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REPORT OF PROFESSIONAL

INTERNSHIP I

LIONEL MENDES DIAS

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The Health School

Polytechnic Institute of Guarda

1ST CYCLE DEGREE ON PHARMACY
4TH ACADEMIC YEAR / 1ST SEMESTER

REPORT OF PROFESSIONAL INTERNSHIP I

THE INSTITUTE OF CANCER RESEARCH
CLINICAL PHARMACOLOGY - DMPK

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ABSTRACT

Metabolomics is an analytical tool used to detect metabolites in biofluids or tissues and thereby identify and quantify metabolites commonly detected in the human serum metabolome. Cancer cells possess a unique metabolic phenotype that makes it possible to identify fingerprints, profiles or signatures to detect cancer and evaluate the pharmacodynamic effects of therapy. Krebs cycle acids have the potential to be used as biomarkers when used with other metabolites profile. Problems resulting from the analysis of fatty acids by LC-MS have been resolved with the use of N-(4-aminomethylphenyl) pyridinium (AMPP) derivatization. Through this project we show that it is possible to derivatize some of the Krebs acids and lactic acid using that same process used for fatty acids. We also proved that is possible to retain derivatized pyruvic acid under LC-MS conditions.

Key words: Cancer; Metabolomics; Krebs cycle acids; Fatty acids and AMPP derivatization.

ACKNOWLEDGEMENTS

QUOTE

"If you rely on others to make you happy, you will be endlessly disappointed. Happiness radiates like the fragrance from a flower, and draws all good things toward you."

Maharishi Mohesh Yogi

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ABBREVIATIONS

ADME - Absorption, Distribution, Metabolism and Excretion;

AMPP - N-(4-aminomethylphenyl) pyridinium;

API - Atmospheric Pressure Ionisation;

ATP - Adenosine Tri-Phosphate;

CID - Collision-Induced Dissociation;

DMPK - Drug Metabolism and PharmacoKinetic teams;

ESI - ElectroSpray Ionization;

FDA - Food and Drug Administration;

GC - Gas Chromatography;

HPLC - High Performance Liquid Chromatography;

ICR - Institute of Cancer Research;

IND - Investigational New Drug;

LC - Liquid Chromatography;

MRM - Multiple Reaction Monitoring;

MS - Mass Spectrometry;

NADPH - Nicotinamide Adenine Dinucleotide Phosphate Hydrogenase;

NDA - New Drug Application;

NMR - Nuclear Magnetic Resonance;

PD - Pharmacodynamic;

PK - Pharmacokinetic;

PPP - Pentose Phosphate Pathway;

SIR - Selected Ion Recording;

Target ID - Target Identification & Discovery;

TCA - Tri-Carboxylic Acid;

UPLC - Ultra Performance Liquid Chromatography.

1 – INTRODUCTION

1.1 - CANCER WORLD IMPACT

All over the world, particularly for developed countries, cancer has become one of the most common health problems. According to the International Agency for Cancer on Research, there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide.[1] The same source states that lung cancer, breast and colorectal are the most frequent cancers in the population during 2012.[1]

Cancer is fundamentally a disease at the cellular level, where the cancer cell fails to respond to the controls that regulate normal cell growth and division. Such tumour progression is thought to occur by a series of clonal selections, involving genetic variation and selection for more rapidly growing cells within the tumour population.[2] Cancers thus develop in a stepwise fashion from initially altered cells, which have begun to proliferate abnormally, to metastatic tumours, which have spread to distant body sites.[2]

In accordance with that described by the World Health Organization, Cancer arises from interactions between genetic factors and external agents. These include physical carcinogens (such as ultraviolet ionizing radiation); chemical carcinogens (such as components of tobacco smoke, food, alcohol and medicines) and biological carcinogens (such as infections from certain viruses, bacteria or parasites).[3] About 30% of cancer deaths are due to the five leading behavioural and dietary factors for cancer where tobacco use is the most important causing 22% of global cancer deaths and 71% of global lung cancer deaths.[1,3] In spite of this generalization, there are a number of occurrences in which an individual's susceptibility to cancer is affected by heredity.

1.2 – CANCER AND NORMAL CELLS

Comparative studies of cell growth and behaviour have led to the recognition of a number of specific properties that distinguish many cancer cells from their normal counterparts.[2]

In contrast to normal differentiated cells, which rely primarily on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes, most cancer cells instead rely on aerobic glycolysis, a phenomenon termed "the Warburg effect" (see Figure 1.1).[4]
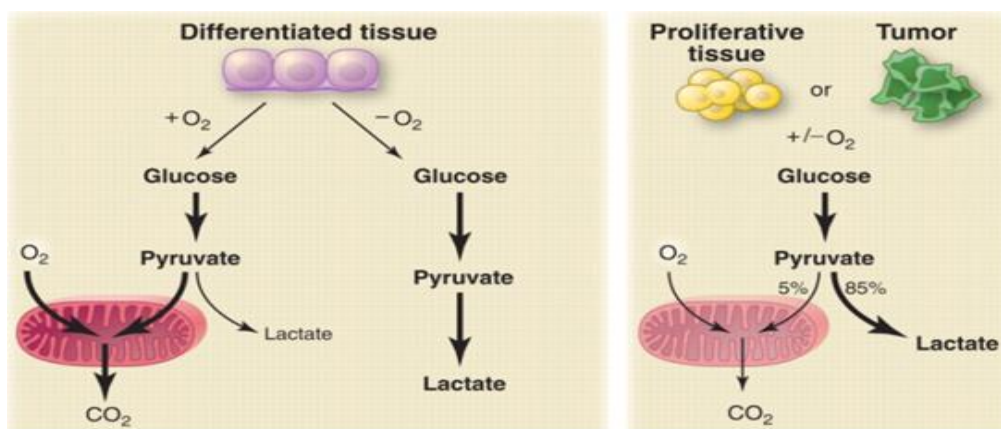
Figure 1.1 – Schematic illustration of the Warburg effect.[4]

