystems, Inc.).

5.2.9. Data analysis

5.2.9.1. LD-REIMS analysis of alveolar macrophages

The data acquired was pre-processed using the same process as described in section 3.2.7. The .csv output from the preprocessing pipeline with the list of the detected *m/z*

values of the different samples and their subgroups was then used for further analysis. The output matrix was log-transformed, and ion intensity boxplots were generated using MetaboAnalyst²¹⁸. Pearson correlation (MetaboAnalyst) was used for discovery of the m/z values highly correlated to BMP(22:6/22:6).

5.2.9.2. DESI MSI and LD-REIMS Imaging

Waters raw data from the mass spectrometry imaging experiments was imported and processed in HDImaging v.1.4 (Waters Corp.), which was also used for the generation and visualisation of the single ion images of both DESI and LD-REIMS imaging data.

5.2.9.3. LC-MS and LC-MS/MS Lipidomics

The LC-MS and LC-MS/MS data were acquired and analysed with MassLynx V4.1 (Waters Corp.), which was also used for visualisation of mass spectra.

5.2.9.4. LC-MS Oxylin assay

The detection and quantification of the oxylin was performed by the National Phenome Centre, which provided the .csv output summarising the oxylin detected and the respective concentrations.

5.3. Results and Discussion

5.4. PUFAs and eicosanoid upregulation

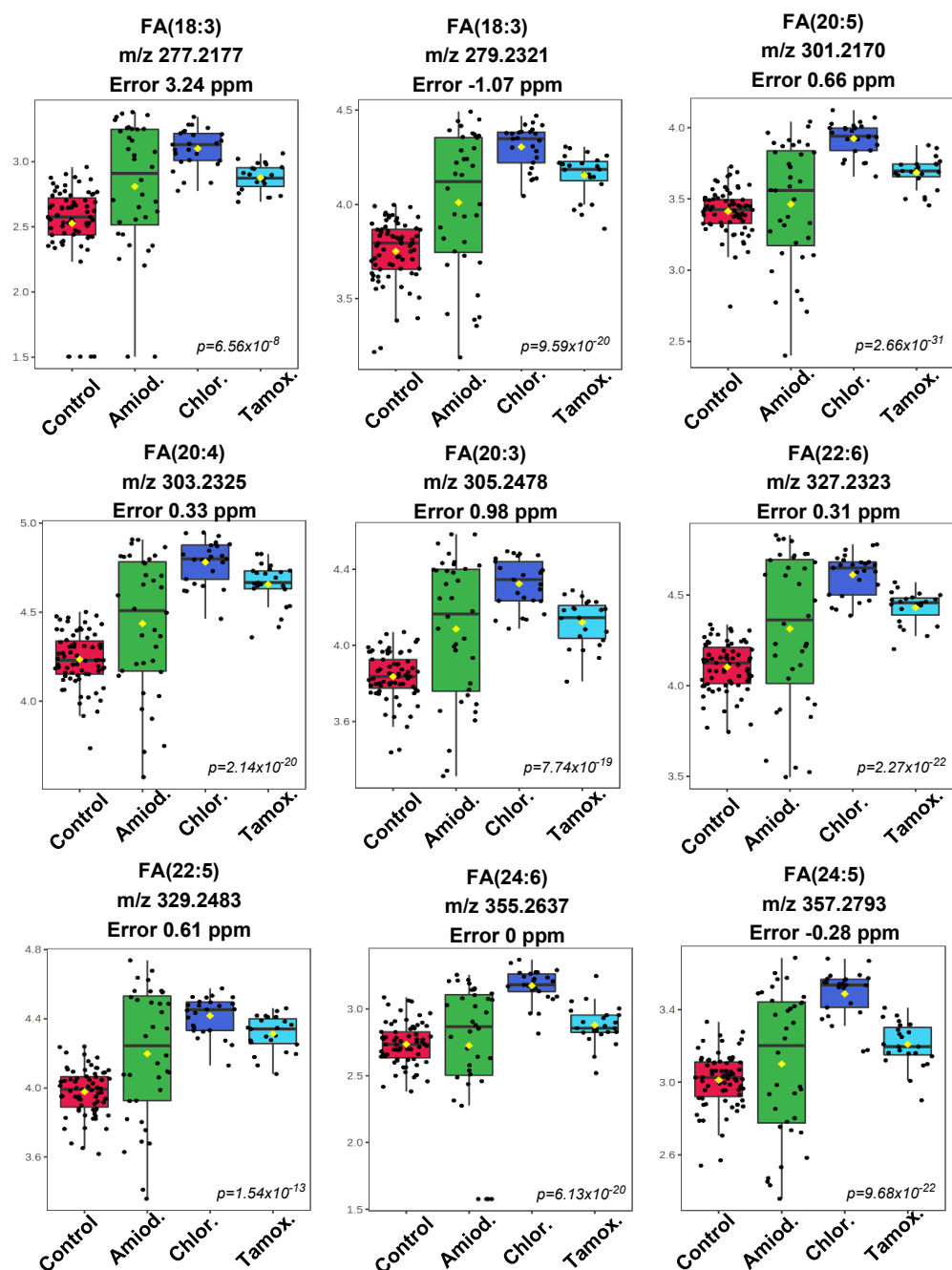


Figure 46. PUFA upregulation in alveolar macrophages treated with 5 µg/µL of Amiodarone (green), Chlorcyclizine (blue) and Tamoxifen (pale blue), in comparison to control – untreated – cells (red).

Arachidonic acid (FA(20:4)) holds an important role in the cellular metabolism, as a precursor of signalling molecules – specifically the eicosanoids, which take part in inflammatory processes within cells. Interestingly, arachidonic acid and other PUFAs were found to be upregulated in the phospholipidosis induced rat alveolar macrophages – i.e., the cells treated with amiodarone, chlorcyclizine, and tamoxifen - in contrast to the untreated control cells, as demonstrated in Figure 46. The fact that PUFAs are upregulated in treated cells indicates that the cells are preparing for inflammatory response and their oxidation to eicosanoids and other oxylipins. DESI MSI and LD-REIMS imaging of the rat lung tissue treated with amiodarone also showed upregulation of PUFAs when compared to the untreated rat lung tissue (Figure 47). From the single ion images of both DESI MSI and LD-REIMS in negative ion mode, arachidonic acid has the highest intensity in comparison to the other PUFAs observed in the treated tissue, with FA(18:2) and FA(20:3).

LC-MS analysis confirmed the results obtained from the high-throughput LD-REIMS analysis of drug-induced phospholipidosis cells, as well as the MSI analysis of treated lung tissue. Figure 48 shows the differences in the fatty acids between the chromatograms of treated and control alveolar macrophages and lung tissue.

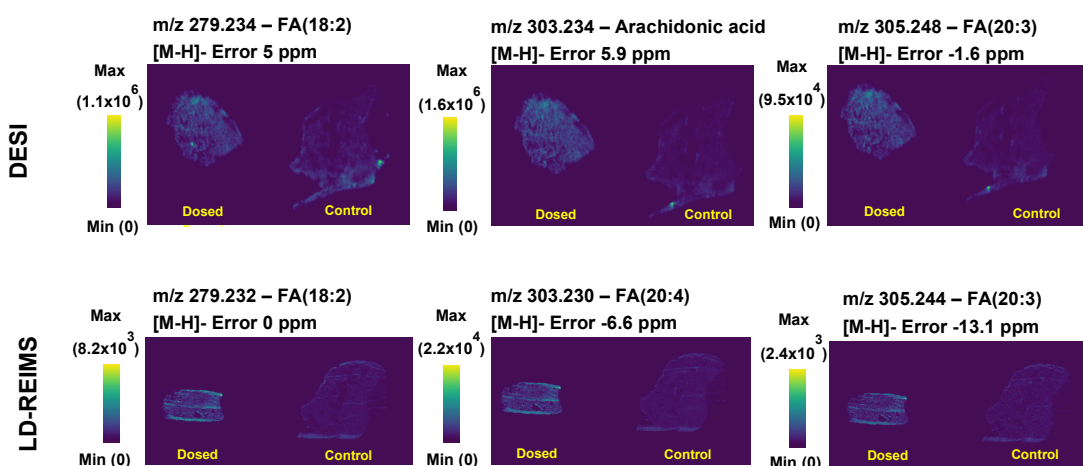


Figure 47. Single ion images of DESI MSI (top) and REIMS Imaging (bottom) of and control rat lung tissue. The upregulation of PUFAs observed in the rat alveolar macrophages is also observed in the tissue, especially the arachidonic acid, which appears to be of higher intensity than the FA(18:2) and FA(20:3) and more prominent in the treated tissue.

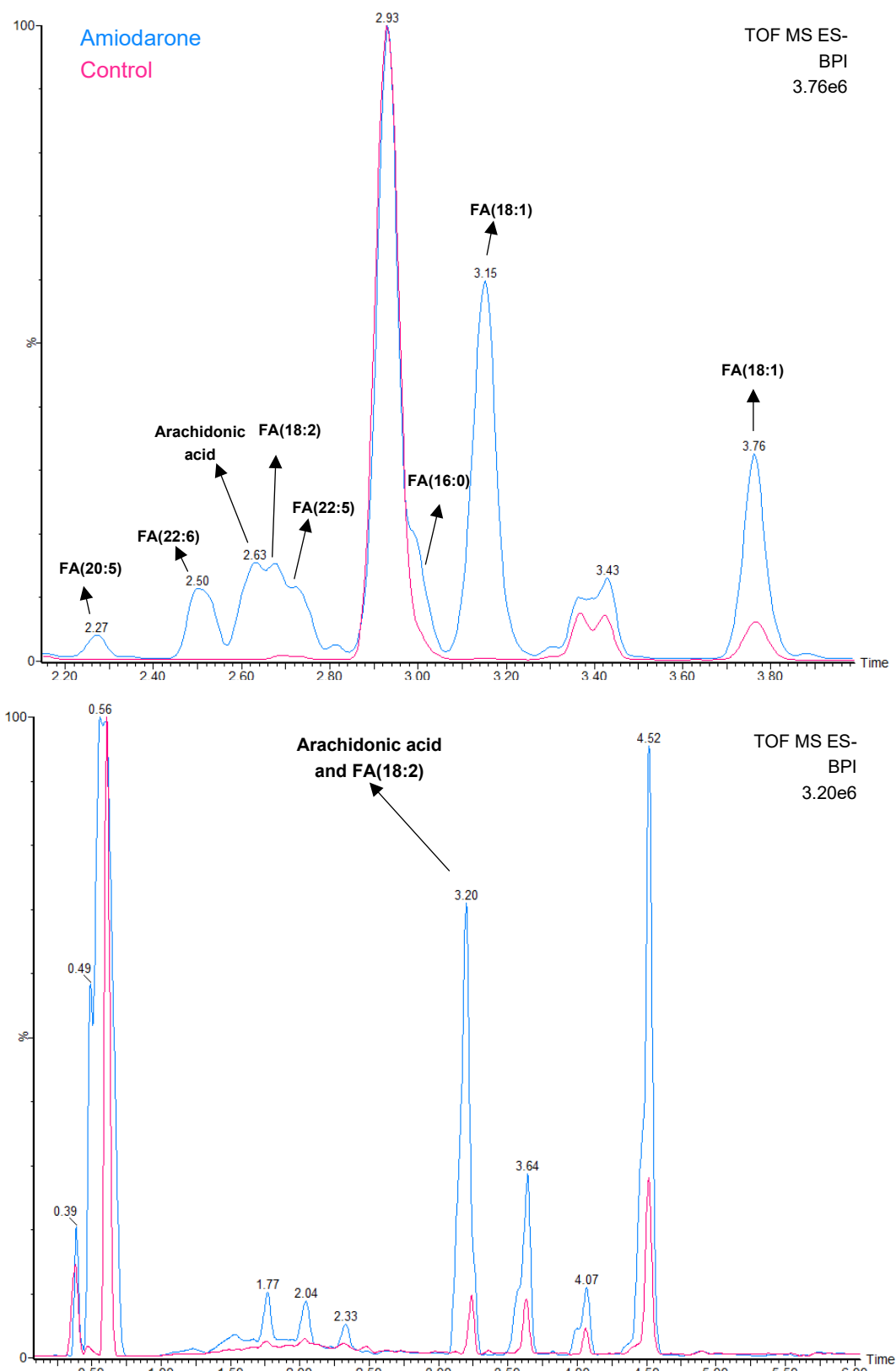


Figure 48. Overlaid chromatograms of treated and control alveolar macrophages (top) and treated and control lung tissue (bottom). The fatty acid region seems to be enhanced in the treated cells and tissue, confirming the observation of the LD-REIMS analysis of cells and the mass spectrometry imaging analysis of the tissue.

Apart from the enhanced PUFAs in the cells and animal tissue treated with amiodarone, eicosanoids are also observed in the treated rat lung tissue, especially when analysed with DESI MSI – but with trace levels also observed with LD-REIMS imaging. To confirm the identity of the m/z values observed with the ambient ionisation MSI, LC-MS/MS was performed at the National Phenome Centre, using a method specifically developed and validated for the identification and quantification of oxylipins and eicosanoids. The LC-MS/MS assay revealed that the oxylipins found in significantly different concentrations in the amiodarone-treated lungs were 5-HETE and 5-oxoETE, which are synthesised by the 5-lipoxygenase (5-LOX) enzyme from arachidonic acid (Figure 50). Unfortunately, the assay cannot identify/quantify 5-HpETE, the precursor of 5-oxoETE and 5-HETE - however, we can tentatively annotate the peak at m/z 335.223 detected with DESI is 5-HpETE, especially since it is within 3.5 ppm error from the molecule's theoretical mass, and it has the same spatial distribution as the other two molecules.

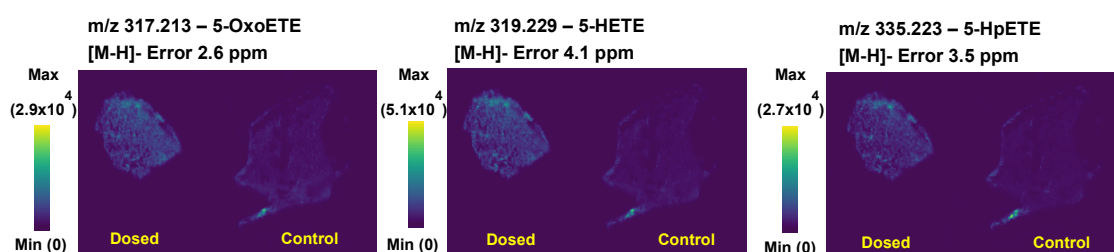


Figure 49. DESI MSI of amiodarone-treated rat lung tissue, revealing the presence of eicosanoids identified by LC-MS/MS as members of the 5-LOX pathway (See Table 16 in Appendix).

5-oxoETE, which appears to be increased fivefold in the treated lung tissue in comparison to the control in the oxylipin assay (Table 16 in Appendix), is very informative regarding the inflammatory processes taking place after drug treatment. 5-oxoETE has been found to induce the infiltration of neutrophils and macrophages in damage-induced tissue²⁴⁷ - in addition, 5-oxoETE activates Ca^{2+} mobilisation and its migration to neutrophils, which correlates with amiodarone's action in blocking K^+ and Ca^{2+} channels²⁴⁸. Thus, the presence of 5-oxoETE could be interpreted as a protective mechanism of the cells to re-establish Ca^{2+} homeostasis by initiating its mobilisation^{249–251}. Intracellular Ca^{2+} plays an important role in cell and lipid metabolism, and its concentration affects the function of phospholipases A2 (PLA2), which are responsible for the hydrolysis of the sn-2 acyl bond in phospholipids for the

release of free fatty acids and lysophospholipids. Specifically, the cytoplasmic PLA2 – cPLA2 – is a Ca^{2+} dependent phospholipase and is one of the main PLA2s catalysing the release of fatty acids from phospholipids, especially those containing polyunsaturated fatty acyl chains – mainly arachidonic acid. The effect of amiodarone on intracellular Ca^{2+} would interfere with the function of cPLA2, thus causing fatty acid deficiency. On the other hand, iPLA2, which belongs to the same enzyme family, is a Ca^{2+} independent phospholipase. It has been reported that, under the effect of a stimuli, iPLA2 releases arachidonic acid in macrophages, while under normal

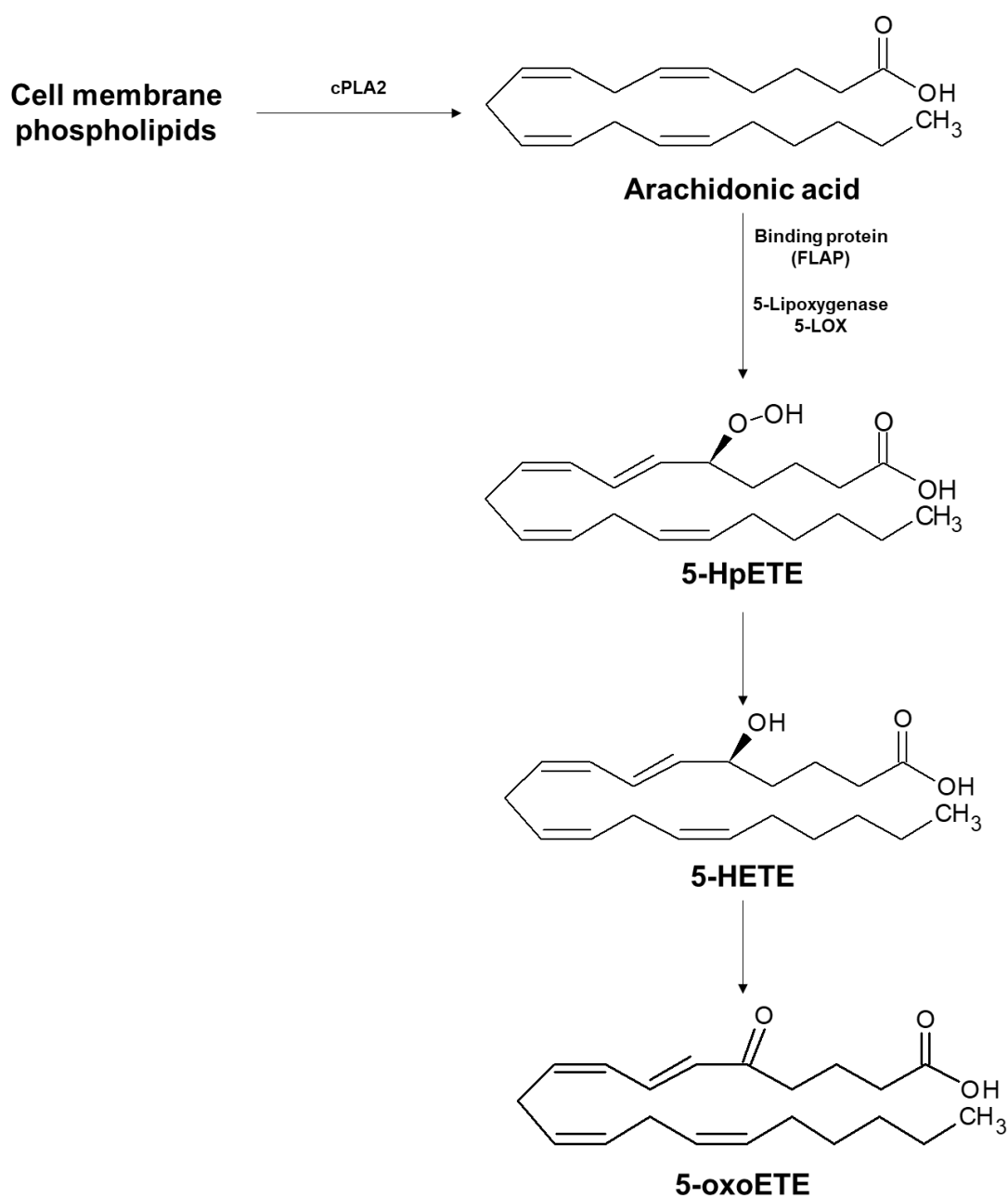


Figure 50. 5-LOX pathway for the generation of 5HpETE, 5-HETE, and 5-oxoETE.

conditions releases preferentially towards FA(22:6)²⁵². Figure 51 demonstrates the western blot quantification of the total cPLA2, the activated phospho-cPLA2, and the calcium-independent iPLA2 in control alveolar macrophages and macrophages dosed with 2.5 and 5 µg/mL amiodarone. Two different amiodarone concentrations were

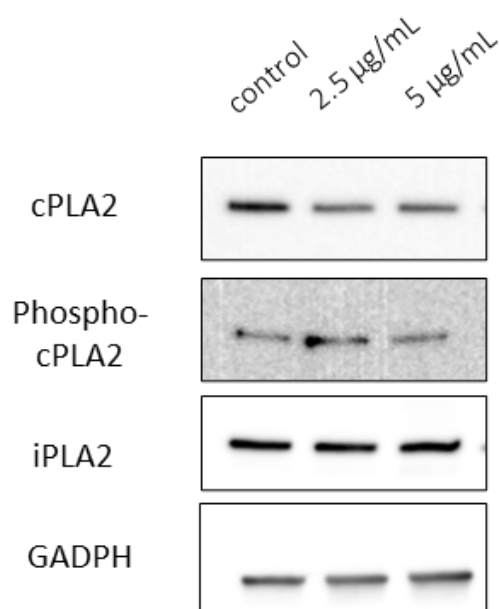


Figure 51. Western blot of calcium-dependent cytoplasmic cPLA2, phospho-PLA2 and calcium independent iPLA2 in control alveolar macrophages and macrophages dosed with 2.5 and 5 µg/mL of amiodarone. GADPH was used a control protein.

used to evaluate changes in enzyme concentration relative to drug concentration. cPLA2 and activated phospho-cPLA2 are evidently decreased in the 5 µg/mL amiodarone-treated alveolar macrophages. This is expected due to the effect of amiodarone in the intracellular Ca^{2+} . However, the levels of calcium-independent iPLA2 seems to be similar for both the control and the 2.5 µg/mL dosed cells, with a subtle increase in the 5 µg/mL dosed cells. iPLA2 could be activated in the amiodarone-dosed cells to release arachidonic acid, while usually it is selectively released by cPLA2²⁵³. However, due to the Ca^{2+} deficiency, the cells could potentially use iPLA2 for the supply of arachidonic acid and its subsequent metabolism towards 5-oxoETE for the resolution of inflammatory events, and the recovery of Ca^{2+} homeostasis.