By examining how Louis Pasteur's observations regarding fermentation of glucose to ethanol might apply to mammalian tissues, Warburg found that unlike most normal tissues, cancer cells tend to "ferment" glucose into lactate even in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation.[4,5] Glycolysis produces only two Adenosine Tri-Phosphate (ATP) molecules per glucose molecule, compared with 38 for complete oxidation.[5] But for a cancer cell, it's actually highly efficient, because it needs to produce ATP as fast as you want to supply growth and proliferation. [4,5] At the same time, the metabolism of cancer cells and all proliferating cells, is adapted to facilitate the uptake and incorporation of nutrients into the biomass (e.g., nucleotides, amino acids, and lipids) needed to produce a new cell (see Figure 1.2). [6]
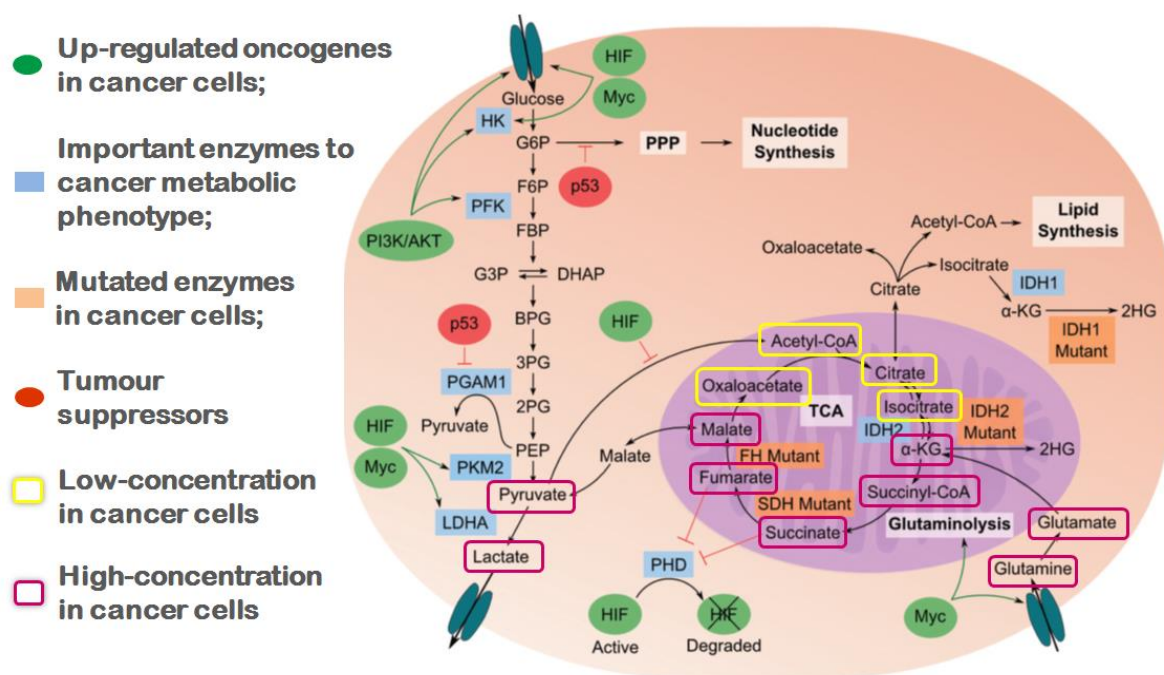
Figure 1.2 – Cancer metabolism illustration.[6] In many cancer cells, glucose is mainly used for the glycolytic pathway, leading to a generation of lactate and important metabolic intermediates such as glucose-6-phosphate for the Pentose Phosphate Pathway (PPP) that generates Nicotinamide Adenine Dinucleotide Phosphate Hydrogenase (NADPH) and ribose for maintaining redox balance and synthesis of nucleic acids.[4,6] The flow of glucose into mitochondria in the form of pyruvate is relatively low in cancer cells. Glutamine is actively metabolized in cancer cells, both in the cytosol and in the mitochondria, where it is catalysed by glutaminase to generate glutamate, which is further converted to alpha-ketoglutarate for utilization through the Tri-Carboxylic Acid (TCA) cycle. Both glycolysis and glutaminolysis provide important metabolic intermediates that serve as substrates for other pathways including the synthesis of nucleic acids, fatty acids, amino acids, and glutathione.[4,6]

1.3 – METABOLOMICS AND CANCER APPLICATIONS

Our understanding of the origin, development and treatment of cancer has greatly been supported by novel "-omics" technologies such as genomics, proteomics and metabolomics that are utilised to quantify and qualify the effect of biological molecules.

Metabolomics is an analytical tool used in conjunction with pattern recognition approaches and bioinformatics to detect metabolites and follow their changes in biofluids or tissue.[7] Since cancer cells are known to possess a highly unique metabolic phenotype, development of specific biomarkers in oncology is possible and might be used in identifying

fingerprints, profiles, or signatures to detect the presence of cancer, determine prognosis, predict response and/or assess the pharmacodynamic (PD) effects of therapy.[6,7]

In order to achieve comprehensive biochemical information on metabolomics samples, targeted and non-targeted NMR (Nuclear Magnetic Resonance), GC-MS (Gas Chromatography Mass Spectrometry) and LC-MS (Liquid Chromatography Mass Spectrometry) methods with computer-aided literature mining have been combined to identify and quantify a set of metabolites commonly detected and in the human serum metabolome (see Table 1.1).[8] Serum plasma is easily obtainable and in large quantities compared with tumours.

Table 1.1 – Chemical classes in the serum metabolome database. [8]

Compound class	Number	Compound class	Number
Acyl glycines	10	Indoles and indole derivatives	12
Acyl phosphates	10	Inorganic ions and gases	20
Alcohol phosphates	2	Keto acids	8
Alcohols and polyols	40	Ketones	6
Aldehydes	3	Leukotrienes	8
Alkanes and alkenes	10	Lipoamides and derivatives	0
Amino acid phosphates	1	Minerals and elements	40
Amino acids	114	Miscellaneous	77
Amino alcohols	14	Nucleosides	24
Amino ketones	14	Nucleotides	24
Aromatic acids	22	Peptides	21
Bile acids	19	Phospholipids	2177
Biotin and derivatives	2	Polyamines	11
Carbohydrates	35	Polyphenols	22
Carnitines	22	Porphyrins	6
Catecholamines and derivatives	21	Prostanoids	23
Cobalamin derivatives	4	Pterins	14
Coenzyme A derivatives	1	Purines and purine derivatives	11
Cyclic amines	9	Pyridoxals and derivatives	7
Dicarboxylic acids	17	Pyrimidines and pyrimidine derivatives	2
Fatty acids	65	Quinones and derivatives	3
Glucuronides	8	Retinoids	11
Glycerolipids	1070	Sphingolipids	3
Glycolipids	15	Steroids and steroid derivatives	109
Hydroxy acids	129	Sugar phosphates	9
		Tricarboxylic acids	2

LC-MS is a technique which combines the separating power of High Performance Liquid Chromatography (HPLC) or Ultra Performance Liquid Chromatography (UPLC) with the detection power of mass spectrometry.

In its simplest form the process of mass analysis in LC-MS involves the separation or filtration of analyte ions (or fragments of analyte ions), created in the Atmospheric Pressure Ionisation (API) interface by ElectroSpray Ionization (ESI) in a high vacuum region of the mass analyser.[9] In API, the solvent molecules are removed from the HPLC eluent and this initiates the charging process of the analyte molecule. The analyte and fragment ions are

plotted in terms of their mass-to-charge ratio (m/z) against the abundance of each mass to yield a mass spectrum of the analyte through a detector.[9,10]

Tandem mass spectrometry (or MS/MS analysis) is used to obtain structural information by fragmenting ions produced in the API interface.[11] This method is used to produce structural information for analyte elucidation or to follow specific fragmentation reactions in order to greatly improve the specificity of the detection technique by identifying either the precursor or product ions.[9,11] Most common instruments use a combination of quadrupoles with a collision cell between the analyzing devices in which the emergent ions from the first analyzer are fragmented prior to secondary mass filtering (see Figure 1.3).[10,11]
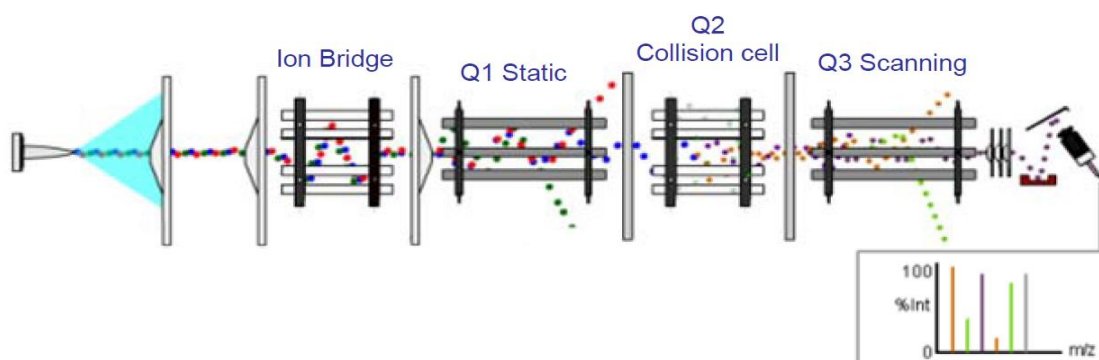
Figure 1.3 – Schematic diagram of multiple reaction monitoring performed on a triple quadrupole mass analyser.[9]

In chromatography, the analyte must be soluble in the mobile phase and the samples have to be prepared in a solvent system that has the same or less organic solvent than the mobile phase.[10] The mobile phase is continuously pumped at a fixed flow rate through the system when the injector is used to introduce a sample into the mobile phase.[12] The amount of water in the mobile phase will determine how strongly a hydrophobic analyte is repelled/attracted to the stationary phase. Optimisation of the HPLC process, is affected by mobile phase composition; bonded phase chemistry, column (reversed-phase column, normal-phase or HILIC column) and packing dimension; injection volume; sample pre-treatment and concentration; flow rate; the column type (e.g. C18 reversed-phase column), temperature and detector parameters.[9,10,12]

1.4 – DRUG DISCOVERY AND DEVELOPMENT

In drug research and development, the patient is the main focus. The mission of drug discovery is to help the patient recover from diseases and improve their quality of life. The drug development process is designed to ensure that innovative new medicines are effective, safe and available for the community in the shortest possible time.[13, 14]

Once a target has been selected and validated, a number of screening assay are developed to evaluate large libraries of compounds. The "hits" resulting from those screens are optimised for potency and selectivity resulting in a lead compound. For a successful optimisation, a complex and iterative process is required involving interplay of multiple parameters including affinity, permeability, solubility, activity, protein binding, cross reactivity and off-target effects. [15]

| Years: | 3 | 1 | 6 | 1.5 |
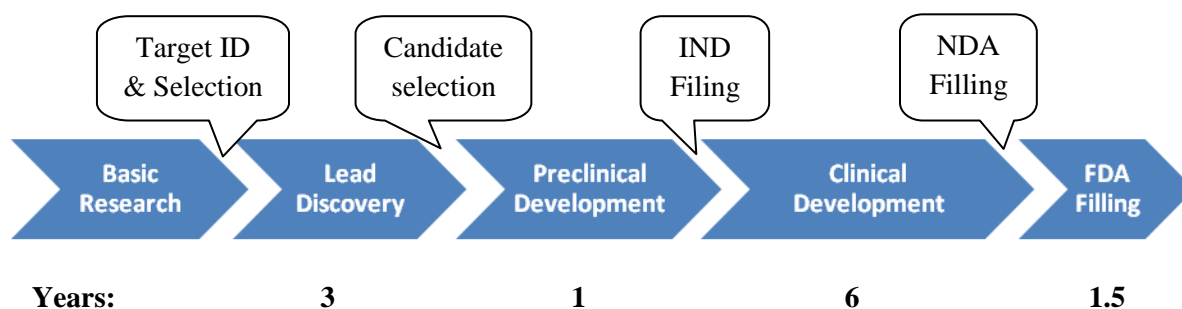
Figure 1.4 - Drug discovery process from Target ID and validation through to filing of a compound and the approximate timescale for these processes.[14] FDA (Food and Drug Administration); IND (Investigational New Drug); NDA (New Drug Application).

The establishment of in vivo activity and optimal scheduling in pre-clinical models is supported by favourable and optimised PK and PD models.

PK is the study of what the body does to an administered drug. Investigation of the time course of drug concentration within the body spaces such as plasma, blood, urine, cerebrospinal fluid and tissues via the processes of Absorption, Distribution, Metabolism and Excretion (ADME) yields parameters such as half-life, volume of distribution and clearance that can aid drug discovery.[17] PD can be defined as the study of the biological effects of drugs, the relationship of the effects to drug exposure and the mechanisms of drug action.[17]

The importance of PK/PD in drug development is becoming increasingly recognised and now permeates the program from preclinical development through to Phase IV clinical trials. In the clinical context tumours cannot be collected therefore circulating biomarkers are very useful. These studies are also carried out in early clinical trials during the clinical

development of these compounds. Only those compounds that deliver the optimal concentrations at the tumour site and result in appropriate target modulation will be developed.

1.5 – IMPROVEMENT OF FATTY ACIDS ANALYSIS BY DERIVATIZATION

The existent diversity of fatty acids on human body with differences at structural levels such as: varied chain lengths, varied degrees of unsaturation and locations of double bonds in chains, makes that group of molecules an important source of information on the human serum metabolome.[18,19]

The earliest quantification methods for free fatty acids relied on gas chromatography (GC) with flame ionization detection and coupled to a mass spectrometer via electron ionization.[18,20] However, this method is limited by the thermal stability and volatility of the compound. In contrast, the ESI-MS coupled with LC has dramatically improved the analysis of fatty acids in comparison to traditional ESI–GC.[20] However, a major obstacle to the ESI technique is that fatty acids undergo less than ideal fragmentation behaviour in negative ion mode via Collision-Induced Dissociation (CID).[18,20] Issues such as low sensitivity and ionization efficiency with weak fragmentations necessitated development of a new method to improve the sensitivity of these groups of molecules. [18,19]

To overcome the low ionization efficiency in the negative ion mode, charge-switch derivatization has been previously used to increase the sensivity of carboxylic acid analysis by LC/MS approaches.[18,19] A major limitation of these methodologies is the relatively harsh conditions usually required for derivatization, which can result in unwanted oxidation, isomerization or degradation of some fatty acids and unwanted tendency of derivatives to fragment via CID.[18] Fragmentation in the derivatization tag is undesirable due to the fact that analytes that form isobaric precursor ions and co-elute during LC will not be distinguished in the mass spectrometer if they give rise to the same detected fragment ion, essentially eliminating any advantage a MS/MS experiment has over a MS one.[18,20]

Recently, Bollinger et al reported the utilization of N-(4-aminomethylphenyl) pyridinium (AMPP) derivatization for eicosanoid analysis by LC-MS/MS with a dramatic improvement in sensitivity and the production of structurally informative fragment ions during CID.[20] This process generates a permanent positive charge with a 10- to 20-fold

improvement in detection sensitivity by LC-ESI-MS and specific MS/MS Fragmentation.[18,21] The AMPP can be coupled to free carboxylic acids through an amide bond linkage and converted to a cationic group so that MS could detect in positive ion mode (see Figure 1.5).[21] To optimize the reaction, the derivatization process makes use of high temperatures and the reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 1-Hydroxy-7-azabenzotriazole (HOAt) or N-hydroxybenzotriazole (HOBt) and N-hydroxybenzotriazole (HOBt) with acetonitrile.[18,20,21]
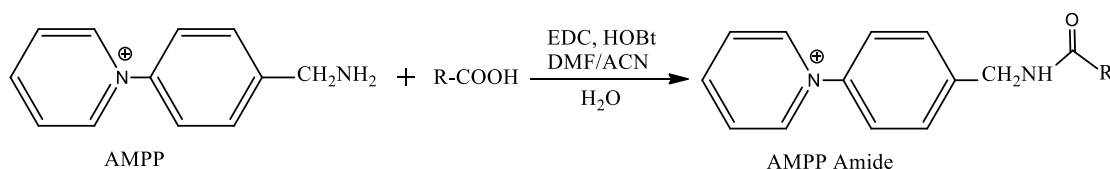
Figure 1.5 – Structure of AMPP and an AMPP amide along with the reagents used for derivatization.[19,21]

The derivatization process is easy, simple and fast to execute and the resulting derivatives can be directly submitted to LC-ESI-MS/MS. One can anticipate that the AMPP derivatization method can be extended to other carboxylic acid analytes for enhanced sensitivity detection.