5.4.1. BMP lipids in phospholipidosis and their differentiation from their PG counterparts

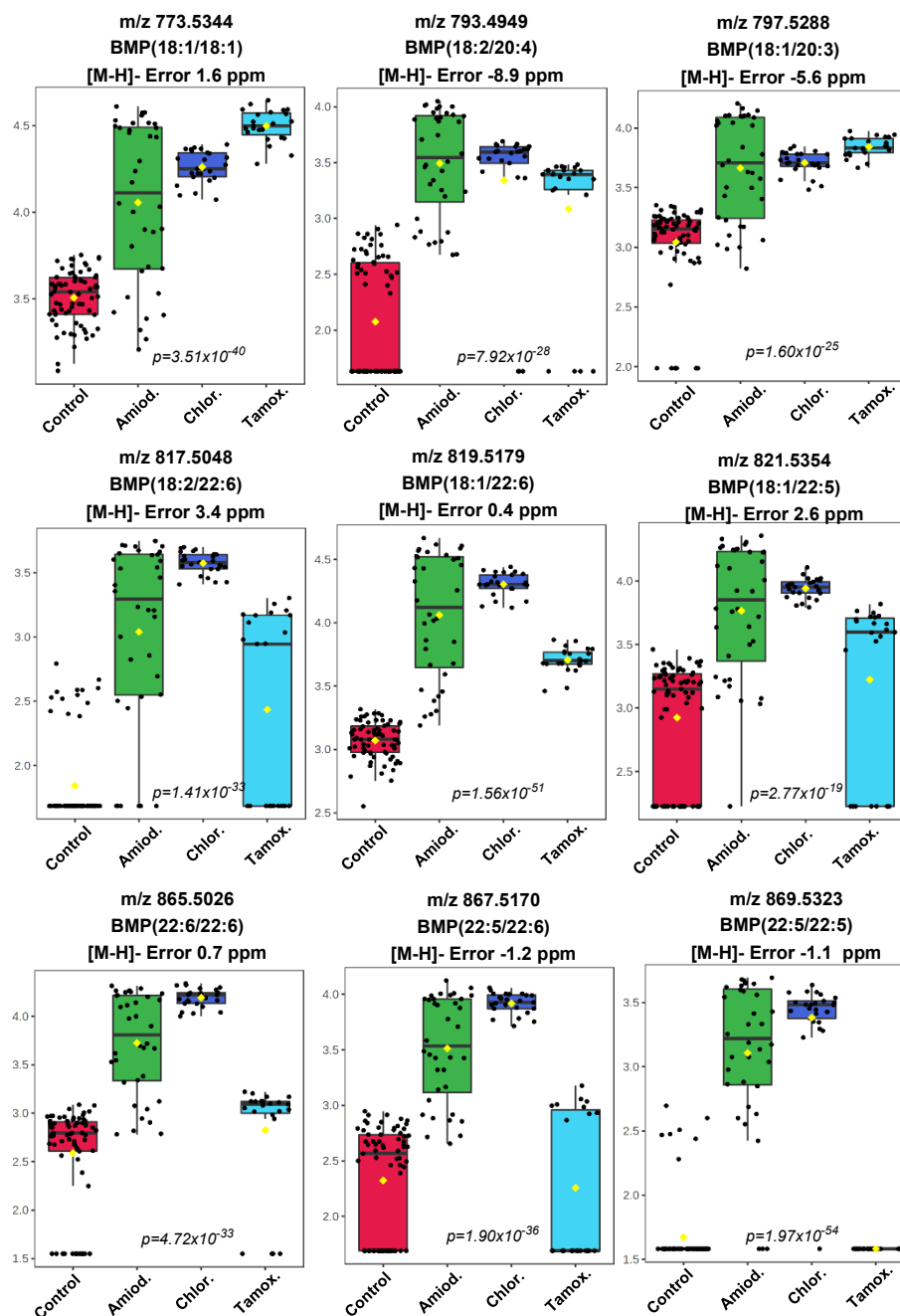


Figure 52. BMP lipids detected with LD-REIMS in amiodarone, chlorcyclizine, and tamoxifen alveolar macrophages. In the control cells these lipids are either not detected or detected in much lower abundance than the phospholipidosis cells.

As mentioned in section 5.1., previous studies have reported BMP(22:6/22:6) as a potential biomarker of phospholipidosis. In this section the detection of BMP lipids in cells using the high-throughput LD-REIMS platform is demonstrated, as well as the detection of their presence in *in vitro* and *ex vivo* drug-induced phospholipidosis models. The identification and separation from their PG counterparts were performed using LC-MS/MS in positive ion mode and manual interpretation of their fragmentation patterns, based on the characteristic fragment ions PGs and BMPs produce in positive ion mode.

BMP	[M-H] ⁻	[M+H] ⁺	[M+Na] ⁺	BMP Characteristic fragments in positive ion mode
BMP(16:1/18:2)	743.486	745.501	767.483	313.274 and 337.274
BMP(18:1/18:1)	773.533	775.549	797.531	339.290
BMP(16:1/22:6)	791.486	793.501	815.483	313.274 and 385.274
BMP(18:2/22:6)	817.502	819.517	841.499	337.274 and 385.274
BMP(18:1/22:6)	819.518	821.533	843.515	339.290 and 385.274
BMP(20:4/22:6)	841.502	843.517	865.499	361.274 and 385.274
BMP(20:3/22:6)	843.518	845.533	867.515	363.29 and 385.274
BMP(22:6/22:6)	865.502	867.517	889.499	385.274
BMP(22:5/22:6)	867.518	869.533	891.515	385.274 and 387.290

Table 12. BMP lipids identified with LC-MS/MS in negative and positive ion mode, and the BMP lipid class characteristic fragment ions in positive ion mode.

LD-REIMS data of the alveolar macrophages revealed *m/z* values – matching BMP species or their PG isomers – that were either not present in the control alveolar macrophages (Figure 52), or present in very low abundance when compared to the phospholipidosis-induced cells. These *m/z* values were confirmed as BMP lipids in the CAD induced phospholipidosis cells with LC-MS/MS in positive ion mode, where the two isomers can be distinguished based on their fragmentation patterns. As well as the expected BMP(22:6/22:6), a family of BMP lipids of different polyunsaturated fatty acyl chains – mainly 20:4 or 22:6, but also 16:1, 22:5, 18:2, and 18:1 chains – were

identified, which corroborates with fatty acyl compositions mainly found in BMP lipids²⁵⁴. The identified BMP species found predominantly in the phospholipidosis induced cells and their characteristic fragmentation peaks are summarised in Table 1. Example MS/MS spectra of PG(18:1/18:1) and BMP(18:1/18:1) as $[M+Na]^+$ adducts demonstrate the different fragmentation patterns of the two isomers in Figure 53.

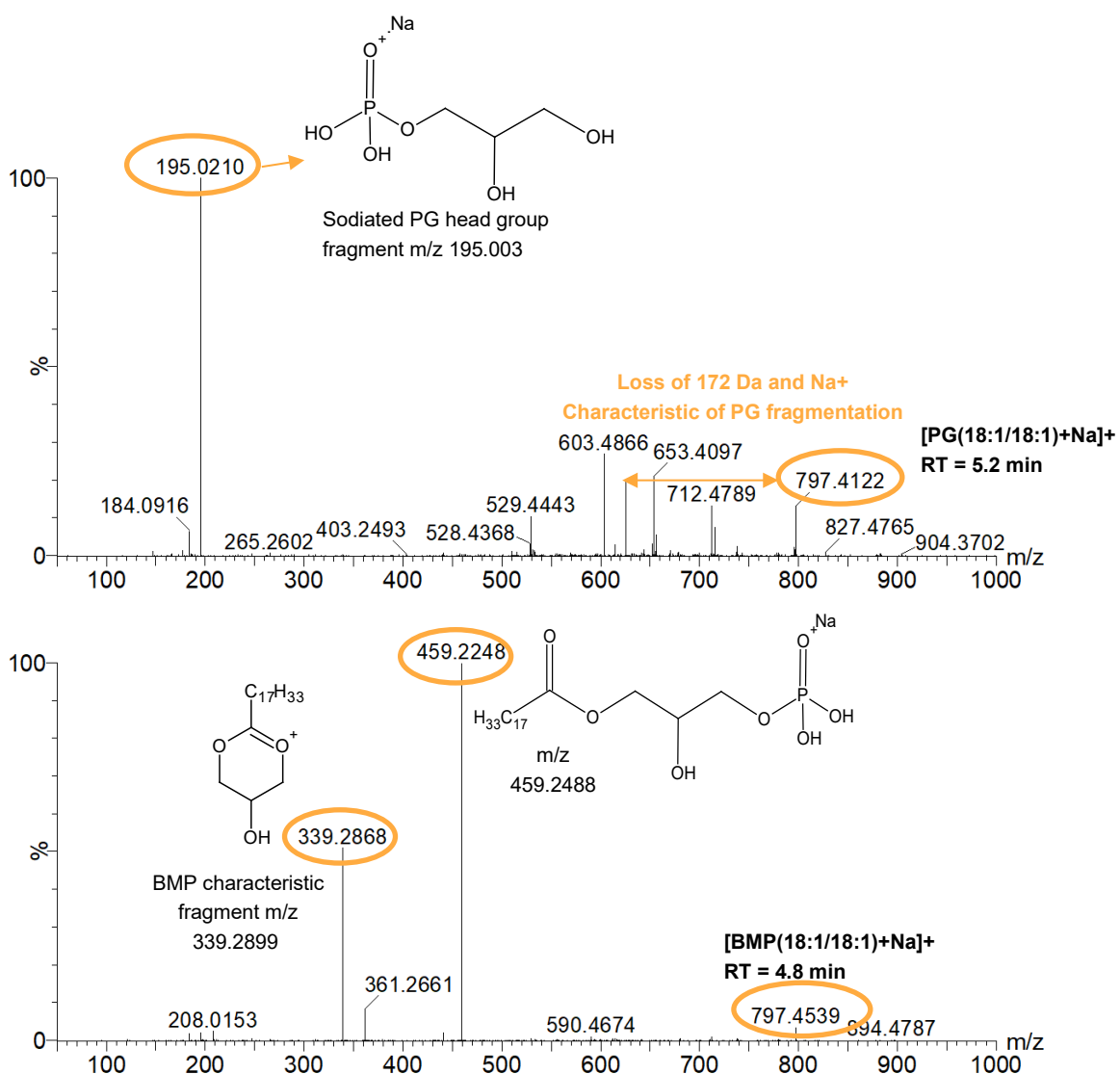


Figure 53. Representative positive ion mode LC-MS/MS spectra of BMP(18:1/18:1) and PG(18:1/18:1).

The two different molecules have a difference of 0.4 minutes in retention time, with the BMP lipid eluting first in reversed phase LC. Each molecule can be differentiated by its fragmentation pattern, characteristic of its class.

In addition to the BMP species detected in the phospholipidosis induced cells with LD-REIMS, glycerophosphoglycerol (GPG) was also detected, following the same trend as the BMP lipids – i.e., high abundance in the CAD treated alveolar macrophages, and very low abundance in the control cells. GPG is a main precursor in the phospholipid biosynthesis²⁵⁵; as shown in Figure 54, its structure – two glycerol groups attached to each side of a phosphate moiety – resembles the core structure of the BMP and PG lipids, meaning that it can play a role in metabolism of these lipids.

BMP lipids are critical for the mobilisation of cholesterol and its transfer from the inner endosomal and lysosomal membranes to the cytoplasm, where its presence is in turn crucial for the activity of hydrolases and the subsequent degradation of the inner membrane lipids. They are also responsible for the negative charging of inner lysosomal membrane and the binding of enzymes. Therefore, dysregulation of the BMP homeostasis would cause complications in the transportation of cholesterol from the lysosomes to the cytoplasm, and consequently in the degradation of the membrane lipids²⁴⁴.

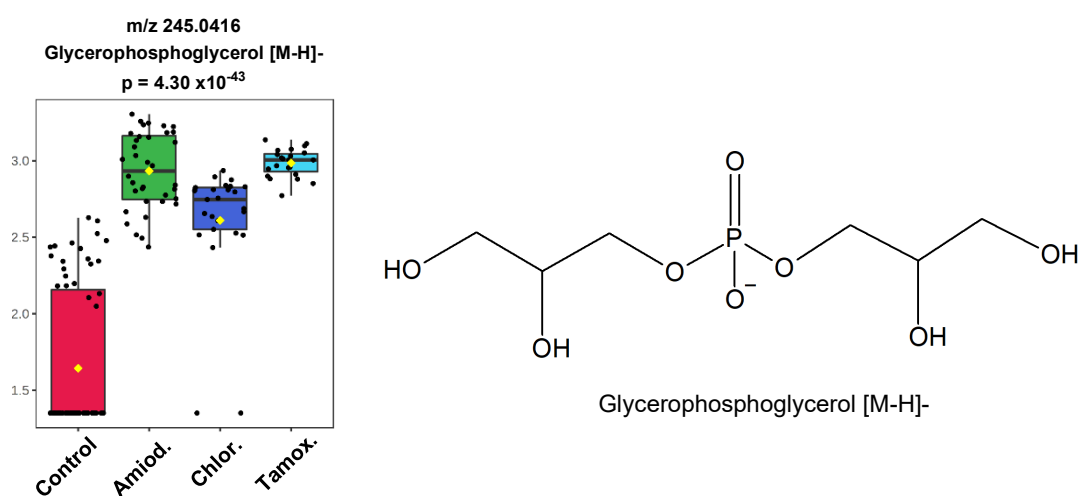
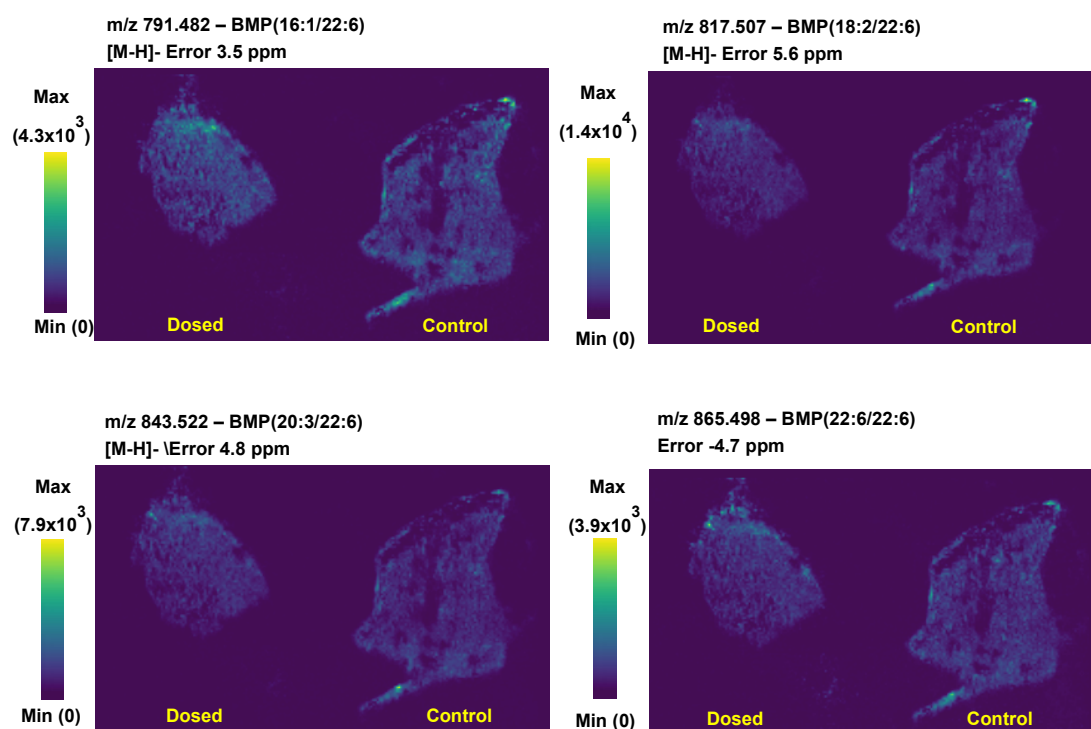


Figure 54. Glycerophosphoglycerol (GPG) detected in the phospholipidosis induced cells, potentially originating from the biosynthesis or catabolism of BMPs and/or PG lipids.

DESI MSI



LD-REIMS Imaging

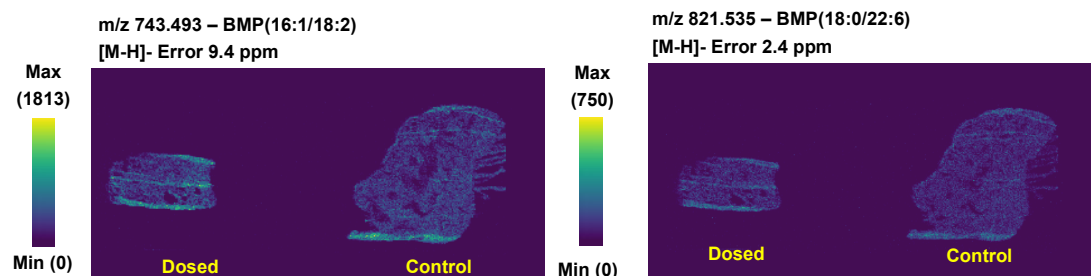


Figure 55. Rat lung tissue treated with amiodarone does not seem to exhibit upregulation in BMP lipids when compared to the control lung tissue, when analysed with DESI MSI and LD-REIMS imaging.

It is interesting that BMP lipids can be detected in the amiodarone treated lung tissue, but without a noticeable increase when compared to the control lung tissue (Figure 55). Differences between the *in vitro* and *in/ex vivo* models are expected, due to the complexity of the lungs and the presence of pulmonary surfactant and other types of cells, including Type II cells (spherical pneumocytes) – the lack of lung surfactant in the *in vitro* models can alter the function of the alveolar macrophages, since one main function of pulmonary cells is the degradation of the pulmonary surfactant and its

components (including cholesterol), which the Type II cells synthesise and secrete in the alveoli^{256,257}.

5.4.2. Sphingomyelin accumulation in the CAD treated macrophages

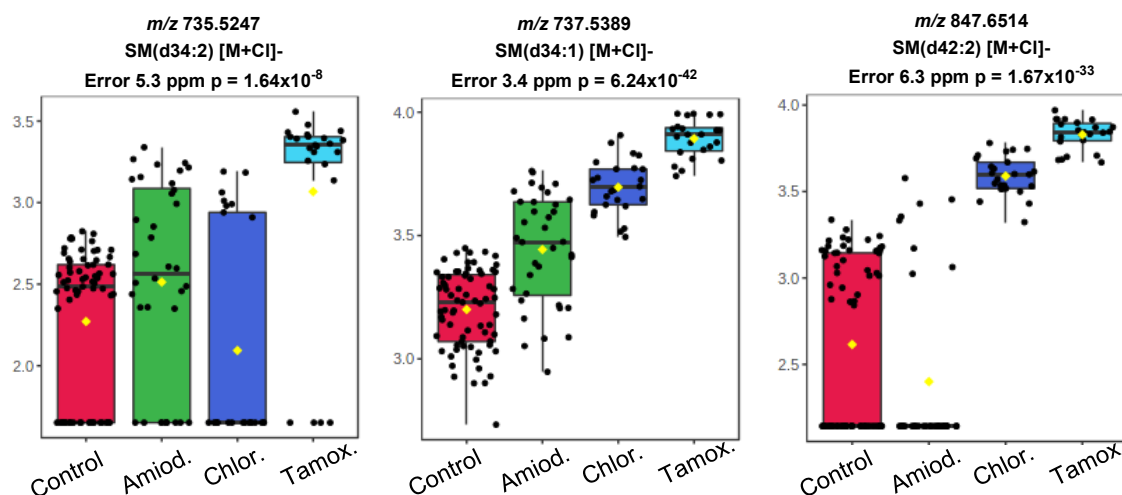


Figure 56. Sphingomyelins (SM) detected as chlorinated adducts in the alveolar macrophages treated with CADs and untreated (control).

Detection of sphingomyelins with increased intensities in the CAD treated alveolar macrophages (mainly chlorcyclizine and tamoxifen), as shown in Figure 56, provides more evidence of drug induced phospholipidosis and lysosomal disorder caused by these drugs. As mentioned in section 5.1 CADs can accumulate in the lysosomes since the acidic environment protonates the amine group of the CADs, thus not being able to diffuse through the lysosomal membrane and exit the lysosome. The accumulation of CADs in the lysosomes results in the reduction of the negative surface potential created by anionic phospholipids (such as BMPs), resulting in the release of catabolic hydrolases, including the lysosomal acid sphingomyelinase. The release of the acid sphingomyelinase from the lysosomal surface leads to the reduction of the catabolism of sphingomyelins and thus the accumulation of these lipids in the lysosomes²⁵⁸.

5.4.3. Presence of PG ether lipids in the lung tissue

As described in Chapter 1, ether lipids are a special class of phospholipids which have an ether bond at the sn-1 position of the glycerol group to link the glycerol moiety to a hydrocarbon chain, while the sn-2 position is occupied by a fatty acyl chain, similar to other phospholipids²⁵⁹.