1.6 – AIM OF THE PROFESSIONAL INTERNSHIP

This internship is within the DMPK department at the Institute of Cancer Research (ICR) in Sutton, Greater London, United Kingdom (UK). It has a planned duration of one academic year and began on 2th September 2013. Dr. Florence I Raynaud is my coordinator in this internship, Yasmin Asad and Ching Thai are my supervisors together with the DMPK team.

As part of this internship, I am participating in this research project called "AMPP Project" to investigate whether we can with the purpose of increase the sensitivity and selectivity detection of carboxylic acids presents in the Krebs cycle using the derivatization process with AMPP as demonstrated with fatty acids and explained in the literature above.

The main aim of this project is to achieve a better way to quantify and qualify Krebs acids with the LC-MS method in order to measure them in human plasma and obtain more information about the human metabolome, the individual response to a new treatment, the prognostic of cancer disease and many more options to be used in health care.

2 – MATERIAL

In order to accomplish this project, we used a Waters Xevo TQ-S Mass Spectrometer coupled to an Acquity UPLC H-class system. Other equipment and materials such as LC columns that were used are described in Appendix A.

Two groups of different reagents were used in the experiments. The Cayman kit is the same kit used in the literature for AMPP derivatization of fatty acids. This includes 20mM of AMPP derivatizing reagent in acetonitrile; 20mM of HOBt in acetonitrile; 640mM in of EDC in water and 4:1 of acetonitrile/DMF. This kit is from Cayman Chemical, Cambridge, UK, as are all the fatty acids used: Prostaglandin E2 (PGE2) and 12(S)-Hydroxy-eicosatetraenoic acid (12S-HETE). The Internal kit (AMPP IC) contains the AMPP compound prepared by a chemist at ICR as well as the other reagents such as HOBt, EDC and acetonitrile /DMF from the company Sigma-Aldrich, Gillingham, UK. All other chemicals such as lactic acid and Krebs acids used were obtained from Sigma-Aldrich.

3– METHODS

3.1 – DERIVATIZATION

The derivatization process is performed according to the described in the literature for AMPP reagents of Cayman Kit[21] regarding AMPP derivatization of fatty acids. The cayman kit reagents supplied, in solution, ready to use whereas the AMPP IC (as described in the section 2- material) requires preparation before use in the derivatization process.(Table 1.2)

Table 1.2 – Conditions of reagents preparation. [21]

Reagent	Concentration	Solvent
AMPP	20mM	Purified water
EDC.HCL	640mM	Purified water
HOAt or HOBt	20mM	Acetonitrile
DMF/ACN	1:4	Acetonitrile

100µM stock solutions of fatty acids, Krebs acids and lactic acid were prepared in absolute ethanol. 10µL of diluted stock were placed in a vial and evaporated at 60ºC for approximately 15 minutes. Addition of 20µL of DMF/ acetonitrile, 20µL of EDC.HCL, 10µL of HOAt or HOBt and 30µL of AMPP to the same vial. The vial is closed and maintained in the dri block at 60ºC for 30 minutes in order to optimize the derivatization reaction. Once derivatized, solutions can be stored at -20ºC for further use.

3.1.1 – Confirmation of fatty acids derivatization

To confirm the efficacy of the derivatization in fatty acids and to compare the efficiency of both groups of reagents (Cayman Kit and AMPP IC), we prepared 0.3μM solutions of each fatty acid (12S-HETE and PGE$_2$). We also compared the efficiency of the reagents, HOAt and HOBt in derivatization using AMPP IC. Using a 96-well U bottom plate we distributed 150μL of each sample and ran in LC-MS. The LC and MS conditions were taken from the literature [19].

3.1.2 – Derivatization of krebs cycle acids and lactic acid

After confirming the derivatization process in fatty acids we repeated the process for derivatization of all of the Krebs acids and lactic acid. 50nM solutions of lactic acid, pyruvic acid, oxaloacetic acid, α-ketoglutaric acid, glutamic acid, citric acid, malic acid and fumaric acid. Were derivatised using the AMPP IC reagents solutions were then optimized on the mass spectrometer.

Once a standard curve of pyruvic acid (derivatized by AMPP IC) was prepared from concentrations of 100μM, 50μM, 20μM, 10μM, 5μM, 2μM, 1μM, 0.5μM and 0.2μM using serial dilutions with acetonitrile as solvent. Solutions were distributed in a 96-well U bottom plate (150μL of each concentration) and ran on LC-MS.

3.2 – MASS SPECTOMETRY OPTIMIZATION AND MULTIPLE REACTION MONITORING

To obtain the most useful results from MS system, the controls of the instrument need to be adjusted to achieve the best resolution and highest sensitivity possible.[22] In order to provide the ability to manually or automatically mass calibrate the instrument and to set up MS methods and conditions, we used the manual tuning and the IntelliStart[TM] program of MassLynx[TM] software on Xevo TQS.

The MS experiment file or MS methods is a group of settings defining the scans that the MS should perform during an acquisition.[22,23] We select the process modes of MS more convenient such as: MS scan or full scan; MRM for quantify target compounds and Selected Ion Recording (SIR) in target analysis for defined analytes.[23] Is in that process that we use the information of the MS tuning to optimize the data acquisition. A generalized workflow about IntelliStart[TM] is shown by the diagram below:

Calculate Mass/Charge (M/Z) values
Depending on the size and charge of the product ion, the possible m/z values for the percursor need to be calculated.

Enter M/Z values into IntelliStart
IntelliStart allows multiple m/z values to be entered for a compound, to search with different charged states.

Run IntelliStart
It will select the product ions at higher m/z values than precursor ions and opimize the ionization mode, MRM transition, cone voltage and collision energy.

IntelliStart report automatically generated
The report is saved into a specific sub-directory for future reference and generated settiings used to created an MS method.