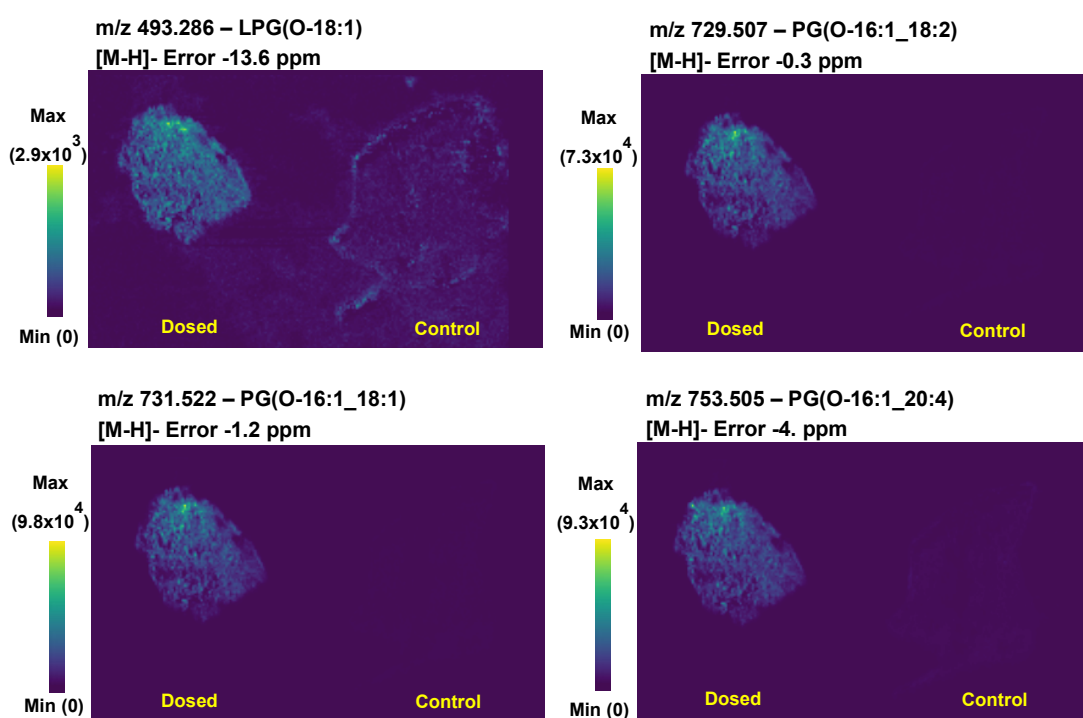


Figure 57. PG ether lipids detected in the amiodarone treated rat lungs with DESI MSI. Fatty alkyl and fatty acyl chain length confirmed with LC-MS/MS (Table 13).

m/z values matching the theoretical mass of PG ether lipids as deprotonated ions were observed in the amiodarone treated rat lungs, and LC-MS/MS spectra confirmed their identity and their fatty alkyl and acyl chain composition. Figures 57 and 58 demonstrate single ion images of these molecules in DESI MSI and LD-REIMS imaging respectively of the amiodarone treated and control lung tissue. In most of the cases, these species are not present in the control lung tissue (see extracted chromatogram of m/z 731.522 in the control amiodarone treated lung tissue in Figure 59), except for trace levels of LPG(O-18:1) detected in DESI MSI (Figure 57), and PG(O-18:2_20:4) detected in the LD-REIMS imaging (Figure 58).

Interestingly, these species were not observed in the phospholipidosis induced macrophages, which potentially means that they are either not coming from these cells or that there are more complex mechanisms taking place in the lungs that induce their production.

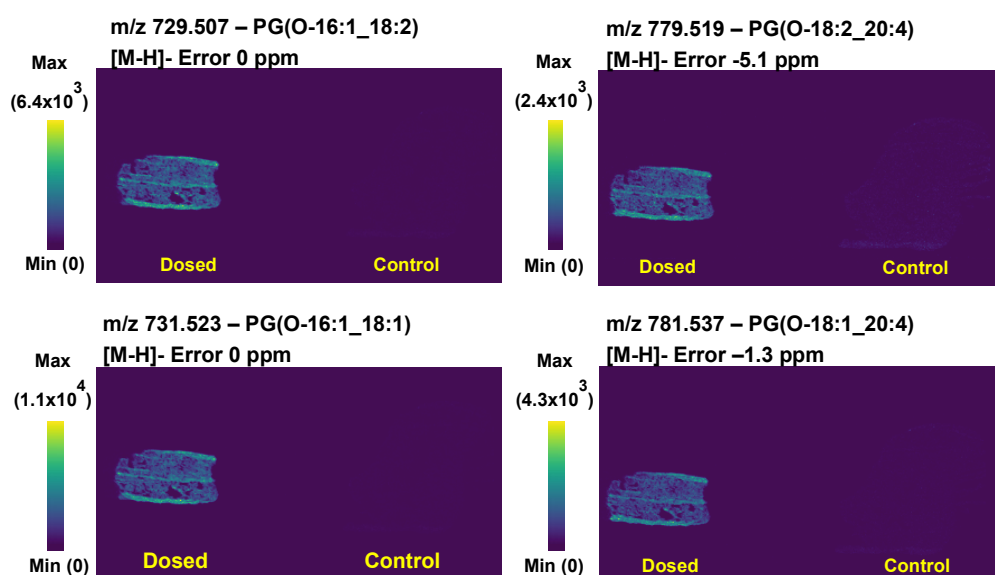


Figure 58. PG ether lipids detected in the amiodarone treated rat lungs with LD-REIMS -imaging. Fatty alkyl and fatty acyl chain length was confirmed with LC-MS/MS (Table 2).

MS/MS analysis confirmed the identity of these lipids and their fatty alkyl and fatty acyl chain composition as demonstrated in Figures 60 and 61 and Table 13. It is interesting that in all the species detected the fatty acyl composition consists of polyunsaturated fatty acyl chains, which correlates well with the PUFA increase in the amiodarone dosed lung tissue described in section 5.3.1., while the fatty alkyl chain composition is, in all the cases, either 16:1 or 18:1. The existence of a double bond in the fatty alkyl chain could potentially mean that the ether bond of these lipids which connects the hydrocarbon chain to the sn-1 position of the glycerol moiety is a vinyl ether bond, making these lipids plasmalogens – a subcategory of ether lipids – however, this has not been confirmed with MS/MS.

PG ether lipids have not been reported as components of mammalian cells or tissues (PE and PC ether lipids are far more common and contribute significantly to the lipidome of mammalian cells), however, they have been reported in clostridia, where their structure was determined with the analysis of their acid hydrolysis products^{259–}

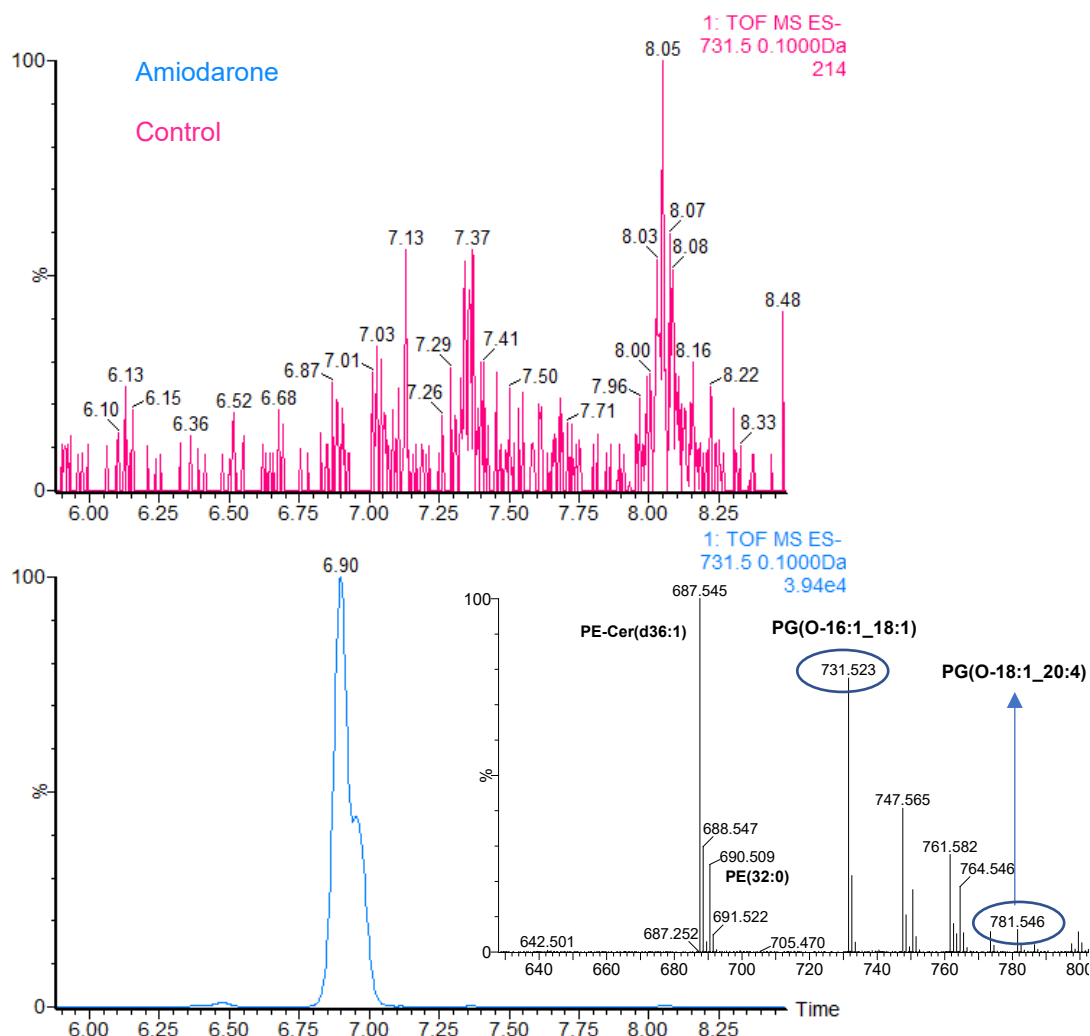


Figure 59. Extracted chromatogram of m/z 731.5 in amiodarone treated rat lungs and control lung tissue. The high abundance of the lipid in amiodarone treated tissue, not present in the control.

²⁶¹. Therefore, their presence in lung tissue cannot be readily explained by lipid pathways that already exist. A possible source for their presence in lung tissue could be pulmonary surfactant, in which PG lipids are the second most abundant class of lipids²⁶². PG lipids are necessary in lung surfactants because of their acidic character and their negative charge – which helps them interact with the positively charged proteins SP-B and SP-C also present there. In addition, the acidic character of PGs in combination with dipalmitoylphosphatidylcholine (DPPC) – which is also in high concentration in lung surfactant – has enhanced adsorption activity in the lung surfactant compared to other lipid mixtures, e.g., PC lipids²⁶².

PG ether lipid	[M-H]-	Fragment ions
PG(O-16:1_16:1)	703.492	467.277* and 253.218**
PG(O-16:1_18:3)	727.491	467.277* and 277.218**
PG(O-16:1_18:2)	729.507	467.277* and 279.232**
PG(O-16:1_18:1)	731.522	467.277* and 281.249**
PG(O-16:1_20:4)	753.505	467.277* and 303.232**
PG(O-18:1_18:2)	757.535	495.308* and 279.232**
PG(O-18:1/18:1)	759.545	495.308* and 281.249**
PG(O-18:1_20:4)	781.530	495.308* and 303.232**
*m/z after the neutral loss of the fatty acyl chain (LPG ether backbone) **fatty acyl chain fragment		

Table 13. Fatty alkyl and fatty acyl chain length of PG ether lipids, confirmed with LC-MS/MS.

Unfortunately, due to the lack of chemical standards of these compounds it is not easy to confirm with absolute certainty their identity – however, accurate mass, MS/MS analysis, and their presence in the lungs – an organ that exhibits high abundance of PG lipids – imply that they could be PG ether lipids. Further investigation using more biological replicates is needed for the confirmation of these species and their origin in the amiodarone treated tissue.

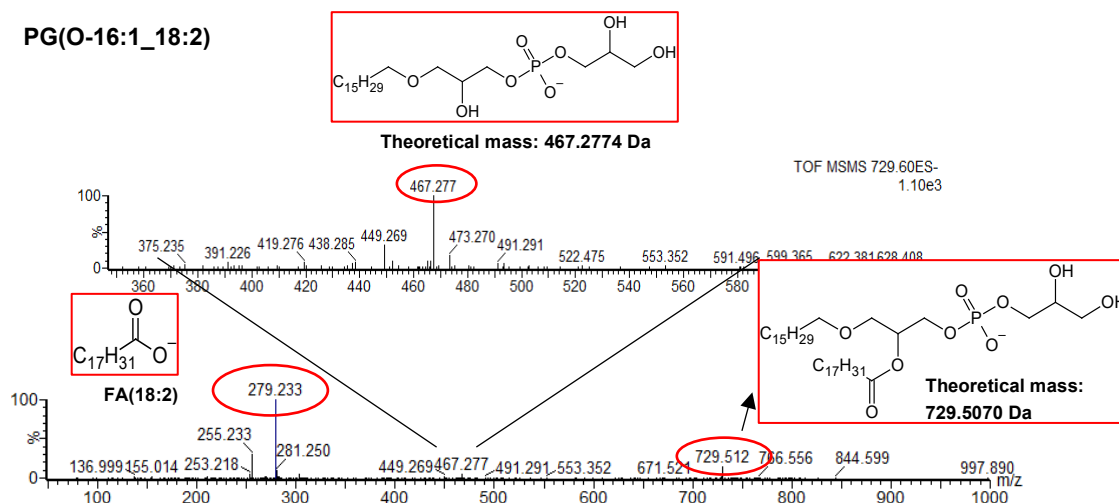


Figure 60. MS/MS spectrum of m/z 729.507, which produces the LPG ether backbone, m/z 467.277 matching the mass of LPG(O-16:1) with the loss of the fatty acyl chain from the sn-2 position of the glycerol moiety, and the fatty acyl anion at m/z 279.233 corresponding to the fatty acyl chain 18:2.

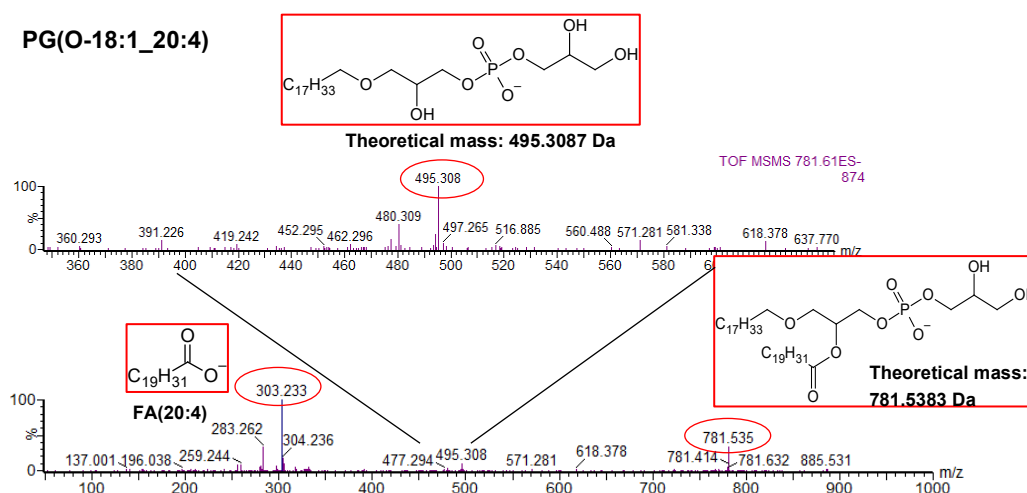


Figure 61. MS/MS spectrum of m/z 781.538, which produces the LPG ether backbone, m/z 495.308 matching the mass of LPG(O-18:1) with the loss of the fatty acyl chain from the sn-2 position of the glycerol moiety, and the fatty acyl anion at m/z 303.233 corresponding to the fatty acyl chain 20:4, i.e., the arachidonic acid.

5.5. Conclusion

As demonstrated in this chapter, high-throughput LD-REIMS analysis on cells and mass spectrometry imaging of tissue can easily detect key features of phospholipidosis and give insights in important biological functions taking place *in vitro* and *in vivo*. These observations, such as the increased concentration of PUFAs and the wide range of BMP lipids, were confirmed using three different cationic amphiphilic drugs – amiodarone, chlorcyclizine, and tamoxifen – dosed into rat alveolar macrophages and partially confirmed in the tissue analysis. Mass spectrometry imaging using DESI MSI and LD-REIMS imaging confirmed the observation of increased PUFA abundance in the phospholipidosis induced tissue, while PG ether lipids were detected almost exclusively in the CAD-treated rat lungs, which however were not detected in the *in vitro* phospholipidosis model. Additionally, the increase of sphingomyelins in the CAD treated alveolar macrophages indicate lysosomal disorder – probably involving lysosomal acid sphingomyelinase – and drug induced phospholipidosis. By comparison to electron microscopy, which is currently the gold standard for phospholipidosis detection²⁶³, high-throughput LD-REIMS analysis can give fast results with minimal sample preparation, which could facilitate the early detection of phospholipidosis inducing drug candidates and their elimination in the early stages of development, while ambient ionisation MSI techniques (DESI MSI and LD-REIMS imaging) can also be used for the detection of phospholipidosis in tissues.

Chapter 6

Conclusion and Future Work

Chapter 6: Conclusion and Future Work

6.1. Conclusion

In this project, the development, optimisation, and validation of an automated LD-REIMS-based platform was demonstrated, used for the metabolomic and lipidomic characterisation of cells with minimal or no sample preparation, able to measure the cells' metabolome and lipidome, in a high-throughput manner. LD-REIMS proved to be a suitable platform for the analysis of various types of cells without the requirement for co-crystallisation with a “matrix” compound or the use of conductive target plates – such as in the case of MALDI, the cryofixation of cells as in DESI^{134,135} or the lysis of the cells required for acoustic mist ionisation (AMI)^{136–138}.

Methodologies developed and optimised for the analysis of cells, either in a pelleted form or analysed as a monolayer of living or frozen cells, directly from the cell culture plate. A panel of 5 breast and 5 colorectal cancer cell lines was used for all method development and validation studies, as well as for the comparison study between LD-REIMS, and bipolar forceps and cell probe REIMS technologies. For the development, optimisation, and validation of the cell monolayer analysis using LD-REIMS the same panel of breast and colorectal cancer cell lines was used, while the method was successfully tested against an already proven biological hypothesis, showing an increase in polyunsaturated fatty acids (PUFAs) in PIK3CA mutant cells, as well as their decrease after treatment with the cPLA2 inhibitor. Finally, two application studies were conducted exploring the applicability of LD-REIMS for biopharmaceutical drug development, and for detection of drug-induced phospholipidosis in alveolar macrophages.

Comparison of LD-REIMS cell pellet analysis to bipolar forceps and cell probe REIMS modalities demonstrated the superiority of the automated LD-REIMS analysis, with very good repeatability between the spectra acquired for each cell line (correlation coefficient (r) = 0.86 and standard deviation (SD) = 0.108, as opposed to bipolar forceps REIMS where r = 0.77 and SD = 0.189). The LD-REIMS-based approaches outperformed the bipolar forceps and cell probe REIMS modalities in terms of classification, molecular coverage, and repeatability, generating mass

spectrometric data mainly featuring deprotonated intact lipids and metabolites, while the data was replicable with high correlation and low standard deviation.

LD-REIMS analysis of the cell monolayer was demonstrated on living or snap-frozen cells directly from the cell culture plate. Differences recorded between the mass spectra generated from cell pellets and the cell monolayer were analysed by LD-REIMS. The cell monolayer exhibited even better performance (in terms of classification and repeatability) than the cell pellet analysis, while it also featured more small metabolites from metabolic pathways in the cells – such as glucose, glutamate, and amino acids – that were not able to be detected previously. LD-REIMS analysis on the cell monolayer directly from the culture well plate could have a great potential for the analysis of adherent cells, and - since it enables the analysis of living cells – allows real-time measurements. Suspension cell lines still present a difficulty since the cells are not growing as a cell monolayer and be directly analysed, thus their analysis for the present time is performed in the pelleted form of these cells, which is still much faster and less laborious than the current “gold standard”, i.e., LC-MS.

As mentioned above, two application studies were performed to assess the applicability of the method, addressing different cell-related challenges. The first study comprised of the use of LD-REIMS in the classification of genetically modified CHO cells producing biopharmaceuticals – in the present study, the therapeutic monoclonal antibody adalimumab – as high- or low-producing cell lines. The method differentiated successfully high and low producers, revealing significant biological differences between the two groups. Specifically, low producing cell lines were found to be richer in glycerophospholipids containing polyunsaturated fatty acyl chains, which could make their cell membrane more prone to peroxidation by radical oxygen species. In addition, low producers were found to have higher abundance of small metabolites, mainly amino acids, potentially indicating that these cells cannot exploit the nutrients from the growth medium efficiently, which could ultimately disturb the production of the monoclonal antibody. The classification performance of high and low producing cell lines, as well as the biological information revealed in this study, proves the applicability of LD-REIMS to biopharmaceutical production. LD-REIMS could be adapted and used to distinguish low-producing cell lines in the early stages of their development, stopping the production and avoid spending time and resources on a cell line that, ultimately, would not be able to produce the desired

biopharmaceutical compound. In addition, the throughput capabilities of LD-REIMS allow the implementation of the system for the screening of thousands of cell lines and their fast classification as low or high producers, based on their metabolomic and lipidomic profile.

Another application in which LD-REIMS could be implemented is for the fast screening of drugs inducing phospholipidosis. As shown in Chapter 5, the method revealed significant changes in the lipid metabolism of alveolar macrophages treated with drugs known to cause phospholipidosis, i.e., amiodarone, chlorcyclizine, and tamoxifen, with significant upregulation of PUFAs, especially arachidonic acid, and the presence of a family of BMP lipids, with different combinations of polyunsaturated fatty acyl chains, indicating lysosomal disorder. Therefore, based on these observations, LD-REIMS could be used for the fast detection of phospholipidosis induced by drug candidates in *in vitro* models, replacing the time-consuming and laborious electron microscopy methods currently used.

6.2. Future Work

Further experiments for the standardisation of the method are necessary for the method's implementation in GLP environments. Validation of the system should be performed, including inter-laboratory reproducibility studies and implementation of quality control procedures, potentially including the use of standard compounds. Additionally, a comprehensive comparison to LC-MS methodologies, which is currently the gold standard for metabolomics and lipidomics, should be performed to demonstrate the accuracy of LD-REIMS and its molecular coverage. Moreover, different fragmentation techniques could be used to confirm the identifications of certain ions, e.g., the PG ether lipids and the eicosanoids.

Additional method development should be undertaken for the analysis of suspension cell lines in their native state, without the need for centrifugation or other sample preparation steps. Poly-L-lysine coated well plates could be used for the attachment of the suspension cells²⁶⁴ on the bottom of the well and their analysis as a cell monolayer, although this could disturb the cells' metabolism as their natural state is to grow without constraint in suspension in suspension. Substrates to hold the cells on a

3D state could be implemented to avoid this issue, however, here as well, their effect on cell metabolism should be carefully considered.

Further experiments on the genetically modified CHO cells are needed to confirm the hypotheses described in chapter 4, especially regarding the fluidity of the membrane in the high and low producing cell lines, as well as the membrane's vulnerability against ROS. Additionally, the applicability of LD-REIMS could be explored in other genetically modified systems and biopharmaceuticals, such as vaccines, organoids, bacteria, etc.

Finally, a more thorough investigation is needed to confirm the mechanism involved in drug induced phospholipidosis, including experiments such as western blot to investigate the activity of the lysosomal acid sphingomyelinase in the drug induced phospholipidosis models, as well as confirmation of the presence of PG ether lipids in the phospholipidosis induced animal tissue with standard compounds.

Bibliography

1. Nature Education. Contents of Essentials of Cell Biology | Learn Science at Scitable. *Scitable* (2014).
2. Bruce Alberts. *Molecular Biology of THE CELL, 6th ed. Journal of Chemical Information and Modeling* (2017).
3. Watson, H. Biological membranes. *Essays Biochem.* (2015)
doi:10.1042/BSE0590043.
4. Yang, M. & Quayle, J. M. Assessing the real-time metabolism and bioenergetics of single cells using fluorescence biosensors. in *Clinical Bioenergetics* (2021). doi:10.1016/b978-0-12-819621-2.00011-5.
5. Astashkina, A., Mann, B. & Grainger, D. W. A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity. *Pharmacology and Therapeutics* (2012)
doi:10.1016/j.pharmthera.2012.01.001.
6. Ward, S. L. ‘Omics’, Bioinformatics, Computational Biology. *AltTox.org* <http://alttox.org/mapp/emerging-technologies/omics-bioinformatics-computational-biology/> (2014).
7. Subramanian, I., Verma, S., Kumar, S., Jere, A. & Anamika, K. Multi-omics Data Integration, Interpretation, and Its Application. *Bioinformatics and Biology Insights* (2020) doi:10.1177/1177932219899051.
8. Horgan, R. P. & Kenny, L. C. ‘Omic’ technologies: genomics, transcriptomics, proteomics and metabolomics. *Obstet. Gynaecol.* (2011)
doi:10.1576/toag.13.3.189.27672.
9. Baghel, R., Maan, K., Haritwal, T. & Rana, P. Integration of epigenomics and metabolomics: From biomarkers discovery to personalized medicine. in *Epigenetics and Metabolomics* (2021). doi:10.1016/b978-0-323-85652-2.00002-6.
10. Idle, J. R. & Gonzalez, F. J. Metabolomics. *Cell Metabolism* (2007)
doi:10.1016/j.cmet.2007.10.005.

11. Madsen, R., Lundstedt, T. & Trygg, J. Chemometrics in metabolomics-A review in human disease diagnosis. *Anal. Chim. Acta* (2010) doi:10.1016/j.aca.2009.11.042.
12. Beger, R. A Review of Applications of Metabolomics in Cancer. *Metabolites* (2013) doi:10.3390/metabo3030552.
13. Xiao, J. F., Zhou, B. & Ransom, H. W. Metabolite identification and quantitation in LC-MS/MS-based metabolomics. *TrAC - Trends in Analytical Chemistry* (2012) doi:10.1016/j.trac.2011.08.009.
14. Emwas, A. H. M. The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. *Methods Mol. Biol.* (2015) doi:10.1007/978-1-4939-2377-9_13.
15. McGill, D. Development of novel untargeted SPE-NMR protocols for metabolite annotation. (Imperial College London, 2019).
16. Günther, H. NMR Spectroscopy, Second Edition. Basic Principles, Concepts and Applications in Chemistry. *Wiley Sons* (1995).
17. Reo, N. V. NMR-BASED METABOLOMICS. *Drug Chem. Toxicol.* **25**, 375–382 (2002).
18. Mal, T. K., Tian, Y. & Patterson, A. D. Sample preparation and data analysis for NMR-based metabolomics. in *Methods in Molecular Biology* (2021). doi:10.1007/978-1-0716-0849-4_16.
19. Analytical Sensitivity. *Segen's Medical Dictionary* <https://medical-dictionary.thefreedictionary.com/analytical+sensitivity> (2011).
20. Tsedilin, A. M. *et al.* How Sensitive and Accurate are Routine NMR and MS Measurements? *Mendeleev Commun.* (2015) doi:10.1016/j.mencom.2015.11.019.
21. Aalberg, L., Clark, C. R. & DeRuiter, J. Chromatographic and mass spectral studies on isobaric and isomeric substances related to 3,4-methylenedioxymethamphetamine. *J. Chromatogr. Sci.* (2004) doi:10.1093/chromsci/42.9.464.

22. Kostecki, P., Morrison, R. & Dragun, J. HYDROCARBONS. in *Encyclopedia of Soils in the Environment* (ed. Hillel, D.) 217–226 (Elsevier, 2005). doi:<https://doi.org/10.1016/B0-12-348530-4/00098-9>.
23. Büyükköroğlu, G., Dora, D. D., Özdemir, F. & Hızıl, C. Chapter 15 - Techniques for Protein Analysis. in *Omics Technologies and Bio-Engineering* (eds. Barh, D. & Azevedo, V.) 317–351 (Academic Press, 2018). doi:<https://doi.org/10.1016/B978-0-12-804659-3.00015-4>.
24. Mellon, F. A. MASS SPECTROMETRY | Principles and Instrumentation. in *Encyclopedia of Food Sciences and Nutrition (Second Edition)* (ed. Caballero, B.) 3739–3749 (Academic Press, 2003). doi:<https://doi.org/10.1016/B0-12-227055-X/00746-X>.
25. Liigand, P. *et al.* Think Negative: Finding the Best Electrospray Ionization/MS Mode for Your Analyte. *Anal. Chem.* (2017) doi:10.1021/acs.analchem.7b00096.
26. Volmer, D. A. & Jessome, L. L. Ion Suppression: A Major Concern in Mass Spectrometry. *LCGC North Am.* (2006).
27. Mei, H. *et al.* Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: Application to drug discovery. *Rapid Commun. Mass Spectrom.* (2003) doi:10.1002/rcm.876.
28. Furey, A., Moriarty, M., Bane, V., Kinsella, B. & Lehane, M. Ion suppression; a critical review on causes, evaluation, prevention and applications. *Talanta* **115**, 104–122 (2013).
29. German, J. B., Gillies, L. A., Smilowitz, J. T., Zivkovic, A. M. & Watkins, S. M. Lipidomics and lipid profiling in metabolomics. *Current Opinion in Lipidology* (2007) doi:10.1097/MOL.0b013e328012d911.
30. Rangholia, N., Leisner, T. M. & Holly, S. P. Bioactive ether lipids: Primordial modulators of cellular signaling. *Metabolites* **11**, 1–22 (2021).
31. Han, X. & Gross, R. W. Shotgun lipidomics: Electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from

- crude extracts of biological samples. *Mass Spectrom. Rev.* **24**, 367–412 (2005).
32. Van Meer, G. Cellular lipidomics. *EMBO J.* (2005) doi:10.1038/sj.emboj.7600798.
 33. Züllig, T., Trötz Müller, M. & Köfeler, H. C. Lipidomics from sample preparation to data analysis: a primer. *Analytical and Bioanalytical Chemistry* (2020) doi:10.1007/s00216-019-02241-y.
 34. Goto-Inoue, N., Hayasaka, T., Zaima, N. & Setou, M. Imaging mass spectrometry for lipidomics. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* (2011) doi:10.1016/j.bbalip.2011.03.004.
 35. Li, J., Vosegaard, T. & Guo, Z. Applications of nuclear magnetic resonance in lipid analyses: An emerging powerful tool for lipidomics studies. *Progress in Lipid Research* (2017) doi:10.1016/j.plipres.2017.09.003.
 36. Murphy, R. C. & Gaskell, S. J. New applications of mass spectrometry in lipid analysis. *Journal of Biological Chemistry* (2011) doi:10.1074/jbc.R111.233478.
 37. Rustan, A. C. & Drevon, C. A. Fatty Acids: Structures and Properties. in *Encyclopedia of Life Sciences* (2005). doi:10.1038/npg.els.0003894.
 38. Schönfeld, P. & Wojtczak, L. Short- and medium-chain fatty acids in energy metabolism: The cellular perspective. *Journal of Lipid Research* (2016) doi:10.1194/jlr.R067629.
 39. Labatut, R. A. & Pronto, J. L. Chapter 4 - Sustainable Waste-to-Energy Technologies: Anaerobic Digestion. in *Sustainable Food Waste-To-energy Systems* (eds. Trabold, T. A. & Babbitt, C. W.) 47–67 (Academic Press, 2018). doi:https://doi.org/10.1016/B978-0-12-811157-4.00004-8.
 40. Kihara, A. Very long-chain fatty acids: Elongation, physiology and related disorders. *Journal of Biochemistry* (2012) doi:10.1093/jb/mvs105.
 41. Buckley, M. T. *et al.* Selection in Europeans on fatty acid desaturases associated with dietary changes. *Mol. Biol. Evol.* (2017) doi:10.1093/molbev/msx103.

42. Saini, R. K. & Keum, Y. S. Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance — A review. *Life Sciences* (2018) doi:10.1016/j.lfs.2018.04.049.
43. Innes, J. K. & Calder, P. C. Omega-6 fatty acids and inflammation. *Prostaglandins Leukotrienes and Essential Fatty Acids* (2018) doi:10.1016/j.plefa.2018.03.004.
44. Chavan-Gautam, P., Rani, A. & Freeman, D. J. Chapter Six - Distribution of Fatty Acids and Lipids During Pregnancy. in (ed. Makowski, G. S.) vol. 84 209–239 (Elsevier, 2018).
45. Howe, G. A. & Schilmiller, A. L. Oxylin metabolism in response to stress. *Current Opinion in Plant Biology* (2002) doi:10.1016/S1369-5266(02)00250-9.
46. Mosblech, A., Feussner, I. & Heilmann, I. Oxylin: Structurally diverse metabolites from fatty acid oxidation. *Plant Physiology and Biochemistry* (2009) doi:10.1016/j.plaphy.2008.12.011.
47. Chapman, K. D. Emerging physiological roles for N-acylphosphatidylethanolamine metabolism in plants: Signal transduction and membrane protection. *Chemistry and Physics of Lipids* (2000) doi:10.1016/S0009-3084(00)00198-5.
48. Coras, R. *et al.* Pro- and anti-inflammatory eicosanoids in psoriatic arthritis. *Metabolomics* (2019) doi:10.1007/s11306-019-1527-0.
49. Vance, J. E. Phosphatidylserine and phosphatidylethanolamine in mammalian cells: Two metabolically related aminophospholipids. *Journal of Lipid Research* (2008) doi:10.1194/jlr.R700020-JLR200.
50. Leventis, P. A. & Grinstein, S. The distribution and function of phosphatidylserine in cellular membranes. *Annual Review of Biophysics* (2010) doi:10.1146/annurev.biophys.093008.131234.
51. Athenstaedt, K. & Daum, G. Phosphatidic acid, a key intermediate in lipid metabolism. *European Journal of Biochemistry* (1999) doi:10.1046/j.1432-1327.1999.00822.x.

52. English, D. Phosphatidic acid: A lipid messenger involved in intracellular and extracellular signalling. *Cellular Signalling* (1996) doi:10.1016/0898-6568(95)00076-3.
53. Kooijman, E. E. & Burger, K. N. J. Biophysics and function of phosphatidic acid: A molecular perspective. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* (2009) doi:10.1016/j.bbalip.2009.04.001.
54. van der Veen, J. N. *et al.* The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. *Biochimica et Biophysica Acta - Biomembranes* (2017) doi:10.1016/j.bbamem.2017.04.006.
55. Cui, Z. & Houweling, M. Phosphatidylcholine and cell death. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* (2002) doi:10.1016/S1388-1981(02)00328-1.
56. Marrink, S. J. & Mark, A. E. Molecular view of hexagonal phase formation in phospholipid membranes. *Biophys. J.* (2004) doi:10.1529/biophysj.104.048710.
57. Patel, D. & Witt, S. N. Ethanolamine and Phosphatidylethanolamine: Partners in Health and Disease. *Oxidative Medicine and Cellular Longevity* (2017) doi:10.1155/2017/4829180.
58. Uphoff, A., Hermansson, M., Haimi, P. & Somerharju, P. Analysis of complex lipidomes. in *Medical Applications of Mass Spectrometry* (2008). doi:10.1016/B978-044451980-1.50013-6.
59. Alcorn, J. L. Pulmonary Surfactant Trafficking and Homeostasis. in *Lung Epithelial Biology in the Pathogenesis of Pulmonary Disease* (2017). doi:10.1016/B978-0-12-803809-3.00004-X.
60. Furse, S. Is phosphatidylglycerol essential for terrestrial life? *Journal of Chemical Biology* (2017) doi:10.1007/s12154-016-0159-3.
61. Dean, J. M. & Lodhi, I. J. Structural and functional roles of ether lipids. *Protein and Cell* (2018) doi:10.1007/s13238-017-0423-5.
62. IUPAC-IUB, C. on B. N. The nomenclature of lipids. *Chemistry and Physics of Lipids* (1978) doi:10.1016/0009-3084(78)90063-4.

63. Miura, Y. & Fulco, A. J. ω -1, ω -2 and ω -3 Hydroxylation of long-chain fatty acids, amides and alcohols by a soluble enzyme system from *Bacillus megaterium*. *Biochim. Biophys. Acta (BBA)/Lipids Lipid Metab.* (1975) doi:10.1016/0005-2760(75)90089-2.
64. Jeck, V., Korf, A., Vosse, C. & Hayen, H. Localization of double-bond positions in lipids by tandem mass spectrometry succeeding high-performance liquid chromatography with post-column derivatization. *Rapid Commun. Mass Spectrom.* (2019) doi:10.1002/rcm.8262.
65. Postle, A. D. LIPIDS | Composition and Role of Phospholipids. in *Encyclopedia of Human Nutrition (Second Edition)* (ed. Caballero, B.) 132–142 (Elsevier, 2005). doi:https://doi.org/10.1016/B0-12-226694-3/00193-9.
66. Murphy, R. C. *Tandem Mass Spectrometry of Lipids: molecular analysis of complex lipids. Tandem mass spectrometry of lipids* (2014).
67. Liebisch, G. *et al.* Update on LIPID MAPS classification, nomenclature, and shorthand notation for MS-derived lipid structures. *J. Lipid Res.* (2020) doi:10.1194/jlr.S120001025.
68. Bélanger, J. M. R., Jocelyn Paré, J. R. & Sigouin, M. Chapter 2 High performance liquid chromatography (HPLC): Principles and applications. *Tech. Instrum. Anal. Chem.* (1997) doi:10.1016/S0167-9244(97)80011-X.
69. Chromatography. *Chemistry LibreTexts* 55254 <https://chem.libretexts.org/@go/page/55254> (2020).
70. Liquid Chromatography. *Chemistry LibreTexts* 309 <https://chem.libretexts.org/@go/page/309> (2020).
71. Zhang, T., Creek, D. J., Barrett, M. P., Blackburn, G. & Watson, D. G. Evaluation of coupling reversed phase, aqueous normal phase, and hydrophilic interaction liquid chromatography with orbitrap mass spectrometry for metabolomic studies of human urine. *Anal. Chem.* (2012) doi:10.1021/ac2030738.
72. Lindahl, A., Sääf, S., Lehtiö, J. & Nordström, A. Tuning Metabolome Coverage in Reversed Phase LC-MS Metabolomics of MeOH Extracted

- Samples Using the Reconstitution Solvent Composition. *Anal. Chem.* (2017) doi:10.1021/acs.analchem.7b00475.
73. Theodoridis, G. A., Gika, H. G., Plumb, R. & Wilson, I. D. Chapter 9 - Liquid Chromatographic Methods Combined with Mass Spectrometry in Metabolomics. in *Proteomic and Metabolomic Approaches to Biomarker Discovery* (eds. Issaq, H. J. & Veenstra, T. D.) 145–161 (Academic Press, 2013). doi:<https://doi.org/10.1016/B978-0-12-394446-7.00009-1>.
 74. Watson, T. J. & Sparkman, D. O. Introduction. in *Introduction to Mass Spectrometry* 1–52 (John Wiley & Sons, Ltd, 2007). doi:<https://doi.org/10.1002/9780470516898.ch11>.
 75. Hoffmann, E. De & Stroobant, V. *Mass Spectrometry Principles and Applications Third Edition. Mass spectrometry reviews* (2007).
 76. Lopez-Avila, V., Roach, P. J. & Urdahl, R. Chapter 14 - Environmental Applications of Soft Ionization with GC–TOFMS and GC–QTOFMS. in *Advanced Techniques in Gas Chromatography–Mass Spectrometry (GC–MS–MS and GC–TOF–MS) for Environmental Chemistry* (eds. Ferrer, I. & Thurman, E. M.) vol. 61 325–346 (Elsevier, 2013).
 77. Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. & Whitehouse, C. M. Electrospray ionization for mass spectrometry of large biomolecules. *Science* (1989) doi:10.1126/science.2675315.
 78. Yamashita, M. & Fenn, J. B. Electrospray ion source. Another variation on the free-jet theme. *J. Phys. Chem.* (1984) doi:10.1021/j150664a002.
 79. Konermann, L., Ahadi, E., Rodriguez, A. D. & Vahidi, S. Unraveling the mechanism of electrospray ionization. *Anal. Chem.* (2013) doi:10.1021/ac302789c.
 80. Wilm, M. Principles of electrospray ionization. *Molecular and Cellular Proteomics* (2011) doi:10.1074/mcp.M111.009407.
 81. Dewald, H. D. Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation and Applications (ed. Cole, Richard B.). *J. Chem. Educ.* (1999) doi:10.1021/ed076p33.1.

82. Kebarle, P. & Verkerk, U. H. Electrospray: From Ions in solution to Ions in the gas phase, what we know now. *Mass Spectrom. Rev.* (2009) doi:10.1002/mas.20247.
83. Constantopoulos, T. L., Jackson, G. S. & Enke, C. G. Challenges in achieving a fundamental model for ESI. *Anal. Chim. Acta* (2000) doi:10.1016/S0003-2670(99)00600-5.
84. Kebarle, P. & Verkerk, U. H. A Brief Overview of the Mechanisms Involved in Electrospray Mass Spectrometry. in *Reactive Intermediates: MS Investigations in Solution* (2010). doi:10.1002/9783527628728.ch1.
85. Kebarle, P. & Peschke, M. On the mechanisms by which the charged droplets produced by electrospray lead to gas phase ions. *Anal. Chim. Acta* (2000) doi:10.1016/S0003-2670(99)00598-X.
86. Kloepper, A., Quintana, J. B. & Reemtsma, T. Operational options to reduce matrix effects in liquid chromatography- electrospray ionisation-mass spectrometry analysis of aqueous environmental samples. *J. Chromatogr. A* (2005) doi:10.1016/j.chroma.2004.11.101.
87. Krueve, A., Kaupmees, K., Liigand, J. & Leito, I. Negative electrospray ionization via deprotonation: Predicting the ionization efficiency. *Anal. Chem.* (2014) doi:10.1021/ac404066v.
88. Chen, C., Gonzalez, F. J. & Idle, J. R. LC-MS-based metabolomics in drug metabolism. *Drug Metabolism Reviews* (2007) doi:10.1080/03602530701497804.
89. Chen, G. & Pramanik, B. N. Application of LC/MS to proteomics studies: current status and future prospects. *Drug Discovery Today* (2009) doi:10.1016/j.drudis.2009.02.007.
90. Roux, A., Lison, D., Junot, C. & Heilier, J. F. Applications of liquid chromatography coupled to mass spectrometry-based metabolomics in clinical chemistry and toxicology: A review. *Clinical Biochemistry* (2011) doi:10.1016/j.clinbiochem.2010.08.016.
91. Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. & Whitehouse, C. M.