Check LC-MS
The MS method developed can be used to evaluate the genereted MRM against criteria such as intensiy, signal/noise and specificity.
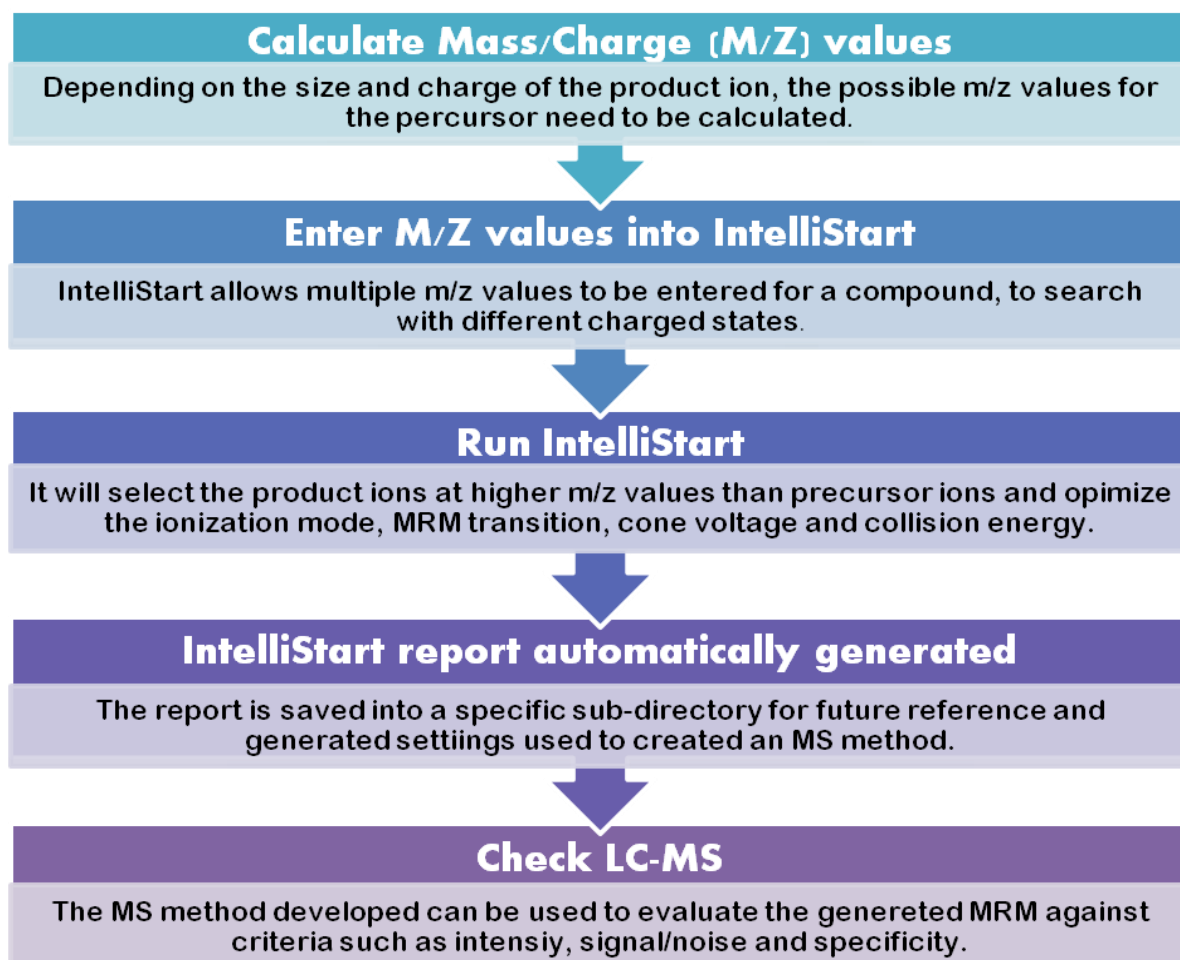
Figure 1.6 – Schematic process of MS tune with IntelliStart[TM].[23]

To calculate the derivatied product m/z, we based on the following mathematical equation:

$$\text{Product (m/z)} - H_2O \text{ (m/z)} + \text{AMPP (m/z)} = \text{Derivatized Product (m/z)}$$

To use the formula we just need to know that AMPP is 185.11m/z, the water is 18.01m/z and the information present in the table above:
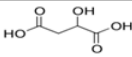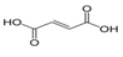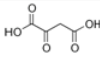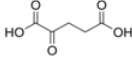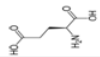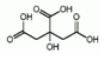
Name	Structure	Formula	Molecular mass
Pyruvic Acid		$C_3H_4O_3$	88.06
Lactic Acid		$C_3H_6O_3$	90.08
Malic Acid		$C_4H_6O_5$	134.09
Fumaric Acid		$C_4H_4O_4$	116.07
Succinic Acid		$C_4H_6O_4$	118.09
Oxaloacetic Acid		$C_4H_4O_5$	132.07
α-Ketoglutaric Acid		$C_5H_6O_5$	146.11
Glutamic Acid		$C_5H_9NO_4$	147.13
Citric Acid		$C_6H_8O_7$	192.124

Table 1.3 – Krebs acids and lactic acid structure and properties.

After making the calculations we get the following results present in the next table:

PA + AMPP = 255.15 m/z	LA + AMPP = 257.18 m/z
OA + AMPP = 299.16 m/z OA + 2AMPP = 452.288 m/z	MA + AMPP = 301.119 m/z MA + 2AMPP = 468.27 m/z
GA + AMPP = 314.22 m/z GA + 2AMPP = 481.31 m/z	FA + AMPP = 283.16 m/z FA + 2AMPP = 450.25 m/z
KA + AMPP = 313.2 m/z KA + 2AMPP = 480.29 m/z	SA + AMPP = 285.19 m/z SA + 2AMPP= 452.288 m/z
CA + AMPP = 359.214 m/z CA + 2AMPP = 526.304 m/z CA + 3AMPP = 693.394 m/z	

Table 1.4 – Derivatized product m/z calculation of krebs cycle acids and lactic acid. Pyruvic Acid (PA); Lactic Acid (LA); Oxaloacetic Acid (OA); Malic Acid (MA); Glutamic Acid (GA); Fumaric Acid (FA); Ketoglutaric acid (KA); Succinic Acid (SA); Citric Acid (CA).

3.3 – CHROMATOGRAPHY

The LC needs to be optimized before use and for that we create an inlet method. An inlet method is a group of settings that control the LC during data acquisition to provide complete control of an experiment, and uses parameters including flow rate, run time, mobile phase composition, gradient, sample and column temperature.[22]

For the first experiments we used a flow rate of 0.4 mL/min and a run time of 15:00 minutes. In order to try to get more interaction between analyte/column to increase the retention time, we tried several different columns such as a C18-XB, Polar-RP, Hilic Silica 3μm, Hilic 100A and BEH Hilic 1.7μm. We tried different solvents (ammonium acetate, ammonium formate, water + 0,1% formic acid and acetonitrile) in different gradients but the analytes were not retained

When we ran the standard calibration of pyruvic acid we used a C18 reverse-phase column with a flow rate of 0.6 mL/min and a run time of 3.50 minutes. The gradient used is shown in Table 1.5.

	%A	%B
Initial	98%	2%
2:00	80%	20%
2:10	0%	100%
3:50	98%	2%

A- 10mM of ammonium acetate in water;
B- Acetonitrile.

Table 1.5 – Gradient used for pyruvic acid chromatography.

4 – RESULTS

4.1 – CONFIRMATION OF FATTY ACIDS DERIVATIZATION

	Name	Sample Text	RT	Response	Transition	Peak Area	Vial
1	13_0850001	wash	9.11	9.137	519>239	9.137	4:1,H
2	13_0850002	wash	9.12	5.216	519>239	5.216	4:2,H
1	13_0897_003	PGE2 kit	7.11	19131.81	519 > 239	19131.809	2:1,A
2	13_0897_004	PGE2 kit	7.1	18775.33	519 > 239	18775.330	2:1,A
3	13_0897_005	wash	7.1	2.469	519 > 239	2.469	4:1,H
4	13_0897_006	wash	7.09	1.742	519 > 239	1.742	4:2,H
5	13_0897_007	PGE2 IC	7.11	15495.01	519 > 239	15495.009	2:2,A
6	13_0897_008	PGE2 IC	7.11	16093.54	519 > 239	16093.542	2:2,A
7	13_0897_009	wash	7.1	3.033	519 > 239	3.033	4:1,H
12	13_0897_014	wash	8.76	0.794	487 > 347	0.794	4:2,H
13	13_0897_015	12S HETE kit	8.67	98190.45	487 > 347	98190.453	2:1,B
14	13_0897_016	12S HETE kit	8.67	97444.18	487 > 347	97444.180	2:1,B
15	13_0897_017	wash	8.75	5.145	487 > 347	5.145	4:1,H
16	13_0897_018	wash	8.76	2.955	487 > 347	2.955	4:2,H
17	13_0897_019	12S HETE IC	8.67	88492.78	487 > 347	88492.781	2:2,B
18	13_0897_020	12S HETE IC	8.68	89310.73	487 > 347	89310.727	2:2,B
19	13_0897_021	wash	8.75	3.493	487 > 347	3.493	4:1,H

Table 1.6 – TargetLynx[TM] **Analysis of LC-MS/MS results of the experiment using fatty acids.**

In table 1.6 we can see the LC-MS/MS results processed by TargetLynx[TM] program of MassLynx[TM] software. The results compare the process of AMPP derivatization between the Cayman Kit reagents (as described as "kit" in the table) and the reagent prepared at ICR, the Internal Compounds (as described as "IC" in the table) using two fatty acids, PGE_2 and 12S-HETE. This table shows us important information about the retention time of the analyte, the transition used to identify the compound in the instrument (Transition) and the Peak Area of the chromatogram.