- Electrospray ionization—principles and practice. *Mass Spectrom. Rev.* (1990) doi:10.1002/mas.1280090103.
92. Thomson, B. A. Atmospheric pressure ionization and liquid chromatography/mass spectrometry—together at last. *J Am Soc Mass Spectrom* **9**, 187–193 (1998).
 93. Vestal, M. L. Studies of ionization mechanisms involved in thermospray LC-MS. *Int. J. Mass Spectrom. Ion Phys.* (1983) doi:10.1016/0020-7381(83)80086-2.
 94. *The IUPAC Compendium of Chemical Terminology. The IUPAC Compendium of Chemical Terminology* (2019). doi:10.1351/goldbook.
 95. Mulvaney, R. L. 2 - Mass Spectrometry. in *Nitrogen Isotope Techniques* (eds. Knowles, R. & Blackburn, T. H.) 11–57 (Academic Press, 1993). doi:https://doi.org/10.1016/B978-0-08-092407-6.50007-9.
 96. Inczedy, J. & IUPAC. *Compendium of Analytical Nomenclature: Definitive Rules 1997*. (Blackwell Science, 1997).
 97. Murray, K. K. *et al.* Definitions of terms relating to mass spectrometry (IUPAC Recommendations 2013). *Pure Appl. Chem.* (2013) doi:10.1351/PAC-REC-06-04-06.
 98. Thurman, M. E. & Ferrer, I. Comprehensive Analytical Chemistry, Volume 61. in 471–490 (2013).
 99. Beynon, J. H. Recommendations for symbolism and nomenclature for mass spectroscopy: International union of pure and applied chemistry physical chemistry division commission on Molecular Structure and Spectroscopy. *Pure Appl. Chem.* **50**, 65–74 (1978).
 100. Want, E. J. LC-MS Untargeted Analysis. in *Metabolic Profiling: Methods and Protocols* (eds. Theodoridis, G. A., Gika, H. G. & Wilson, I. D.) 99–116 (Springer New York, 2018). doi:10.1007/978-1-4939-7643-0_7.
 101. Lin, L. *et al.* Types, principle, and characteristics of tandem high-resolution mass spectrometry and its applications. *RSC Advances* (2015) doi:10.1039/c5ra22856e.

102. Gillette, M. A. & Carr, S. A. Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. *Nature Methods* (2013) doi:10.1038/nmeth.2309.
103. Chicooree, N., Unwin, R. D. & Griffiths, J. R. The application of targeted mass spectrometry-based strategies to the detection and localization of post-translational modifications. *Mass Spectrometry Reviews* (2015) doi:10.1002/mas.21421.
104. Allen, D. R. & McWhinney, B. C. Quadrupole Time-of-Flight Mass Spectrometry: A Paradigm Shift in Toxicology Screening Applications. *Clin. Biochem. Rev.* (2019) doi:10.33176/AACB-19-00023.
105. Cotter, R. J. Time-of-flight mass spectrometry for the structural analysis of biological molecules. *Anal. Chem.* **64**, 1027A-1039A (1992).
106. Guilhaus, M. MASS SPECTROMETRY | Time-of-Flight. in *Encyclopedia of Analytical Science (Second Edition)* (eds. Worsfold, P., Townshend, A. & Poole, C.) 412–423 (Elsevier, 2005). doi:https://doi.org/10.1016/B0-12-369397-7/00347-2.
107. Guilhaus, M., Selby, D. & Mlynski, V. Orthogonal acceleration time-of-flight mass spectrometry. *Mass Spectrom. Rev.* (2000) doi:10.1002/(SICI)1098-2787(2000)19:2<65::AID-MAS1>3.0.CO;2-E.
108. Sheil, M. M. Critical moments in time: Reflections on the development of orthogonal acceleration time-of-flight mass spectrometry. *J. Am. Soc. Mass Spectrom.* (2012) doi:10.1007/s13361-012-0398-7.
109. Li, W., Zhang, J. & Tse, F. L. S. *Handbook of LC-MS bioanalysis: Best Practices, experimental protocols, and regulations. Handbook of LC-MS Bioanalysis: Best Practices, Experimental Protocols, and Regulations* (2013). doi:10.1002/9781118671276.
110. Rombouts, C., De Spiegeleer, M., Van Meulebroek, L., De Vos, W. H. & Vanhaecke, L. Validated comprehensive metabolomics and lipidomics analysis of colon tissue and cell lines. *Anal. Chim. Acta* (2019) doi:10.1016/j.aca.2019.03.020.

111. Yusufi, F. N. K. *et al.* Mammalian Systems Biotechnology Reveals Global Cellular Adaptations in a Recombinant CHO Cell Line. *Cell Syst.* **4**, 530-542.e6 (2017).
112. Yeo, H. C., Chen, S., Ho, Y. S. & Lee, D. Y. An LC–MS-based lipidomics pre-processing framework underpins rapid hypothesis generation towards CHO systems biotechnology. *Metabolomics* (2018) doi:10.1007/s11306-018-1394-0.
113. Sarvin, B. *et al.* Fast and sensitive flow-injection mass spectrometry metabolomics by analyzing sample-specific ion distributions. *Nat. Commun.* (2020) doi:10.1038/s41467-020-17026-6.
114. Peng, W. *et al.* LC-MS/MS metabolome analysis detects the changes in the lipid metabolic profiles of dMMR and pMMR cells. *Oncol. Rep.* (2018) doi:10.3892/or.2018.6510.
115. BLIGH, E. G. & DYER, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* (1959) doi:10.1139/o59-099.
116. Wenk, M. R. The emerging field of lipidomics. *Nat. Rev. Drug Discov.* **4**, 594–610 (2005).
117. Nanita, S. C. & Kaldon, L. G. Emerging flow injection mass spectrometry methods for high-throughput quantitative analysis. *Anal. Bioanal. Chem.* (2016) doi:10.1007/s00216-015-9193-1.
118. Dunn, W. B., Bailey, N. J. C. & Johnson, H. E. Measuring the metabolome: Current analytical technologies. *Analyst* (2005) doi:10.1039/b418288j.
119. Fuhrer, T., Heer, D., Begemann, B. & Zamboni, N. High-throughput, accurate mass metabolome profiling of cellular extracts by flow injection-time-of-flight mass spectrometry. *Anal. Chem.* (2011) doi:10.1021/ac201267k.
120. Khoo, S. H. G. & Al-Rubeai, M. Metabolomics as a complementary tool in cell culture. *Biotechnol. Appl. Biochem.* (2007) doi:10.1042/ba20060221.
121. Karas, M. & Krüger, R. Ion formation in MALDI: The cluster ionization mechanism. *Chemical Reviews* (2003) doi:10.1021/cr010376a.

122. Jaskolla, T. W. & Karas, M. Compelling evidence for lucky survivor and gas phase protonation: The unified MALDI analyte protonation mechanism. *J. Am. Soc. Mass Spectrom.* (2011) doi:10.1007/s13361-011-0093-0.
123. Neumann, E. K., Comi, T. J., Rubakhin, S. S. & Sweedler, J. V. Lipid Heterogeneity between Astrocytes and Neurons Revealed by Single-Cell MALDI-MS Combined with Immunocytochemical Classification. *Angew. Chemie - Int. Ed.* (2019) doi:10.1002/anie.201812892.
124. Miura, D., Fujimura, Y., Tachibana, H. & Wariishi, H. Highly sensitive matrix-assisted laser desorption ionization-mass spectrometry for high-throughput metabolic profiling. *Anal. Chem.* (2010) doi:10.1021/ac901083a.
125. Shrivas, K. & Setou, M. Chapter 4 - Imaging Mass Spectrometry: Sample Preparation, Instrumentation, and Applications. in *Advances in Imaging and Electron Physics* (ed. Hawkes, P. W.) vol. 171 145–193 (Elsevier, 2012).
126. Walker, A. V. Secondary Ion Mass Spectrometry. in *Encyclopedia of Spectroscopy and Spectrometry (Second Edition)* (ed. Lindon, J. C.) 2504–2510 (Academic Press, 2010). doi:https://doi.org/10.1016/B978-0-12-374413-5.00022-1.
127. West, A. Chapter 3 - Experimental Methods to Investigate Self-Assembly at Interfaces. in *Self-Assembly Processes at Interfaces* (ed. Ball, V.) vol. 21 131–241 (Elsevier, 2018).
128. Passarelli, M. K. *et al.* The 3D OrbiSIMS - Label-free metabolic imaging with subcellular lateral resolution and high mass-resolving power. *Nat. Methods* (2017) doi:10.1038/nmeth.4504.
129. Schaepe, K. *et al.* Assessment of different sample preparation routes for mass spectrometric monitoring and imaging of lipids in bone cells via ToF-SIMS. *Biointerphases* **10**, 19016 (2015).
130. Huang, M.-Z., Yuan, C.-H., Cheng, S.-C., Cho, Y.-T. & Shiea, J. Ambient Ionization Mass Spectrometry. *Annu. Rev. Anal. Chem.* **3**, 43–65 (2010).
131. Takáts, Z., Wiseman, J. M., Gologan, B. & Cooks, R. G. Mass spectrometry sampling under ambient conditions with desorption electrospray ionization.

- Science* (80-.). (2004) doi:10.1126/science.1104404.
132. Takáts, Z., Wiseman, J. M. & Cooks, R. G. Ambient mass spectrometry using desorption electrospray ionization (DESI): Instrumentation, mechanisms and applications in forensics, chemistry, and biology. *J. Mass Spectrom.* **40**, 1261–1275 (2005).
 133. Domin, M. & Cody, R. *Ambient Ionization Mass Spectrometry*. (Royal Society of Chemistry, 2014).
 134. Bodzon-Kulakowska, A., Drabik, A., Marszalek, M., Kotlinska, J. H. & Suder, P. DESI analysis of mammalian cell cultures - Sample preparation and method optimisation. *J. Mass Spectrom.* (2014) doi:10.1002/jms.3381.
 135. Jayashree, B. *et al.* Monitoring of changes in lipid profiles during PLK1 knockdown in cancer cells using DESI MS. *Anal. Bioanal. Chem.* (2016) doi:10.1007/s00216-016-9665-y.
 136. Sinclair, I. *et al.* Novel Acoustic Loading of a Mass Spectrometer: Toward Next-Generation High-Throughput MS Screening. *J. Lab. Autom.* (2016) doi:10.1177/2211068215619124.
 137. Sinclair, I. *et al.* Acoustic mist ionization platform for direct and contactless ultrahigh-throughput mass spectrometry analysis of liquid samples. *Anal. Chem.* (2019) doi:10.1021/acs.analchem.9b00142.
 138. Bachman, M., Sinclair, I., Ivanov, D. & Wingfield, J. Information-rich high-throughput cellular assays using acoustic mist ionisation mass spectrometry. *Analyst* (2021) doi:10.1039/d0an01519a.
 139. Jones, E. A. *et al.* Matrix assisted rapid evaporative ionization mass spectrometry. *Anal. Chem.* (2019) doi:10.1021/acs.analchem.9b01441.
 140. Balog, J. *et al.* In Vivo Endoscopic Tissue Identification by Rapid Evaporative Ionization Mass Spectrometry (REIMS). *Angew. Chemie* **127**, 11211–11214 (2015).
 141. Schäfer, K. C. *et al.* In vivo, in situ tissue analysis using rapid evaporative ionization mass spectrometry. *Angew. Chemie - Int. Ed.* **48**, 8240–8242 (2009).

142. Takats, Z., Strittmatter, N. & McKenzie, J. S. Ambient Mass Spectrometry in Cancer Research. *Advances in Cancer Research* vol. 134 231–256 (2017).
143. Balog, J. *et al.* Evaporative Ionization Mass Spectrometry. **82**, 7343–7350 (2010).
144. Dhanda, J. *et al.* iKnife Rapid Evaporative Ionisation Mass Spectrometry (REIMS) Technology In Head and Neck Surgery. A Ex Vivo Feasibility Study. *Br. J. Oral Maxillofac. Surg.* (2017) doi:10.1016/j.bjoms.2017.08.293.
145. St John, E. R. *et al.* Rapid evaporative ionisation mass spectrometry of electrosurgical vapours for the identification of breast pathology: Towards an intelligent knife for breast cancer surgery. *Breast Cancer Res.* (2017) doi:10.1186/s13058-017-0845-2.
146. Golf, O. *et al.* Rapid evaporative ionization mass spectrometry imaging platform for direct mapping from bulk tissue and bacterial growth media. *Anal. Chem.* **87**, 2527–2534 (2015).
147. Strittmatter, N. *et al.* Analysis of intact bacteria using rapid evaporative ionisation mass spectrometry. *Chem. Commun.* **49**, 6188–6190 (2013).
148. Bolt, F. *et al.* Automated High-Throughput Identification and Characterization of Clinically Important Bacteria and Fungi using Rapid Evaporative Ionization Mass Spectrometry. *Anal. Chem.* **88**, 9419–9426 (2016).
149. Strittmatter, N. *et al.* Shotgun Lipidomic Profiling of the NCI60 Cell Line Panel Using Rapid Evaporative Ionization Mass Spectrometry. *Anal. Chem.* (2016) doi:10.1021/acs.analchem.6b00187.
150. Koundouros, N. *et al.* Metabolic Fingerprinting Links Oncogenic PIK3CA with Enhanced Arachidonic Acid-Derived Eicosanoids. *Cell* (2020) doi:10.1016/j.cell.2020.05.053.
151. Paraskevaïdi, M. *et al.* Laser-assisted rapid evaporative ionisation mass spectrometry (LA-REIMS) as a metabolomics platform in cervical cancer screening. *EBioMedicine* (2020) doi:10.1016/j.ebiom.2020.103017.
152. Bergman, N., Shevchenko, D. & Bergquist, J. Approaches for the analysis of low molecular weight compounds with laser desorption/ionization techniques

- and mass spectrometry. *Analytical and Bioanalytical Chemistry* (2014) doi:10.1007/s00216-013-7471-3.
153. Strittmatter, N. *et al.* Shotgun Lipidomic Profiling of the NCI60 Cell Line Panel Using Rapid Evaporative Ionization Mass Spectrometry. *Anal. Chem.* **88**, 7507–7514 (2016).
 154. Plekhova, V. *et al.* Rapid ex vivo molecular fingerprinting of biofluids using laser-assisted rapid evaporative ionization mass spectrometry. *Nature Protocols* (2021) doi:10.1038/s41596-021-00580-8.
 155. Cameron, S. J. S. *et al.* Utilisation of Ambient Laser Desorption Ionisation Mass Spectrometry (ALDI-MS) Improves Lipid-Based Microbial Species Level Identification. *Sci. Rep.* **9**, 1–8 (2019).
 156. Cameron, S. J. S. *et al.* Sample Preparation Free Mass Spectrometry Using Laser-Assisted Rapid Evaporative Ionization Mass Spectrometry: Applications to Microbiology, Metabolic Biofluid Phenotyping, and Food Authenticity. *J. Am. Soc. Mass Spectrom.* (2021) doi:10.1021/jasms.0c00452.
 157. Zhao, X. *et al.* Data analysis of MS-based clinical lipidomics studies with crossover design: A tutorial mini-review of statistical methods. *Clinical Mass Spectrometry* (2019) doi:10.1016/j.clinms.2019.05.002.
 158. Kwon, D., Vannucci, M., Joon, J. S., Jeong, J. & Pfeiffer, R. M. A novel wavelet-based thresholding method for the pre-processing of mass spectrometry data that accounts for heterogeneous noise. *Proteomics* (2008) doi:10.1002/pmic.200701010.
 159. Bauer, C., Cramer, R. & Schuchhardt, J. Evaluation of peak-picking algorithms for protein mass spectrometry. *Methods Mol. Biol.* (2011) doi:10.1007/978-1-60761-987-1_22.
 160. Antoniadis, A., Bigot, J. & Lambert-Lacroix, S. Peaks detection and alignment for mass spectrometry data. *J. la Société Française Stat.* (2010).
 161. Stanford, T. E., Bagley, C. J. & Solomon, P. J. Informed baseline subtraction of proteomic mass spectrometry data aided by a novel sliding window algorithm. *Proteome Sci.* (2016) doi:10.1186/s12953-016-0107-8.

162. Alfassi, Z. B. On the normalization of a mass spectrum for comparison of two spectra. *J. Am. Soc. Mass Spectrom.* (2004) doi:10.1016/j.jasms.2003.11.008.
163. Xi, B., Gu, H., Baniyadi, H. & Raftery, D. Statistical analysis and modeling of mass spectrometry-based metabolomics data. *Methods Mol. Biol.* (2014) doi:10.1007/978-1-4939-1258-2_22.
164. Purohit, P. V., Rocke, D. M., Viant, M. R. & Woodruff, D. L. Discrimination models using variance-stabilizing transformation of metabolomic NMR data. *Omi. A J. Integr. Biol.* (2004) doi:10.1089/1536231041388348.
165. van den Berg, R. A., Hoefsloot, H. C. J., Westerhuis, J. A., Smilde, A. K. & van der Werf, M. J. Centering, scaling, and transformations: Improving the biological information content of metabolomics data. *BMC Genomics* (2006) doi:10.1186/1471-2164-7-142.
166. Alghamdi, A., Gray, A. & Watson, D. INVESTIGATION OF METABOLOMICS TECHNIQUES BY ANALYSIS OF MS PROPOLIS DATA: WHICH PRE-TREATMENT METHOD IS BETTER? *Adv. Appl. Stat.* (2019) doi:10.17654/as058010013.
167. McCue, C. Chapter 7 - Identification, Characterization, and Modeling. in *Data Mining and Predictive Analysis (Second Edition)* (ed. McCue, C.) 137–155 (Butterworth-Heinemann, 2015). doi:https://doi.org/10.1016/B978-0-12-800229-2.00007-9.
168. Bujak, R., Struck-Lewicka, W., Markuszewski, M. J. & Kaliszan, R. Metabolomics for laboratory diagnostics. *Journal of Pharmaceutical and Biomedical Analysis* (2015) doi:10.1016/j.jpba.2014.12.017.
169. Talabis, M. R. M., McPherson, R., Miyamoto, I., Martin, J. L. & Kaye, D. Chapter 1 - Analytics Defined. in *Information Security Analytics* (eds. Talabis, M. R. M., McPherson, R., Miyamoto, I., Martin, J. L. & Kaye, D.) 1–12 (Syngress, 2015). doi:https://doi.org/10.1016/B978-0-12-800207-0.00001-0.
170. Percival, B., Gibson, M., Leenders, J., Wilson, P. B. & Grootveld, M. Chapter 1: Univariate and Multivariate Statistical Approaches to the Analysis and Interpretation of NMR-based Metabolomics Datasets of Increasing

- Complexity. in *RSC Theoretical and Computational Chemistry Series* (2021). doi:10.1039/9781788015882-00001.
171. Bartel, J., Krumsiek, J. & Theis, F. J. Statistical methods for the analysis of high-throughput metabolomics data. *Computational and Structural Biotechnology Journal* (2013) doi:10.5936/csbj.201301009.
 172. Vinaixa, M. *et al.* A guideline to univariate statistical analysis for LC/MS-based untargeted metabolomics-derived data. *Metabolites* (2012) doi:10.3390/metabo2040775.
 173. Jolliffe, I. T. Principal Component Analysis, Second Edition. *Encycl. Stat. Behav. Sci.* (2002) doi:10.2307/1270093.
 174. Worley, B. & Powers, R. Multivariate Analysis in Metabolomics. *Curr. Metabolomics* (2013) doi:10.2174/2213235x11301010092.
 175. Gromski, P. S. *et al.* A tutorial review: Metabolomics and partial least squares-discriminant analysis – a marriage of convenience or a shotgun wedding. *Anal. Chim. Acta* **879**, 10–23 (2015).
 176. Mohanty, N., John, A. L.-S., Manmatha, R. & Rath, T. M. Chapter 10 - Shape-Based Image Classification and Retrieval. in *Handbook of Statistics* (eds. Rao, C. R. & Govindaraju, V.) vol. 31 249–267 (Elsevier, 2013).
 177. Vaibhaw, Sarraf, J. & Pattnaik, P. K. Chapter 2 - Brain–computer interfaces and their applications. in *An Industrial IoT Approach for Pharmaceutical Industry Growth* (eds. Balas, V. E., Solanki, V. K. & Kumar, R.) 31–54 (Academic Press, 2020). doi:https://doi.org/10.1016/B978-0-12-821326-1.00002-4.
 178. Hastie, T; Tibshirani, R; Friedman, J. The Elements of Statistical Learning Second Edition. *Math. Intell.* (2017).
 179. Bartosik, A. & Whittingham, H. Chapter 7 - Evaluating safety and toxicity. in *The Era of Artificial Intelligence, Machine Learning, and Data Science in the Pharmaceutical Industry* (ed. Ashenden, S. K.) 119–137 (Academic Press, 2021). doi:https://doi.org/10.1016/B978-0-12-820045-2.00008-8.
 180. Edgar, T. W. & Manz, D. O. Chapter 4 - Exploratory Study. in *Research*

- Methods for Cyber Security* (eds. Edgar, T. W. & Manz, D. O.) 95–130 (Syngress, 2017). doi:<https://doi.org/10.1016/B978-0-12-805349-2.00004-2>.
181. Sinnott, R. O., Duan, H. & Sun, Y. Chapter 15 - A Case Study in Big Data Analytics: Exploring Twitter Sentiment Analysis and the Weather. in *Big Data* (eds. Buyya, R., Calheiros, R. N. & Dastjerdi, A. V.) 357–388 (Morgan Kaufmann, 2016). doi:<https://doi.org/10.1016/B978-0-12-805394-2.00015-5>.
 182. Belyadi, H. & Haghighat, A. Chapter 5 - Supervised learning. in *Machine Learning Guide for Oil and Gas Using Python* (eds. Belyadi, H. & Haghighat, A.) 169–295 (Gulf Professional Publishing, 2021). doi:<https://doi.org/10.1016/B978-0-12-821929-4.00004-4>.
 183. Caie, P. D., Dimitriou, N. & Arandjelović, O. Chapter 8 - Precision medicine in digital pathology via image analysis and machine learning. in *Artificial Intelligence and Deep Learning in Pathology* (ed. Cohen, S.) 149–173 (Elsevier, 2021). doi:<https://doi.org/10.1016/B978-0-323-67538-3.00008-7>.
 184. Misra, S. & Li, H. Chapter 9 - Noninvasive fracture characterization based on the classification of sonic wave travel times. in *Machine Learning for Subsurface Characterization* (eds. Misra, S., Li, H. & He, J.) 243–287 (Gulf Professional Publishing, 2020). doi:<https://doi.org/10.1016/B978-0-12-817736-5.00009-0>.
 185. Martin, M. & Govaerts, B. *Feature Selection in metabolomics with PLS-derived methods*. (UCLouvain, 2019).
 186. Han, Y., Huang, L. & Zhou, F. A dynamic recursive feature elimination framework (dRFE) to further refine a set of OMIC biomarkers. *Bioinformatics* (2021) doi:10.1093/bioinformatics/btab055.
 187. Oyejide, L., Mendes, O. R. & Mikaelian, I. Molecular Pathology: Applications in Nonclinical Drug Development. in *A Comprehensive Guide to Toxicology in Preclinical Drug Development* (2013). doi:10.1016/B978-0-12-387815-1.00010-1.
 188. Misra, P. & Yadav, A. S. Improving the classification accuracy using recursive feature elimination with cross-validation. *Int. J. Emerg. Technol.*

(2020).