Average retention time: 7.11 min for PGE2 (kit and IC) and 8.67 min for 12S-HETE (kit and IC).

Peak area average: PGE2 kit - 18953.57; PGE2 IC - 15794.275; 12S HETE kit - 97817.315; 12S HETE IC - 88901.754.
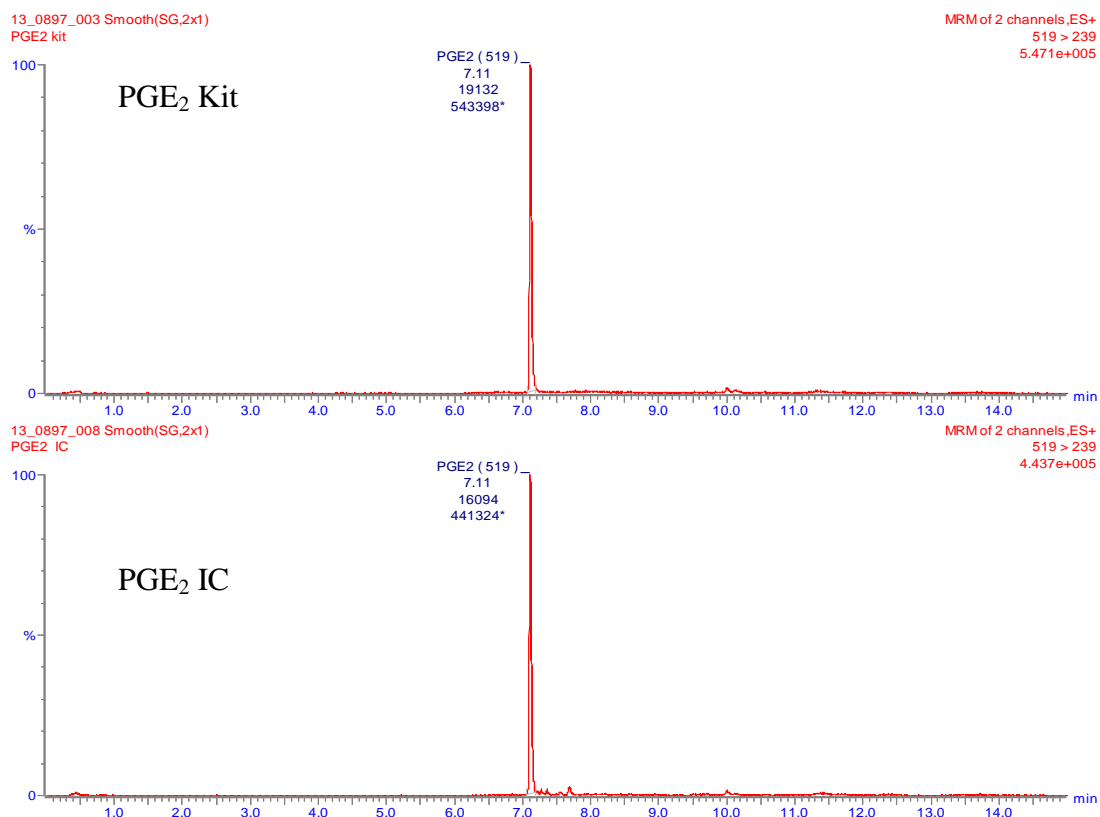
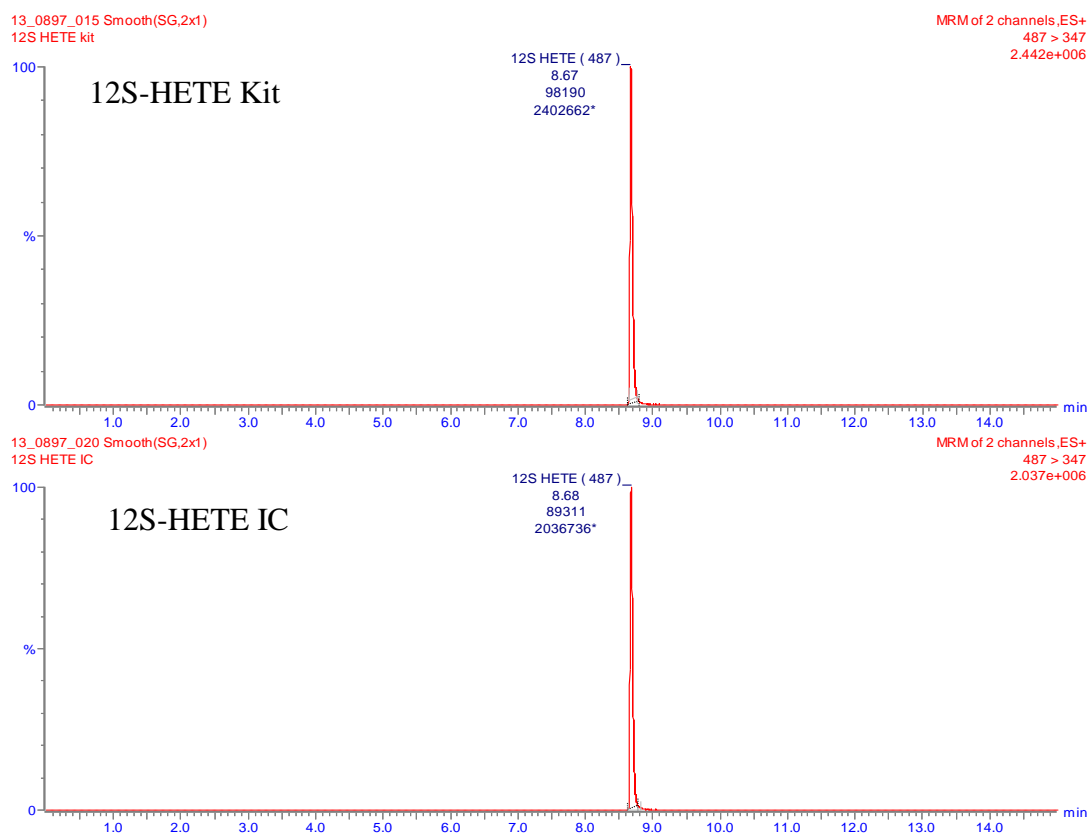**Figure 1.7 – Chromatograms of PGE₂, Kit and IC.**

Figure 1.8 – Chromatograms of12S-HETE, Kit and IC.

4.2.1 – Mass Spectometry results

Compound	N° of -COOH	N° of - AMPP	Parents Formula + AMPP	Derivatized Product Calculated (m/z)	Derivatized Product Find (m/z)	Daughters		
Pyruvic Acid	1	1	$C_{15}H_{15}O_2N_2$	255.15	256.15	89.87	169.01	185.11
Lactic Acid	1	1	$C_{15}H_{17}O_2N_2$	257.18	258.29	86.04	103.13	58.10
Malic Acid	2	2	$C_{28}H_{28}O_3N_4$	468.28	469.07	132.33	157.05	169.27
Fumaric Acid	2	2	$C_{28}H_{26}O_2N_4$	450.25	451.37	89.86	169.04	183.08
Succinic Acid	2	2	$C_{28}H_{28}O_2N_4$	452.29	452.36	142.10	199.17	297.27
Oxaloacetic Acid	2	2	$C_{28}H_{26}O_3N_4$	466.25	466.47	86.59	156.11	142.10
α-Ketoglutaric Acid	2	2	$C_{29}H_{28}O_3N_4$	480.29	481.02	87.73	186.57	189.39
Glutamic Acid	2	1	$C_{17}H_{20}O_3N_3$	314.22	315.31	86.47	103.10	142.15
Citric Acid	3							

Table 1.7 – IntelliStart[TM] Analysis results for the experiment with the krebs cycle acids and lactic acid.