189. Watson, D. G. A rough guide to metabolite identification using high resolution liquid chromatography mass spectrometry in metabolomic profiling in metazoans. *Computational and Structural Biotechnology Journal* (2013) doi:10.5936/csbj.201301005.
190. Kind, T. & Fiehn, O. Seven Golden Rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. *BMC Bioinformatics* (2007) doi:10.1186/1471-2105-8-105.
191. Wang, L. *et al.* Metabolic pathway extension approach for metabolomic biomarker identification. *Anal. Chem.* (2017) doi:10.1021/acs.analchem.6b03757.
192. Sumner, L. W. *et al.* Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* (2007) doi:10.1007/s11306-007-0082-2.
193. Schymanski, E. L. *et al.* Identifying small molecules via high resolution mass spectrometry: Communicating confidence. *Environmental Science and Technology* (2014) doi:10.1021/es5002105.
194. Schrimpe-Rutledge, A. C., Codreanu, S. G., Sherrod, S. D. & McLean, J. A. Untargeted Metabolomics Strategies—Challenges and Emerging Directions. *J. Am. Soc. Mass Spectrom.* (2016) doi:10.1007/s13361-016-1469-y.
195. Harvey, D. Chemistry modern analytical chemistry. *Mod. Anal. Chem.* (2000).
196. Zheng, X., Sacra, S., Verhagen, M. F. & Davies, C. An efficient analytical development strategy for rapid development of biopharmaceuticals. *Am. Pharm. Rev.* (2019).
197. Eurachem. *The Fitness for Purpose of Analytical Methods. Eurachem Guide, ISBN: 0-94948926-12-0* (1998).
198. University of Tartu. LC-MS Method Validation: Repeatability, intermediate precision and reproducibility. https://sisu.ut.ee/lcms_method_validation/41-precision-trueness-accuracy.

199. Strittmatter, N. *et al.* Characterization and identification of clinically relevant microorganisms using rapid evaporative ionization mass spectrometry. *Anal. Chem.* **86**, 6555–6562 (2014).
200. Savitzky, A. & Golay, M. J. E. Smoothing and Differentiation of Data by Simplified Least Squares Procedures. *Anal. Chem.* (1964) doi:10.1021/ac60214a047.
201. Gibb, S. & Strimmer, K. Maldiquant: A versatile R package for the analysis of mass spectrometry data. *Bioinformatics* (2012) doi:10.1093/bioinformatics/bts447.
202. Gibb, S. & Strimmer, K. Mass Spectrometry Analysis Using MALDIquant. in *Statistical Analysis of Proteomics, Metabolomics, and Lipidomics Data Using Mass Spectrometry* (2017). doi:10.1007/978-3-319-45809-0_6.
203. Pang, Z. *et al.* MetaboAnalyst 5.0: Narrowing the gap between raw spectra and functional insights. *Nucleic Acids Res.* (2021) doi:10.1093/nar/gkab382.
204. Sarafian, M. H. *et al.* Objective set of criteria for optimization of sample preparation procedures for ultra-high throughput untargeted blood plasma lipid profiling by ultra performance liquid chromatography-mass spectrometry. *Anal. Chem.* (2014) doi:10.1021/ac500317c.
205. Berman, E. S. F. *et al.* Preparation of Single Cells for Imaging/Profiling Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* (2008) doi:10.1016/j.jasms.2008.05.006.
206. Essential Cell Biology . Fourth Edition. By Bruce Alberts, Dennis Bray, Karen Hopkin, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter. New York: Garland Science (Taylor & Francis Group). \$151.00. xxiii + 726 p. + A:1 - A:58; *Q. Rev. Biol.* (2015) doi:10.1086/682638.
207. Ahn, H. J. *et al.* Characteristics of the cell membrane fluidity, actin fibers, and mitochondrial dysfunctions of frozen-thawed two-cell mouse embryos. *Mol. Reprod. Dev.* (2002) doi:10.1002/mrd.10040.
208. Stephenson, D. T., Lemere, C. A., Selkoe, D. J. & Clemens, J. A. Cytosolic

- phospholipase A2 (cPLA2) immunoreactivity is elevated in Alzheimer's disease brain. *Neurobiol. Dis.* (1996) doi:10.1006/nbdi.1996.0005.
209. Makurvet, F. D. Biologics vs. small molecules: Drug costs and patient access. *Med. Drug Discov.* (2021) doi:10.1016/j.medidd.2020.100075.
 210. Ecker, D. M., Jones, S. D. & Levine, H. L. The therapeutic monoclonal antibody market. *mAbs* (2015) doi:10.4161/19420862.2015.989042.
 211. Walsh, G. Biopharmaceutical benchmarks 2018. *Nat. Biotechnol.* (2018) doi:10.1038/nbt.4305.
 212. Mullard, A. FDA approves 100th monoclonal antibody product. *Nature reviews. Drug discovery* (2021) doi:10.1038/d41573-021-00079-7.
 213. Liu, X. Y., Pop, L. M. & Vitetta, E. S. Engineering therapeutic monoclonal antibodies. *Immunological Reviews* (2008) doi:10.1111/j.1600-065X.2008.00601.x.
 214. Zhu, J. Mammalian cell protein expression for biopharmaceutical production. *Biotechnol. Adv.* (2012) doi:10.1016/j.biotechadv.2011.08.022.
 215. Gupta, S. K. *et al.* Metabolic engineering of CHO cells for the development of a robust protein production platform. *PLoS One* (2017) doi:10.1371/journal.pone.0181455.
 216. Ali, A. S. *et al.* Lipidomics of CHO Cell Bioprocessing: Relation to Cell Growth and Specific Productivity of a Monoclonal Antibody. *Biotechnol. J.* (2018) doi:10.1002/biot.201700745.
 217. Zhang, Y. *et al.* High-Throughput Lipidomic and Transcriptomic Analysis to Compare SP2/0, CHO, and HEK-293 Mammalian Cell Lines. *Anal. Chem.* **89**, 1477–1485 (2017).
 218. Chong, J., Wishart, D. S. & Xia, J. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Curr. Protoc. Bioinforma.* (2019) doi:10.1002/cpbi.86.
 219. Khaselev, N. & Murphy, R. C. Susceptibility of plasmenyl glycerophosphoethanolamine lipids containing arachidonate to oxidative

- degradation. *Free Radic. Biol. Med.* **26**, 275–284 (1999).
220. Maeba, R., Sawada, Y., Shimasaki, H., Takahashi, I. & Ueta, N. Ethanolamine plasmalogens protect cholesterol-rich liposomal membranes from oxidation caused by free radicals. *Chem. Phys. Lipids* **120**, 145–151 (2002).
 221. Skaff, O., Pattison, D. I. & Davies, M. J. The vinyl ether linkages of plasmalogens are favored targets for myeloperoxidase-derived oxidants: A kinetic study. *Biochemistry* **47**, 8237–8245 (2008).
 222. Morand, O. H., Zoeller, R. A. & Raetz, C. R. H. Disappearance of plasmalogens from membranes of animal cells subjected to photosensitized oxidation. *J. Biol. Chem.* **263**, 11597–11606 (1988).
 223. Broniec, A. *et al.* Interactions of plasmalogens and their diacyl analogs with singlet oxygen in selected model systems. *Free Radic. Biol. Med.* **50**, 892–898 (2011).
 224. Koundouros, N. & Poulogiannis, G. Reprogramming of fatty acid metabolism in cancer. *British Journal of Cancer* (2020) doi:10.1038/s41416-019-0650-z.
 225. Gardocki, M. E., Jani, N. & Lopes, J. M. Phosphatidylinositol biosynthesis: Biochemistry and regulation. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* (2005) doi:10.1016/j.bbalip.2005.05.006.
 226. Falkenburger, B. H., Jensen, J. B., Dickson, E. J., Suh, B. C. & Hille, B. Phosphoinositides: Lipid regulators of membrane proteins. in *Journal of Physiology* (2010). doi:10.1113/jphysiol.2010.192153.
 227. Stanishneva-Konovalova, T. B. & Sokolova, O. S. Molecular dynamics simulations of negatively charged DPPC/DPPI lipid bilayers at two levels of resolution. *Comput. Theor. Chem.* (2015) doi:10.1016/j.comptc.2014.04.036.
 228. Pöyry, S. & Vattulainen, I. Role of charged lipids in membrane structures — Insight given by simulations. *Biochim. Biophys. Acta - Biomembr.* (2016) doi:10.1016/j.bbamem.2016.03.016.
 229. Anand, A. *et al.* Cell death induced by cationic amphiphilic drugs depends on lysosomal Ca²⁺ release and cyclic AMP. *Mol. Cancer Ther.* (2019)

doi:10.1158/1535-7163.MCT-18-1406.

230. Hopkins, E., Sanvictores, T. & Sharma, S. Physiology, Acid Base Balance. in (2022).
231. Funk, R. S. & Krise, J. P. Cationic amphiphilic drugs cause a marked expansion of apparent lysosomal volume: Implications for an intracellular distribution-based drug interaction. *Mol. Pharm.* (2012) doi:10.1021/mp200641e.
232. Winchester, B., Vellodi, A. & Young, E. The molecular basis of lysosomal storage diseases and their treatment. in *Biochemical Society Transactions* (2000). doi:10.1042/bst0280150.
233. Platt, F. M., d'Azzo, A., Davidson, B. L., Neufeld, E. F. & Tiffit, C. J. Lysosomal storage diseases. *Nature Reviews Disease Primers* (2018) doi:10.1038/s41572-018-0025-4.
234. Schmitz, G. & Muller, G. Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids. *Journal of Lipid Research* (1991) doi:10.1016/s0022-2275(20)41642-6.
235. Bernhard, W. Regulation of Surfactant-Associated Phospholipid Synthesis and Secretion. in *Fetal and Neonatal Physiology* (2017). doi:10.1016/b978-0-323-35214-7.00082-2.
236. Fisher, A. B. Lung Lipid Composition and Surfactant Biology. in *Comparative Biology of the Normal Lung: Second Edition* (2015). doi:10.1016/B978-0-12-404577-4.00022-9.
237. Halliwell, W. H. Cationic amphiphilic drug-induced phospholipidosis. in *Toxicologic Pathology* (1997). doi:10.1177/019262339702500111.
238. Reasor, M. J. Phospholipidosis in the alveolar macrophage induced by cationic amphiphilic drugs. *Fed. Proc.* (1984).
239. Showalter, M. R. *et al.* The emerging and diverse roles of bis(Monoacylglycero) phosphate lipids in cellular physiology and disease. *International Journal of Molecular Sciences* (2020) doi:10.3390/ijms21218067.

240. Shahane, S. A. *et al.* Detection of phospholipidosis induction: A cell-based assay in high-throughput and high-content format. *J. Biomol. Screen.* (2014) doi:10.1177/1087057113502851.
241. van de Water, F. M., Havinga, J., Ravesloot, W. T., Horbach, G. J. M. J. & Schoonen, W. G. E. J. High content screening analysis of phospholipidosis: Validation of a 96-well assay with CHO-K1 and HepG2 cells for the prediction of in vivo based phospholipidosis. *Toxicol. Vitro.* (2011) doi:10.1016/j.tiv.2011.05.026.
242. Thai, T. Q. *et al.* Rapid specimen preparation to improve the throughput of electron microscopic volume imaging for three-dimensional analyses of subcellular ultrastructures with serial block-face scanning electron microscopy. *Med. Mol. Morphol.* (2016) doi:10.1007/s00795-016-0134-7.
243. Mortuza, G. B., Neville, W. A., Delaney, J., Waterfield, C. J. & Camilleri, P. Characterisation of a potential biomarker of phospholipidosis from amiodarone-treated rats. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **1631**, 136–146 (2003).
244. Gallala, H. D. & Sandhoff, K. Biological function of the cellular lipid bmp-bmp as a key activator for cholesterol sorting and membrane digestion. *Neurochem. Res.* (2011) doi:10.1007/s11064-010-0337-6.
245. Newman, C. F. *et al.* Intracellular Drug Uptake - A Comparison of Single Cell Measurements Using ToF-SIMS Imaging and Quantification from Cell Populations with LC/MS/MS. *Anal. Chem.* (2017) doi:10.1021/acs.analchem.7b01436.
246. Wolfer, A. M., Gaudin, M., Taylor-Robinson, S. D., Holmes, E. & Nicholson, J. K. Development and Validation of a High-Throughput Ultrahigh-Performance Liquid Chromatography-Mass Spectrometry Approach for Screening of Oxylipins and Their Precursors. *Anal. Chem.* **87**, 11721–11731 (2015).
247. Powell, W. S. & Rokach, J. The eosinophil chemoattractant 5-oxo-ETE and the OXE receptor. *Progress in Lipid Research* (2013) doi:10.1016/j.plipres.2013.09.001.

248. Powis, G., Olsen, R., Standing, J. E., Kachel, D. & Martin, W. J. Amiodarone-mediated increase in intracellular free Ca²⁺ associated with cellular injury to human pulmonary artery endothelial cells. *Toxicol. Appl. Pharmacol.* (1990) doi:10.1016/0041-008X(90)90271-U.
249. NATTEL, S., TALAJIC, M., FERMINI, B. & ROY, D. Amiodarone: Pharmacology, Clinical Actions, and Relationships Between Them. *J. Cardiovasc. Electrophysiol.* **3**, 266–280 (1992).
250. Kodama, I., Kamiya, K. & Toyama, J. Cellular electropharmacology of amiodarone. *Cardiovascular Research* (1997) doi:10.1016/S0008-6363(97)00114-4.
251. Petrou, T. *et al.* Intracellular calcium mobilization in response to ion channel regulators via a calcium-induced calcium release mechanism. *J. Pharmacol. Exp. Ther.* (2017) doi:10.1124/jpet.116.236695.
252. Akiba, S. & Sato, T. Cellular function of calcium-independent phospholipase A₂. *Biological and Pharmaceutical Bulletin* (2004) doi:10.1248/bpb.27.1174.
253. Rosa, A. O. & Rapoport, S. I. Intracellular- and extracellular-derived Ca²⁺ influence phospholipase A₂-mediated fatty acid release from brain phospholipids. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* (2009) doi:10.1016/j.bbalip.2009.03.009.
254. Akgoc, Z. *et al.* Bis(monoacylglycero)phosphate: A secondary storage lipid in the gangliosidoses. *J. Lipid Res.* (2015) doi:10.1194/jlr.M057851.
255. Hutschenreuther, A., Birkenmeier, G., Bigl, M., Krohn, K. & Birkemeyer, C. Glycerophosphoglycerol, beta-alanine, and pantothenic acid as metabolic companions of glycolytic activity and cell migration in breast cancer cell lines. *Metabolites* (2013) doi:10.3390/metabo3041084.
256. Baker, A. D. *et al.* PPAR γ regulates the expression of cholesterol metabolism genes in alveolar macrophages. *Biochem. Biophys. Res. Commun.* (2010) doi:10.1016/j.bbrc.2010.02.056.
257. Agudelo, C. W., Samaha, G. & Garcia-Arcos, I. Alveolar lipids in pulmonary disease. A review. *Lipids in Health and Disease* (2020) doi:10.1186/s12944-

020-01278-8.

- 258. Breiden, B. & Sandhoff, K. Emerging mechanisms of drug-induced phospholipidosis. *Biological Chemistry* (2019) doi:10.1515/hsz-2019-0270.
- 259. Dasouki, M. *Chapter 11 - Peroxisomal disorders: Clinical and biochemical laboratory aspects. Biomarkers in Inborn Errors of Metabolism* (2017).
- 260. JOHNSTON, N. C. & GOLDFINE, H. Isolation and characterization of new phosphatidylglycerol acetals of plasmalogens: A family of ether lipids in clostridia. *Eur. J. Biochem.* (1994) doi:10.1111/j.1432-1033.1994.tb19073.x.
- 261. Johnston, N. C., Goldfine, H. & Fischer, W. Novel polar lipid composition of *Clostridium innocuum* as the basis for an assessment of its taxonomic status. *Microbiology* (1994) doi:10.1099/13500872-140-1-105.
- 262. Veldhuizen, E. J. A. & Haagsman, H. P. Role of pulmonary surfactant components in surface film formation and dynamics. *Biochimica et Biophysica Acta - Biomembranes* (2000) doi:10.1016/S0005-2736(00)00256-X.
- 263. Lowe, R., Mussa, H. Y., Nigsch, F., Glen, R. C. & Mitchell, J. B. O. Predicting the mechanism of phospholipidosis. *J. Cheminform.* (2012) doi:10.1186/1758-2946-4-2.
- 264. Mazia, D., Schatten, G. & Sale, W. Adhesion of cells to surfaces coated with polylysine: Applications to electron microscopy. *J. Cell Biol.* (1975) doi:10.1083/jcb.66.1.198.

Appendix

Supplementary material/Chapter 3:

Cell line	Accuracy	Sensitivity	Specificity	Precision	F1
BT549	0.967	1	0.935	0.346	0.514
C2BBE1	0.641	0.315	0.967	0.708	0.436
CL40	1	1	1	1	1
LS180	1	1	1	1	1
LS513	0.798	0.605	0.991	0.929	0.732
MCF7	0.769	0.537	1	1	0.699
MDAMB231	0	0	0.900	0	0
MDAMB468	0.982	1	0.964	0.679	0.809
RKO	0.960	1	0.920	0.192	0.323
T47D	0.939	0.879	1	1	0.935
Average	0.895	0.815	0.968	0.685	0.645

Table 14. Supplementary material of section 3.3.2.2., Figure 27. Accuracy, Sensitivity, Specificity, Precision, and F1 score of each cell line of the testing dataset used to assess the intermediate precision of the LD-REIMS analysis on cell monolayer.

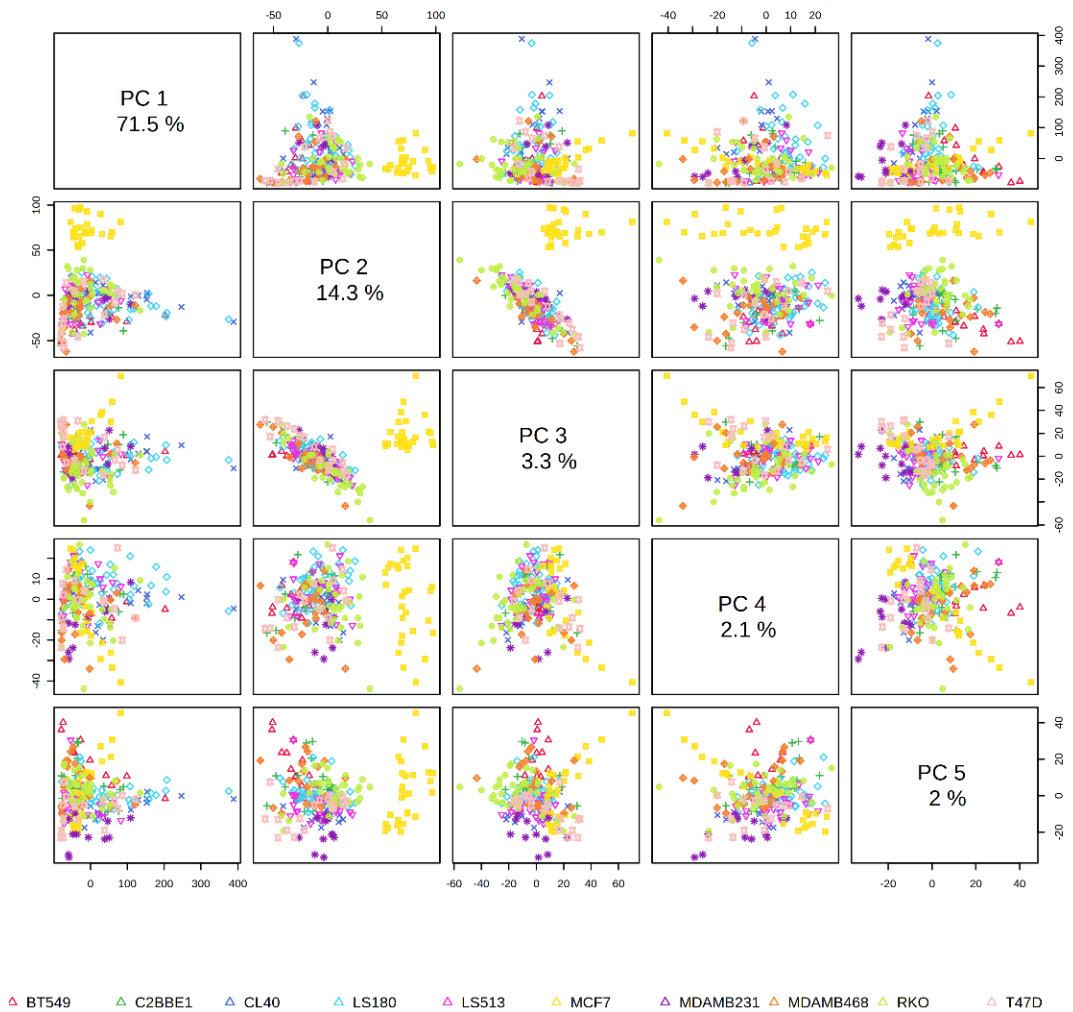


Figure 62. PCA scores plots of the first 5 principal components of the 5 breast and 5 colorectal cancer cell pellets analysed with bipolar forceps REIMS.

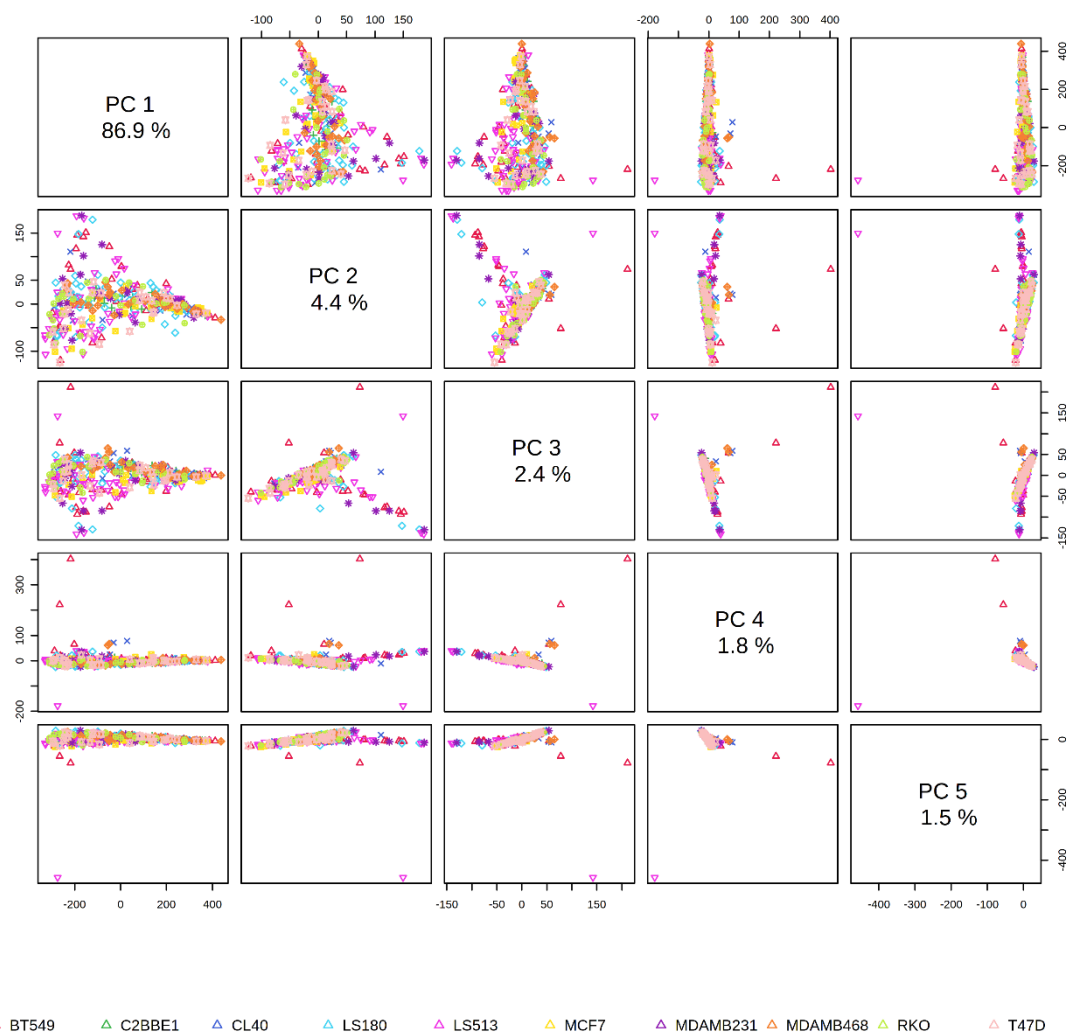


Figure 63. PCA scores plots of the first 5 principal components of the 5 breast and 5 colorectal cancer cell pellets analysed with cell probe REIMS.

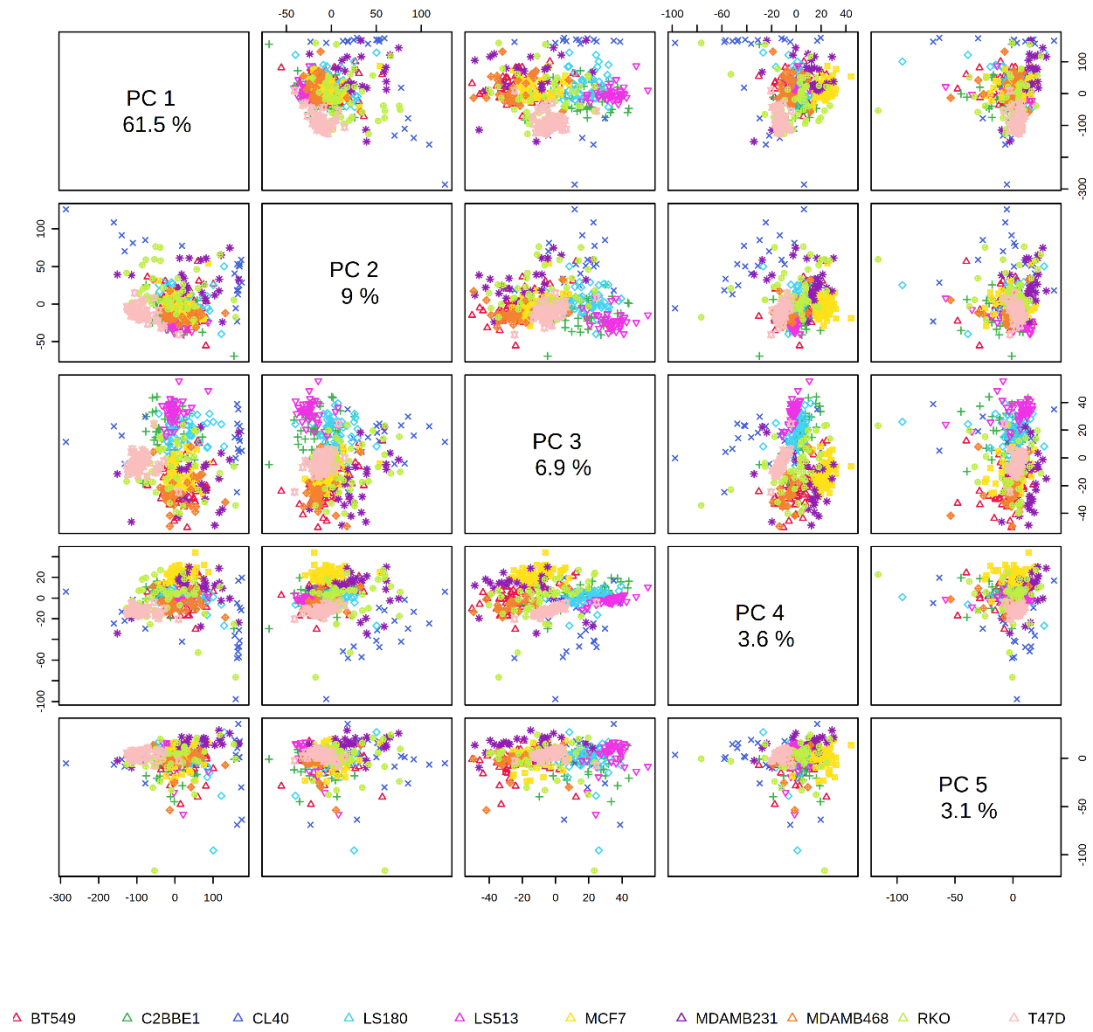


Figure 64. PCA scores plots of the first 5 principal components of the 5 breast and 5 colorectal cancer cell pellets analysed with LD-REIMS

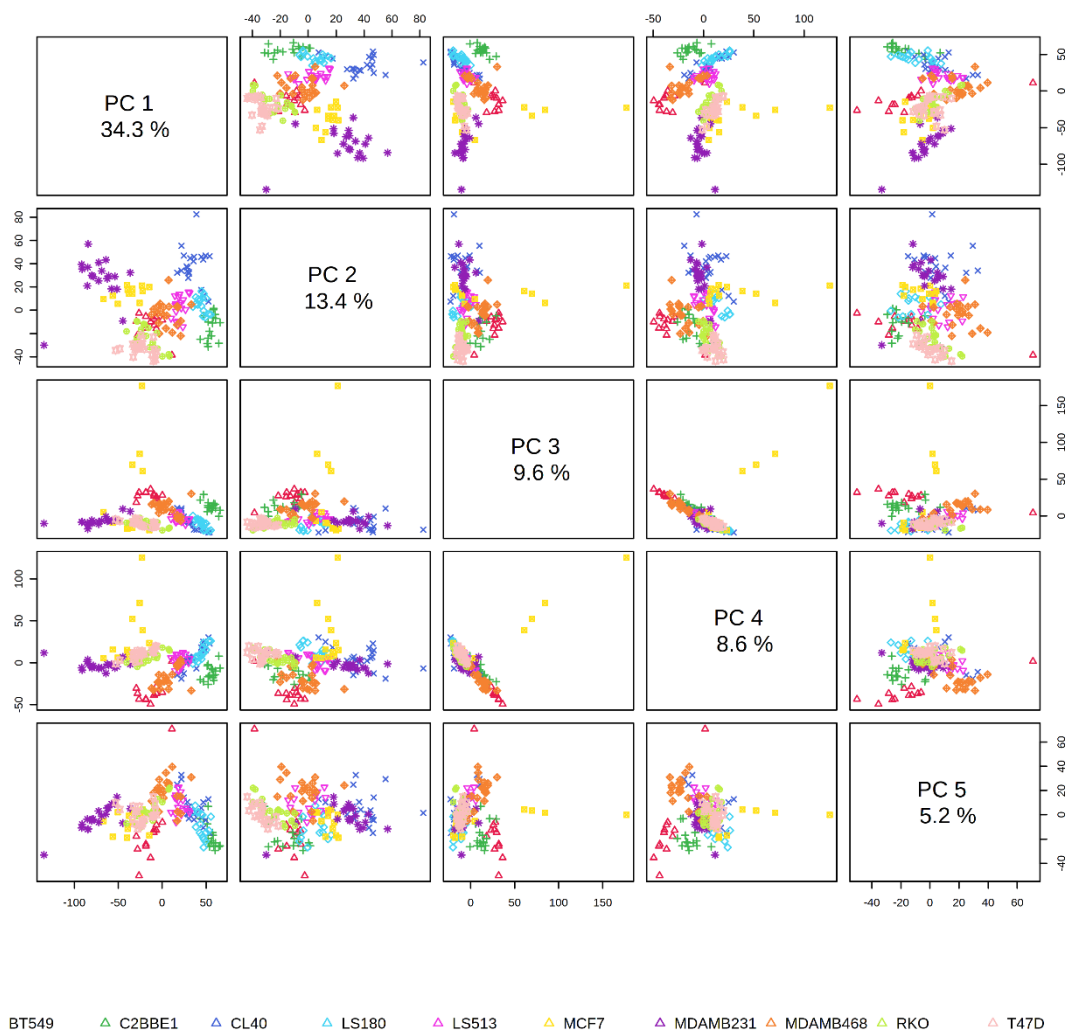


Figure 65. PCA scores plots of the first 5 principal components of the 5 breast and 5 colorectal cancer cell lines analysed with LD REIMS as cell monolayer.

Supplementary material/Chapter 4:

<i>m/z</i>	Annotation	LC-DDA	Detected ion	Theoretical <i>m/z</i>	Error (ppm)	Class	Occurrence
135.030	Threonate	-	[M-H]-	135.029	3.7	Small metabolite	"fed-batch" Day 12
179.056	Glucose	-	[M-H]-	179.056	-0.6	Small metabolite	"fed-batch" Day 6
196.037	N-Methyl-L-glutamate	-	[M+Cl]-	196.038	-5.1	Small metabolite	"non-production"
214.048	D-Glucosamine	-	[M+Cl]-	214.048	-3.3	Small metabolite	"fed-batch" Day 12
215.032	Glucose	-	[M+Cl]-	215.032	0	Small metabolite	"non-production", "fed-batch" Day 6
227.201	FA(14:0)	-	[M-H]-	227.201	-1.3	Fatty acid	"non-production"
253.217	FA(16:1)	-	[M-H]-	253.217	0.4	Fatty acid	"fed-batch" Day 12
255.232	FA(16:0)	-	[M-H]-	255.232	0	Fatty acid	"non-production", "fed-batch" Day 12
279.040	Uridine	-	[M+Cl]-	279.038	5.7	Small metabolite	"non-production"
280.266	Sphingosine	-	[M-H ₂ O-H]-	280.264	8.6	Small metabolite	"fed-batch" Day 12
283.263	FA(18:0)	-	[M-H]-	283.264	-2.1	Fatty acid	"non-production", "fed-batch" Day 6
307.263	FA(20:2)	-	[M-H]-	307.264	-1	Fatty acid	"non-production", "fed-batch" Day 12
309.279	FA(20:1)	-	[M-H]-	309.279	-1.9	Fatty acid	"fed-batch" Day 6
311.293	FA(20:0)	-	[M-H]-	311.295	-5.1	Fatty acid	"fed-batch" Day 6
315.048	Glycerophosphoinositol	-	[M-H ₂ O-H]-	315.048	-1.3	Small metabolite	"fed-batch" Day 12
337.311	FA(22:1)	-	[M-H]-	337.311	0	Fatty acid	"fed-batch" Day 12
339.207	Arachidonic Acid	-	[M+Cl]-	339.209	-7.1	Fatty acid	"fed-batch" Day 12
367.358	FA(24:0)	-	[M-H]-	367.358	0	Fatty acid	"non-production", "fed-batch" Day 6, "fed-batch" Day 12
409.238	LysoPA(16:0)	-	[M-H]-	409.236	6.6	PA	"fed-batch" Day 12
417.242	LysoPA(18:1)	-	[M-H ₂ O-H]-	417.241	3.1	PA	"fed-batch" Day 12
436.285	LysoPE(O-16:1)	-	[M-H]-	436.283	4.8	PE ether lipid	"non-production"
450.262	LysoPE(16:1)	LysoPE(16:1)	[M-H]-	450.262	0.7	PE	"non-production", "fed-batch" Day 6
452.277	LysoPE(16:0)	LysoPE(16:0)	[M-H]-	452.278	-0.9	PE	"non-production"
462.298	LysoPE(O-18:2)	-	[M-H]-	462.299	-1.5	PE ether lipid	"non-production"
464.313	LysoPE(O-18:1)	-	[M-H]-	464.314	-1.5	PE ether lipid	"non-production"
478.294	LysoPE(18:1)	LysoPE(18:1)	[M-H]-	478.293	0.2	PE	"non-production"
480.309	LysoPE(18:0)	LysoPE(18:0)	[M-H]-	480.309	0	PE	"non-production"
483.273	LysoPG(16:0)	LysoPG(16:0)	[M-H]-	483.272	1.4	PG	"fed-batch" Day 12
536.506	Cer(d34:1)	-	[M-H]-	536.504	3.4	Ceramide	"non-production", "fed-batch" Day 6, "fed-batch" Day 12

556.318	LysoPC(18:1)	-	[M+Cl]-	556.317	2.5	PC	"fed-batch" Day 6
572.481	Cer(d34:1)	-	[M+Cl]-	572.481	0	Ceramide	"fed-batch" Day 6, "fed-batch" Day 12
573.431	DG(30:1)	-	[M+Cl]-	573.429	3.8	DG	"fed-batch" Day 12
575.445	DG(30:0)	-	[M+Cl]-	575.444	1	DG	"fed-batch" Day 12
597.305	LysoPI(18:1)	LysoPI(18:1)	[M-H]-	597.304	1.5	PI	"fed-batch" Day 6, "fed-batch" Day 12
599.319	LysoPI(18:0)	LysoPI(18:0)	[M-H]-	599.320	-0.7	PI	"fed-batch" Day 12
601.461	DG(32:1)	-	[M+Cl]-	601.460	2.2	DG	"fed-batch" Day 6, "fed-batch" Day 12
603.475	DG(32:0)	-	[M+Cl]-	603.476	-1.3	DG	"fed-batch" Day 12
620.600	Cer(d40:1)	-	[M-H]-	620.598	3.1	Ceramide	"fed-batch" Day 12
629.495	DG(34:1)	DG(16:0_18:1)	[M+Cl]-	629.491	5.9	DG	"non-production"
645.455	PA(32:1)	-	[M-H]-	645.450	8.2	PA	"fed-batch" Day 6
648.631	Cer(d42:1)	-	[M-H]-	648.630	2.9	Ceramide	"fed-batch" Day 6
657.492	PE-Cer(d34:2)	-	[M-H]-	657.497	-7.3	Ceramide	"non-production", "fed-batch" Day 12
662.480	PE(30:0)	-	[M-H]-	662.476	6.5	PE	"fed-batch" Day 6
671.469	PA(34:2)	-	[M-H]-	671.465	6.3	PA	"fed-batch" Day 6
672.500	PE(O-32:2)	PE(O-16:1_16:1)	[M-H]-	672.497	4.8	PE ether lipid	"fed-batch" Day 12
674.505	PE(O-32:1)	-	[M-H]-	674.513	-11.7	PE ether lipid	"non-production", "fed-batch" Day 6
682.583	Cer(d42:2)	-	[M+Cl]-	682.591	-11.4	Ceramide	"fed-batch" Day 12
686.471	PE(32:2)	-	[M-H]-	686.476	-6.8	PE	"fed-batch" Day 6
687.537	PE-Cer(d36:1)	PE-Cer(d16:1/20:0)	[M-H]-	687.544	-10.8	Ceramide	"fed-batch" Day 12
688.485	PE(32:1)	PE(16:1_16:0)	[M-H]-	688.492	-9.8	PE	"non-production"
698.506	PE(O-34:3)	PE(O-16:2_18:1)	[M-H]-	698.513	-9.7	PE ether lipid	"non-production"
699.497	PA(36:2)	PA(18:1/18:1)	[M-H]-	699.497	0	PA	"fed-batch" Day 12
702.535	PE(O-34:1)	PE(O-16:0_18:1)	[M-H]-	702.544	-12.2	PE ether lipid	"non-production"
714.508	PE(34:2)	PE(16:1_18:1)	[M-H]-	714.507	0.6	PE	"fed-batch" Day 6, "fed-batch" Day 12
715.572	PE-Cer(d38:1)	-	[M-H]-	715.575	-4.8	Ceramide	"non-production", "fed-batch" Day 6
716.523	PE(34:1)	PE(16:0_18:1)	[M-H]-	716.523	0	PE	"non-production", "fed-batch" Day 12
718.534	PE(34:0)	-	[M-H]-	718.539	-7.1	PE	"fed-batch" Day 12
722.505	PE(O-36:5)	-	[M-H]-	722.513	-10.9	PE ether lipid	"fed-batch" Day 6
724.528	PE(O-36:4)	-	[M-H]-	724.528	0.1	PE ether lipid	"non-production"
725.517	PA(38:3)	-	[M-H]-	725.512	5.9	PA	"fed-batch" Day 6
726.543	PE(O-36:3)	PE(O-18:2_18:1)	[M-H]-	726.544	-1.1	PE ether lipid	"non-production"

728.558	PE(O-36:2)	PE(O-18:1_18:1)	[M-H]-	728.559	-2.1	PE ether lipid	"fed-batch" Day 6
730.571	PE(O-36:1)	PE(O-18:1_18:0)	[M-H]-	730.575	-5.7	PE ether lipid	"non-production", "fed-batch" Day 6
732.580	PE(O-36:0)	-	[M-H]-	732.591	-14.1	PE ether lipid	"non-production"
734.533	GlcCer(d34:1)	-	[M+Cl]-	734.534	-1.8	Ceramide	"non-production", "fed-batch" Day 12
737.536	SM(d34:1)	-	[M+Cl]-	737.536	-0.3	SM	"fed-batch" Day 12
742.539	PE(36:2)	PE(18:1/18:1)	[M-H]-	742.539	0	PE	"non-production", "fed-batch" Day 12
744.554	PE(36:1)	PE(18:1_18:0)	[M-H]-	744.554	-0.7	PE	"fed-batch" Day 12
745.502	PG(34:2)	PG(16:1_18:1)	[M-H]-	745.502	-0.3	PG	"fed-batch" Day 6, "fed-batch" Day 12
747.518	PG(34:1)	PG(16:0_18:1)	[M-H]-	747.518	-0.1	PG	"non-production", "fed-batch" Day 12
749.526	PG(34:0)	PG(16:0_18:0)	[M-H]-	749.533	-10.1	PG	"fed-batch" Day 12
752.561	PE(O-38:4)	-	[M-H]-	752.559	2.1	PE ether lipid	"non-production", "fed-batch" Day 12
754.578	PE(O-38:3)	-	[M-H]-	754.575	3.4	PE ether lipid	"non-production"
756.593	PE(O-38:2)	PE(O-18:1_20:1)	[M-H]-	756.591	3	PE ether lipid	"fed-batch" Day 6
758.606	PE(O-38:1)	PE(O-18:1_20:0)	[M-H]-	758.606	0	PE ether lipid	"non-production", "fed-batch" Day 12
759.573	DG(44:6)	-	[M+Cl]-	759.570	3.9	DG	"fed-batch" Day 6, "fed-batch" Day 12
766.519	PC(32:1)	PC(14:0_18:1)	[M+Cl]-	766.515	4.3	PC	"fed-batch" Day 12
770.573	PE(38:2)	PE(18:1_20:1)	[M-H]-	770.570	4.2	PE	"non-production", "fed-batch" Day 12
771.519	PG(36:3)	PG(18:2_18:1)	[M-H]-	771.518	1.7	PG	"fed-batch" Day 12
772.583	PE(38:1)	PE(18:0_20:1)	[M-H]-	772.586	-3.5	PE	"non-production", "fed-batch" Day 12
773.533	PG(36:2)	PG(18:1/18:1)	[M-H]-	773.533	0	PG	"non-production", "fed-batch" Day 12
774.536	PE(O-40:7)	-	[M-H]-	774.544	-9.4	PE ether lipid	"fed-batch" Day 12
775.546	PG(36:1)	PG(18:1_18:0)	[M-H]-	775.549	-3.2	PG	"non-production", "fed-batch" Day 6
776.551	PE(O-40:6)	PE(O-18:0_22:6)	[M-H]-	776.559	-10.4	PE ether lipid	"non-production", "fed-batch" Day 6
780.567	PC(O-34:1)	-	[M+Cl]-	780.567	-1	PC ether lipid	"fed-batch" Day 12
782.652	GlcCer(d40:1)	-	[M-H]-	782.651	1.3	Ceramide	"non-production"
786.525	PS(36:2)	PS(18:1_18:1)	[M-HI]-	786.529	-4.8	PS	"non-production"
788.544	PS(36:1)	PS(18:1_18:0)	[M-H]-	788.544	-0.9	PS	"fed-batch" Day 12
791.588	SM(d38:2)	-	[M+Cl]-	791.583	5.4	SM	"non-production", "fed-batch" Day 6, "fed-batch" Day 12
792.531	PC(34:2)	PC(16:1_18:1)	[M+Cl]-	792.531	-0.3	PC	"non-production", "fed-batch" Day 12
794.546	PC(34:1)	PC(16:0_18:1)	[M+Cl]-	794.547	-0.6	PC	"fed-batch" Day 12
798.531	PC(O-36:6)	-	[M+Cl]-	798.521	12.5	PC ether lipid	"fed-batch" Day 6