In Table 1.7 we are able to compare the derivatized parent structure and m/z of the different acids used and in addition we can confirm how many carboxylic acids were derivitized by the AMPP reagent in the same molecule by comparing the number of –COOH that are present and the number of -AMPP attached to each acid. Krebs cycle acids with one and two carboxylic groups are completely derivatized with the exception of glutamic acid which only shows derivatization of one of its two –COOH groups. Citric acid did not show any results for this analysis.

4.2.2 –Chromatography results of pyruvic acid

	Name	S.N	SC uM	Conc. uM	RT	Response	Transition	Peak Area	%Dev
1	13_0914_009	PA	0.1	0.133	1.98	22427.46	256.14>89.86	22427.46	33.1
2	13_0914_010	PA	0.2	0.251	1.99	55455.21	256.14>89.86	55455.21	25.6
3	13_0914_011	PA	0.5	0.106	2.03	14445.36	256.14>89.86	14445.36	-78.7
4	13_0914_012	PA	1	1.079	1.96	289827.00	256.14>89.86	289827.00	7.9
5	13_0914_013	PA	2	2.415	1.94	666790.75	256.14>89.86	666790.75	20.7
6	13_0914_014	PA	5	5.952	1.95	1626532.75	256.14>89.86	1626532.75	19.0
7	13_0914_015	PA	10	9.114	1.92	2552891.75	256.14>89.86	2552891.75	-8.9
8	13_0914_016	PA	20	17.542	1.90	4927642.50	256.14>89.86	4927642.50	-12.3
9	13_0914_017	PA	50	41.309	1.86	11606263.00	256.14>89.86	11606263.00	-17.4
10	13_0914_018	PA	100	110.898	1.84	31654780.00	256.14>89.86	31654780.00	10.9

Table 1.8 – TargetLynx^{TM} Analysis of LC-MS/MS results for pyruvic acid standard curve.

The pyruvic acid calibration curve is represented in the table 1.8 as LC-MS/MS results. The average retention time of pyruvic acid is 1.94 min. The solution with least Percentage of deviation (%Dev) is the one represented in line 4 by a theoretical concentration (SC) of 1μM and corresponding to 7.9% of deviation. The solution with the most %Dev is the one in line 3 (S.C of 0.5μM) corresponding of -78.7 %. All the other solutions are around 9 to 20 %Dev.
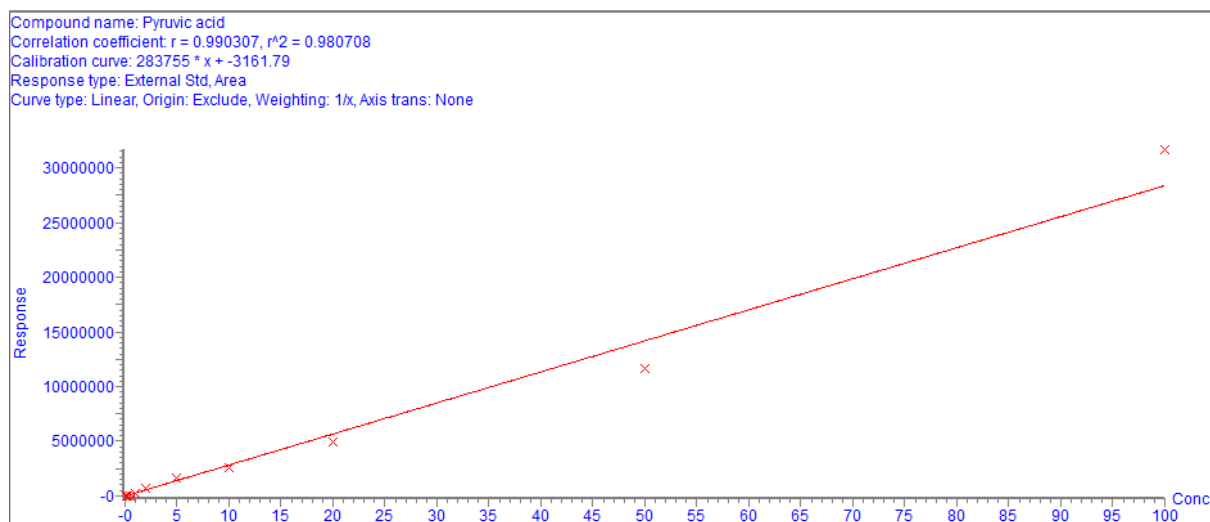

Figure 1.9 – Calibration line of PA results processed by TargetLynx program.

The calibration line of pyruvic acid, a plot of response over the concentration calculated by TargetLynx^{TM} shows a correlation coefficient of r=0.990307 and r^2=0.980708 using a weighting of 1/x for the curve.
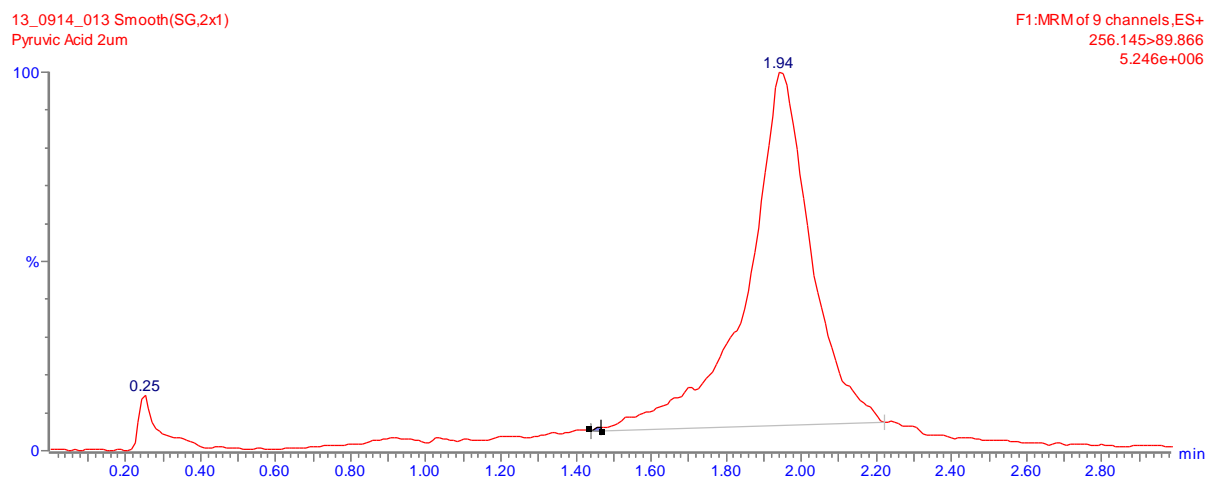

Figure 1.10 – Representative peak of PA (2µM).

5 - DISCUSSION AND CONCLUSION OF RESULTS

From the initiation of this project, we already knew of its complexity and the difficulties that could arise from it for being something new and unknown in the science world community. Nevertheless, if its development is positive, it will be a crucial contribution to the field of metabolomics and for everyone that would like to use LC-ESI-MS/MS to quantify and qualify the Krebs cycle acids.

Initially we wanted to check if the efficacy and efficiency of the derivatization reagents used in this process. We made an experimental comparison between the Cayman kit used in the literature for fatty acids and the reagent synthesized in our laboratories. To have some confidence in the results, we restricted the experiment to fatty acids such as 12S-HETE and PGE2 for use as control since they are securely defined and studied in the literature surrounding this project (see Table 1.6). We also compared the differences in the results when we used HOBt instead of HOAt previously used as one of the reagents present in the AMPP IC. After acquisition, analysis of the respective peak area, showed that there is no significant changes between reagents (internal and Cayman Kit) when HOBt is used as a reagent in the internal kit (see Figure 1.7 and 1.8).

When we started the experiment with kerbs acids and lactic acid, our main objective was to see if we were able to derivatize those compounds and evaluate if one or both carboxylic groups present are derivatized. The results conclude that the derivatization process was successful because we could detect derivatized products and also fragment them with the respective collision energy and cone voltage determined by the IntelliStart™ program in positive ion mode and also verified by manual tuning (see Table 1.7).

According to the results, we conclude that over all of the Krebs acids, just glutamic acid and citric acid did not derivatize all of carboxylic acid moieties present. With glutamic acid we could only find a product mass for that molecule correlating to one AMPP and not two whilst with citric acid we could not get any information about any product mass. Citric acid has three carboxylic groups which makes it a more complex compound to investigate. In the case of glutamic acid, we might explain this result because one end of that molecule is constituted by a carboxylic group and the other an amide group, it is possible that there is a repulsive charge between the nitrogen of the –AMPP derivative and the amide group, preventing its binding and derivatization from occurring.

To try to evaluate other characteristics about our derivatized products and see how they behave when processed by LC-MS, we used some of the Krebs acids and we ran it on the XEVO-TQS. Even after trying different columns such as C18-XB, Polar-RP, Hilic Silica 3μm, Hilic 100A, BEH Hilic 1.7μm and different gradients and solvents such as AA, AF, Water + 0.1% Formic Acid and Acetonitrile, we could not retain the compound. This result may be caused by the characteristics of our acids, they are small molecules and very polar, were not retained by the column and eluted with the solvent front. The Hilic column should be a good choice for this problem because it is a column where the stationary phase is a polar material (Silica, hybrid, cyano, amino, diol, amide) the highly organic mobile phase (acetonitrile) retains the compound and the stronger solvent is water or aqueous mobile phase and thus improves the retention for polar or ionisable compounds such as ours compared with reversed phase columns. Although the attempts with Hilic were not successful, this may be because the conditions were not optimal and it would seem likely that future use of this type of column is warranted since it has shown a great potential for retention of similar compounds.

In order to achieve a conclusion about Krebs acids chromatography, we continue the project by following through the LC-MS analysis of pyruvic acid calibration curve. To give us assurances that we have the correct transitions and that the tuning information obtained by IntelliStart[TM] are correct, we decided to make a calibration curve in order to evaluate if the correct mass was founded in our compound in the sample. After completing the sample analysis, during the data processing we were able to conclude that in fact we had the correct parent and daughter mass for the pyruvic acid as shown by the results (see Table 1.8). According to that described in the human serum metabolome[11], the concentration of pyruvic acid determined by NMR in a healthy subject is around of 34.5μM with a mean deviation of +/-25.2μM and an occurrence of 81%. With this information and regarding the concentrations of 2 and 50μM, we can prove that we are able to derivatized the same concentration in buffer that is the normal concentration of pyruvic acid in plasma.

Analyzing the percentage of deviation, we can see that most solutions are around 9 to 20%, except the one in line 3 of 0.5μM solution corresponding of -78.7% of deviation that may be cause by preparation error. The obtained peak area increased according to the concentration of the sample and the calibration curve obtained shown us a very linear and integrated line from 0.1 to 100nM with an R^2 value of 0.981 (see Figure 1.9). At a

chromatographic level, the peak obtained at 1.94 minutes is not Gaussian but can be improved in the future (see Figure 1.10).

This project has shown some promising results. Once we have completed the process of investigation for AMPP derivatization and Krebs acids detection by LC-MS/MS in buffer, we want to be able to use the same process for quantification in human plasma. We plan to optimise the chromatography for all compounds detected, evaluate the derivatization of plasma samples and show linearity in human plasma with detection of concentration similar to that presented in the literature. Further work may also include exploring commercially available kits for quantitative metabolite detection.

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APPENDIX A

Tables of instruments, chemicals and reagents

INSTRUMENTS

Mettler Toledo MX5 Analytical balance	Mettler Instruments, Switzerland.
TECHNE DB.3 Dri Block	TECHNE, Cambridge, U.K.
Column various: - Kinetex™ HILIC 2.6μ 100A 50x2.1mm; - Kinetex™ C_{18} XB 2.6μ 50x2.1mm; - Kinetex™ C_{18} 2.6u 100A 50x2.1mm - Atlantis™ HILIC Silica 3μm - Acquit UPLC™ BEH Hilic 1.7μm	Phenomenex, Macclesfield, U.K. Waters, Manchester, U.K.
Aquity H-Class LC system	Waters Hertford, U.K.
Xevo TQ-S Mass Spectrometer	Waters, Manchester, U.K.
Gilson Pipettes	Anachem, Luton, U.K.
Whirlimixer Vortex mixer	Fisher Scientific, Loughborough, U.K.
Titramax 100 Plate Mixer	Heidolph Instruments, Germany
Greiner and Abgene 96-Well plates and seals	Fisher Scientific, Loughborough, U.K.
FiveEasy™ FE20 pH meter	Mettler Instruments, Switzerland.

CHEMICALS AND REAGENTS

Acetonitrile	Biosolve, Valkenswaard, The Netherlands
Absolut Ethanol	VWR Prolabo, Fontenay-sous-Bois, France
Water (Ultra Purification System)	Elga Ltd., High Wycombe, U.K.
Formic Acid, 99%	Fisher Scientific, Loughborough, U.K.
Ammonium Acetate (AA) , 98%	Sigma-Aldrich, Gillingham, U.K.
Ammonium Formate (AF) , 99%	Sigma-Aldrich, Gillingham, U.K.
N-(4-aminomethylphenyl) pyridinium (AMPP)	Synthesized at ICR, Sutton Surrey, London, U.K.
1-Hydroxy-7-azabenzotriazole (HOAt)	Sigma-Aldrich, Gillingham, U.K.
N-hydroxybenzotriazole (HOBt)	Sigma-Aldrich, Gillingham, U.K.

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)	Sigma-Aldrich, Gillingham, U.K.
N,N-dimethylformamide (DMF)	Sigma-Aldrich, Gillingham, U.K.

Krebs Cycle Acids
 	
   - Lactic Acid (LA)
   - Pyruvic Acid (PA)
   - Succinic Acid (SA)
   - Oxaloacetic Acid (OA)
   - Malic Acid (MA)
   - Fumaric Acid (FA)
   - A-Ketoglutaric Acid (KA)
   - Glutamic Acid (GA)
   - Citric Acid (CA)
	Sigma-Aldrich, Gillingham, U.K.

Cayman Kit
 	
Contains:
   - AMPP derivatizing reagent – 20mM in ACN.
   - HOBt -20mM in ACN.
   - EDC – 640mM in Water.
   - 4:1 ACN/DMF
	Cayman Chemical, Cambridge, U.K.

Fatty Acids
   - Prostaglandin E_2 (PGE2)
   - 12(S)-Hydroxy-eicosatetraenoic acid (12S-HETE)
	Cayman Chemical, Cambridge, U.K.