798.602	PE(40:2)	PE(18:1_22:1)	[M-H]-	798.601	1	PE	"fed-batch" Day 6, "fed-batch" Day 12
800.496	PE(38:5)	-	[M+Cl]-	800.500	-5.1	PE	"fed-batch" Day 6
800.613	PE(40:1)	PE(18:1_22:0)	[M-H]-	800.617	-5.2	PE	"fed-batch" Day 6
803.577	PG(38:1)	-	[M-H]-	803.580	-4.6	PG	"fed-batch" Day 6
805.611	TG(46:4)	TG(16:0_18:1_22:3)	[M+Cl]-	805.611	-0.5	TG	"fed-batch" Day 12
806.593	PC(O-36:2)	-	[M+Cl]-	806.583	12.8	PC ether lipid	"fed-batch" Day 6
806.611	PE(O-42:5)	-	[M-H]-	806.606	5.1	PE ether lipid	"fed-batch" Day 12
808.666	GlcCer(d42:2)	-	[M-H]-	808.667	-1.2	Ceramide	"non-production"
810.681	GlcCer(d42:1)	-	[M-H]-	810.682	-1.6	Ceramide	"non-production", "fed-batch" Day 12
812.689	GlcCer(42:0)	-	[M-H]-	812.698	-10.6	Ceramide	"fed-batch" Day 6
820.563	PC(36:2)	PC(18:1/18:1)	[M+Cl]-	820.562	1.3	PC	"non-production", "fed-batch" Day 12
822.568	PC(36:1)	PC(18:1_18:0)	[M+Cl]-	822.578	-12.8	PC	"non-production", "fed-batch" Day 12
826.545	PC(O-38:6)	-	[M+Cl]-	826.552	-8.3	PC ether lipid	"fed-batch" Day 6
828.648	PE(42:1)	-	[M-H]-	828.648	-0.6	PE	"fed-batch" Day 12
830.591	PC(O-38:4)	PC(O-18:3_20:1)	[M+Cl]-	830.583	9	PC ether lipid	"fed-batch" Day 6, "fed-batch" Day 12
832.603	PC(O-38:3)	PC(O-18:2_20:1)	[M+Cl]-	832.599	5.6	PC ether lipid	"non-production"
835.534	PI(34:1)	PI(16:0_18:1)	[M-H]-	835.534	0	PI	"non-production", "fed-batch" Day 12
836.538	PS(40:5)	-	[M-H]-	836.544	-8	PS	"fed-batch" Day 12
837.541	PI(34:0)	-	[M-H]-	837.549	-10.3	PI	"fed-batch" Day 12
842.592	PS(40:2)	-	[M-H]-	842.591	0.5	PS	"non-production"
844.608	PS(40:1)	PS(18:0_22:1)	[M-H]-	844.607	1.3	PS	"non-production"
846.624	PS(40:0)	-	[M-H]-	846.622	1.3	PS	"non-production"
846.656	GlcCer(d42:1)	-	[M+Cl]-	846.659	-3.7	Ceramide	"fed-batch" Day 6
848.601	PC(38:2)	PC(18:0_20:2)	[M+Cl]-	848.594	8.7	PC	"fed-batch" Day 12
849.658	SM(42:1)	-	[M+Cl]-	849.662	-4.1	SM	"fed-batch" Day 6, "fed-batch" Day 12
854.552	PE(42:6)	-	[M+Cl]-	854.547	6.1	PE	"non-production"
856.561	PE(42:5)	PE(20:3_22:2)	[M+Cl]-	856.562	-2.1	PE	"fed-batch" Day 6
859.536	PI(36:3)	-	[M-H]-	859.534	2.1	PI	"fed-batch" Day 6, "fed-batch" Day 12
860.608	LacCer(d34:1)	-	[M-H]-	860.610	-2.8	Ceramide	"non-production", "fed-batch" Day 6
861.546	PI(36:2)	PI(18:1/18:1)	[M-H]-	861.549	-3.5	PI	"non-production", "fed-batch" Day 12
863.558	PI(36:1)	PI(18:1_18:0)	[M-H]-	863.565	-7.8	PI	"non-production", "fed-batch" Day 12
865.568	PI(36:0)	-	[M-H]-	865.581	-14.1	PI	"fed-batch" Day 12

870.618	PS(42:2)	-	[M-H]-	870.623	-5.1	PS	"fed-batch" Day 6
874.616	PC(40:3)	-	[M+Cl]-	874.609	8.1	PC	"fed-batch" Day 12
875.620	PA(46:2)	-	[M+Cl]-	875.630	-11.3	PA	"non-production", "fed-batch" Day 12
876.629	PC(40:2)	-	[M+Cl]-	876.625	4.6	PC	"fed-batch" Day 12
878.632	PC(40:1)	PC(18:0_22:1)	[M+Cl]-	878.641	-9.8	PC	"fed-batch" Day 6
887.565	PI(38:3)	-	[M-H]-	887.565	0	PI	"fed-batch" Day 6, "fed-batch" Day 12
889.578	PI(38:2)	-	[M-H]-	889.581	-2.5	PI	"fed-batch" Day 12
892.599	PS(44:5)	-	[M-H]-	892.607	-8.5	PS	"fed-batch" Day 12
923.650	PG(44:1)	-	[M+Cl]-	923.651	-1.1	PG	"fed-batch" Day 6
971.724	TG(60:12)	-	[M+Cl]-	971.726	-1.7	TG	"non-production"
984.765	LacCer(d44:0)	-	[M-H ₂ O-H]-	984.772	-6.3	Ceramide	"non-production", "fed-batch" Day 6, "fed-batch" Day 12
1235.801	Ganglioside GM3 (d40:1)	-	[M-H]-	1235.799	1	Ganglioside	"fed-batch" Day 6
1261.816	Ganglioside GM3 (d42:2)	-	[M-H]-	1261.815	0.5	Ganglioside	"fed-batch" Day 6
1263.835	CL(58:2)	-	[M-H]-	1263.839	-3.4	CL	"non-production", "fed-batch" Day 6
1265.853	Ganglioside GM3 (d42:0)	-	[M-H]-	1265.846	5.5	Ganglioside	"fed-batch" Day 6
1399.959	CL(68:4)	-	[M-H]-	1399.965	-4.1	CL	"non-production", "fed-batch" Day 12
1402.056	PE(O-34:2)	PE(O-18:1_18:1)	[2M-H]-	1402.064	-6.1	PE ether lipid	"non-production"
1418.053	CL(70:0)	-	[M-H ₂ O-H]-	1418.048	3.4	CL	"non-production", "fed-batch" Day 6
1427.990	CL(70:4)	-	[M-H]-	1427.996	-3.9	CL	"non-production", "fed-batch" Day 6, "fed-batch" Day 12
1434.083	CL(70:1)	-	[M-H]-	1434.043	27.8	CL	"non-production"
1442.048	CL(72:2)	-	[M-H ₂ O-H]-	1442.048	0	CL	"non-production"
1448.079	PA(28:4)	-	[2M-H]-	1448.001	53.8	PA	"non-production"
1449.071	CL(72:7)	-	[M-H]-	1449.980	-627.2	CL	"fed-batch" Day 6, "fed-batch" Day 12
1456.026	CL(72:4)	-	[M-H]-	1456.027	-0.5	CL	"fed-batch" Day 6, "fed-batch" Day 12
1464.081	CL(72:0)	-	[M-H]-	1464.090	-5.8	CL	"fed-batch" Day 6
1472.115	CL(70:0)	-	[M+Cl]-	1472.035	54.1	CL	"fed-batch" Day 6
1474.080	CL(74:0)	-	[M-H ₂ O-H]-	1474.110	-20.5	CL	"fed-batch" Day 6, "fed-batch" Day 12
1480.025	CL(74:6)	-	[M-H]-	1480.027	-1.8	CL	"fed-batch" Day 6
1484.032	CL(76:9)	-	[M-H ₂ O-H]-	1484.001	20.7	CL	"fed-batch" Day 6
1494.079	CL(76:4)	-	[M-H ₂ O-H]-	1494.079	0.1	CL	"non-production"
1498.053	CL(72:1)	-	[M+Cl]-	1498.051	1.8	CL	"non-production"

1498.101	CL(76:2)	-	[M-H ₂ O-H] ⁻	1498.110	-6.1	CL	"non-production"
----------	----------	---	-------------------------------------	----------	------	----	------------------

Table 15. Complete list of the RFECV feature annotation of all three CHO culturing conditions, excluding isotope peaks and unidentified features. The annotation was performed based on accurate mass, isotopic pattern, and chemical and biological occurrence. Feature identification is included for the features with MS/MS data available.

Supplementary material/Chapter 5:

Compound	LLOQ conc [pg/mL]	Rat lung control (ng/mL)	Rat lung treated with amiodarone (ng/mL)	Fold change (Amiodarone/control)
12(S)-HEPE	250	<LLOQ	<LLOQ	<LLOQ
15(S)-HEPE	250	<LLOQ	<LLOQ	<LLOQ
5,6-EET	100	0.0192	<LLOQ	<LLOQ
8,9-EET	250	<LLOQ	<LLOQ	<LLOQ
11,12-EET	100	0.0213	0.0267	1.25
14,15-EET	1000	<LLOQ	<LLOQ	<LLOQ
5(S)-HETE	100	0.0946	0.1269	1.34
8(S)-HETE	100	0.0539	0.0826	1.53
11(R)-HETE	100	0.0889	0.1783	2.00
12(R)-HETE	100	0.1694	0.1700	1.00
15(S)-HETE	250	0.1227	0.2179	1.78
16(R)-HETE	100	0.0246	0.0267	1.09
5,6-DHET	100	<LLOQ	<LLOQ	<LLOQ
8,9-DHET	100	<LLOQ	<LLOQ	<LLOQ
11,12-DHET	100	<LLOQ	<LLOQ	<LLOQ
14,15-DHET	100	0.0267	<LLOQ	<LLOQ
5-oxo-EETE	250	0.0473	0.2659	5.62
12-oxo-EETE	500	<LLOQ	0.1995	<LLOQ
14-HDoHE	250	0.0904	<LLOQ	<LLOQ
17(S)-HDoHE	100	0.0878	<LLOQ	<LLOQ
10(S),17(S)- DiHDoHE	100	<LLOQ	<LLOQ	<LLOQ
LTB4	100	<LLOQ	<LLOQ	<LLOQ
12-oxo-LTB4	100	<LLOQ	<LLOQ	<LLOQ
LTC4	500	<LLOQ	<LLOQ	<LLOQ
LTD4	500	<LLOQ	<LLOQ	<LLOQ
LTE4	100	<LLOQ	<LLOQ	<LLOQ
PGD2	100	0.0245	0.0560	2.28
PGE2	100	<LLOQ	<LLOQ	<LLOQ
Lipoxin A4	250	<LLOQ	<LLOQ	<LLOQ
Lipoxin B4	250	<LLOQ	<LLOQ	<LLOQ
6-keto- PGF1alpha	250	0.5650	0.4375	0.77
PGF2alpha	100	<LLOQ	0.04	<LLOQ
8-iso- PGF2alpha	250	<LLOQ	<LLOQ	<LLOQ
15dPGJ2	100	<LLOQ	<LLOQ	<LLOQ
TXB2	250	0.1157	0.1206	1.04
11-dehydro TXB2	100	<LLOQ	<LLOQ	<LLOQ
Resolvin D1	100	<LLOQ	<LLOQ	<LLOQ
Resolvin D2	250	<LLOQ	<LLOQ	<LLOQ

Table 16. Oxylipin quantification results. LLOQ: Lower Limit of Quantification

List of conference presentations

Podium presentations

1. Maneta-Stavrakaki, S. *et al.* High-throughput lipidomics analysis directly on cell culture using Laser-Ablation Rapid Evaporative Mass Spectrometry (LA-REIMS). in *BMSS annual meeting* (2018).
2. Maneta-Stavrakaki, S. *et al.* Laser-Ablation Rapid Evaporative Ionization Mass Spectrometry (LA- REIMS) for fast lipidomic analysis of genetically modified CHO cells in ambient conditions. in *67th ASMS Conference* (2019).
3. Maneta-Stavrakaki, S. *et al.* High-Throughput lipidomic analysis of genetically modified CHO cells under ambient conditions using Laser-Ablation REIMS. in *BMSS annual meeting* (2019).
4. Maneta-Stavrakaki, S. *et al.* Ambient Ionisation Mass Spectrometry Imaging for the investigation of the amiodarone induced phospholipidosis mechanism in an animal model. in *OurCon VII* (2019).

Poster presentations

1. Maneta-Stavrakaki, S. *et al.* Laser-Assisted Rapid Evaporative Ionization Mass Spectrometry (LA- REIMS) for lipidomics analysis of living CHO cells directly from the cell culture. in *The International Mass Spectrometry Conference* (2018).
2. Maneta-Stavrakaki, S. *et al.* High-throughput lipidomics and metabolomics analysis directly on genetically modified CHO cells using Laser-Ablation Rapid Evaporative Mass Spectrometry (LA-REIMS). in *Metabolomics* (2019).

Permissions to reprint

30/11/2021, 20:15

Rightslink® by Copyright Clearance Center



Home

Help

Live Chat

Sign in

Create Account



Fractional Derivatives modeling dielectric properties of biological tissue

Conference Proceedings:

2018 IEEE XXV International Conference on Electronics, Electrical Engineering and Computing (INTERCON)

Author: J.E. Escalante-Martinez

Publisher: IEEE

Date: Aug. 2018

Copyright © 2018, IEEE

Thesis / Dissertation Reuse

The IEEE does not require individuals working on a thesis to obtain a formal reuse license, however, you may print out this statement to be used as a permission grant:

Requirements to be followed when using any portion (e.g., figure, graph, table, or textual material) of an IEEE copyrighted paper in a thesis:

- 1) In the case of textual material (e.g., using short quotes or referring to the work within these papers) users must give full credit to the original source (author, paper, publication) followed by the IEEE copyright line © 2011 IEEE.
- 2) In the case of illustrations or tabular material, we require that the copyright line © (Year of original publication) IEEE appear prominently with each reprinted figure and/or table.
- 3) If a substantial portion of the original paper is to be used, and if you are not the senior author, also obtain the senior author's approval.

Requirements to be followed when using an entire IEEE copyrighted paper in a thesis:

- 1) The following IEEE copyright/ credit notice should be placed prominently in the references: © (year of original publication) IEEE. Reprinted, with permission, from (author names, paper title, IEEE publication title, and month/year of publication)
- 2) Only the accepted version of an IEEE copyrighted paper can be used when posting the paper or your thesis online.
- 3) In placing the thesis on the author's university website, please display the following message in a prominent place on the website: In reference to IEEE copyrighted material which is used with permission in this thesis, the IEEE does not endorse any of (university/educational entity's name goes here)'s products or services. Internal or personal use of this material is permitted. If interested in reprinting/republishing IEEE copyrighted material for advertising or promotional purposes or for creating new collective works for resale or redistribution, please go to http://www.ieee.org/publications_standards/publications/rights/rights_link.html to learn how to obtain a License from RightsLink.

If applicable, University Microfilms and/or ProQuest Library, or the Archives of Canada may supply single copies of the dissertation.

BACK

CLOSE WINDOW



Effect of Average Phospholipid Curvature on Supported Bilayer Formation on Glass by Vesicle Fusion

Author: Chiho Hamai, Tinglu Yang, Sho Kataoka, Paul S. Cremer, Siegfried M. Musser

Publication: Biophysical Journal

Publisher: Elsevier

Date: 15 February 2006

Copyright © 2006 The Biophysical Society. Published by Elsevier Inc. All rights reserved.

Order Completed

Thank you for your order.

This Agreement between Ms. Stefania Maneta-Stavrakaki ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

License Number 5278851023315

[Printable Details](#)

License date Mar 30, 2022

Licensed Content

Licensed Content Publisher	Elsevier
Licensed Content Publication	Biophysical Journal
Licensed Content Title	Effect of Average Phospholipid Curvature on Supported Bilayer Formation on Glass by Vesicle Fusion
Licensed Content Author	Chiho Hamai, Tinglu Yang, Sho Kataoka, Paul S. Cremer, Siegfried M. Musser
Licensed Content Date	Feb 15, 2006
Licensed Content Volume	90
Licensed Content Issue	4
Licensed Content Pages	8

Order Details

Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No

About Your Work

Title	Development and Validation of a High-Throughput Mass Spectrometric Platform for the Molecular Characterisation of Cells
Institution name	Imperial College London
Expected presentation date	May 2022

Additional Data

Portions	Figure 1
----------	----------



Home



Help ▾



Live Chat



Stefania Maneta-Stavrakaki ▾



QqQ and Q-TOF liquid chromatography mass spectrometry direct aqueous analysis of herbicides and their metabolites in water

Author: Luigi Lucini, Roberto Pellegrino, Nicola Cimino, David Kane, Luca Pretali

Publication: International Journal of Mass Spectrometry

Publisher: Elsevier

Date: 3 December 2015

Copyright © 2015 Elsevier B.V. All rights reserved.

Order Completed

Thank you for your order.

This Agreement between Ms. Stefania Maneta-Stavrakaki ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

License Number 5285981056159

[Printable Details](#)

License date Apr 11, 2022

Licensed Content

Licensed Content Publisher	Elsevier
Licensed Content Publication	International Journal of Mass Spectrometry
Licensed Content Title	QqQ and Q-TOF liquid chromatography mass spectrometry direct aqueous analysis of herbicides and their metabolites in water
Licensed Content Author	Luigi Lucini, Roberto Pellegrino, Nicola Cimino, David Kane, Luca Pretali
Licensed Content Date	Dec 3, 2015
Licensed Content Volume	392
Licensed Content Issue	n/a
Licensed Content Pages	7

Order Details

Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No

About Your Work

Title	Development and Validation of a High-Throughput Mass Spectrometric Platform for the Molecular Characterisation of Cells
Institution name	Imperial College London
Expected presentation date	May 2022

Additional Data

Portions	Graphical abstract
----------	--------------------



This is a License Agreement between Stefania Maneta-Stavarakaki ("User") and Copyright Clearance Center, Inc. ("CCC") on behalf of the Rightsholder identified in the order details below. The license consists of the order details, the CCC Terms and Conditions below, and any Rightsholder Terms and Conditions which are included below.

All payments must be made in full to CCC in accordance with the CCC Terms and Conditions below.

Order Date	29-Nov-2021	Type of Use	Republish in a thesis/dissertation
Order License ID	1164857-1	Publisher Portion	Masson; Wiley
ISBN-13	9780471966968		Image/photo/illustration

LICENSED CONTENT

Publication Title	Mass spectrometry : principles and applications	Country	United Kingdom of Great Britain and Northern Ireland
Author/Editor	Hoffmann, Edmond de., Charette, Jean Joseph., Stroobant, Vincent.	Rightsholder	John Wiley & Sons - Books
Date	01/01/1996	Publication Type	Book
Language	English, French		

REQUEST DETAILS

Portion Type	Image/photo/illustration	Distribution	Worldwide
Number of images / photos / illustrations	1	Translation	Original language of publication
Format (select all that apply)	Print, Electronic	Copies for the disabled?	No
Who will republish the content?	Academic institution	Minor editing privileges?	Yes
Duration of Use	Life of current edition	Incidental promotional use?	No
Lifetime Unit Quantity	Up to 499	Currency	GBP
Rights Requested	Main product		

NEW WORK DETAILS

Title	Ms	Institution name	Imperial College London
Instructor name	Stefania Maneta-Stavarakaki	Expected presentation date	2021-12-15

ADDITIONAL DETAILS

The requesting person / organization to appear on the license	Stefania Maneta-Stavarakaki
---	-----------------------------

REUSE CONTENT DETAILS

Title, description or numeric reference of the portion(s)	Mass Spectrometry Principles and Application	Title of the article/chapter the portion is from	Ion Sources
Editor of portion(s)	N/A	Author of portion(s)	Hoffmann, Edmond de.; Charette, Jean Joseph.; Stroobant, Vincent.
Volume of serial or monograph	N/A		

