Development of a chromatographic method for the determination of lactate in urine and saliva

Davide Pellegrini

Corso di laurea magistrale in Chimica

Curriculum analitico

Anno Accademico 2012-2013
Development of a chromatographic method for the determination of lactate in urine and saliva

Candidato: Davide Pellegrini

Relatore interno: Prof.ssa Maria Perla Colombini

Relatore esterno: Dott.ssa Emilia Bramanti

Controrelatore: Prof. Roger Fuoco

Anno Accademico 2012/2013
There is a driving force more powerful than steam, electricity and atomic energy: the will.

Albert Einstein
# Index

**Abstract** ........................................................................................................................................... 06  
**Introduction and aim of the study** ........................................................................................................ 07  
**Chapter 1 State of the art** .................................................................................................................. 09  
  1.1. Biochemistry of lactic acid ........................................................................................................... 09  
  1.2. Lactic acid in sports ..................................................................................................................... 12  
  1.3. Lactic acid in clinics ...................................................................................................................... 14  
  1.4. Lactic acid in food, cosmetics and pharmaceuticals .................................................................. 16  
  1.5 Determination of lactic acid .......................................................................................................... 16  
    1.5.1. Colorimetric methods ........................................................................................................... 17  
    1.5.2. Enzymatic methods ............................................................................................................. 17  
    1.5.3. Biosensors ........................................................................................................................... 18  
    1.5.4. Separation methods ............................................................................................................. 21  
      1.5.4.1. GC-MS ........................................................................................................................... 21  
      1.5.4.2. CE .................................................................................................................................. 22  
      1.5.4.3. IEC ................................................................................................................................. 22  
      1.5.4.4. HPLC-DAD/FD ............................................................................................................. 22  
      1.5.4.5. LC-MS/MS .................................................................................................................... 24  
  1.6. Creatinine ..................................................................................................................................... 25  
    1.6.1. Biochemistry of creatinine ................................................................................................... 25  
    1.6.2. Urinary creatinine relevance ............................................................................................... 26  
    1.6.3. Determination of urinary creatinine ................................................................................... 27  
**Chapter 2 Experimental** .................................................................................................................... 28  
  2.1. Chemicals .................................................................................................................................... 28  
    2.1.1. Standard solutions ................................................................................................................ 28  
  2.2. Biological samples ....................................................................................................................... 29  
  2.3. HPLC-DAD/FD ........................................................................................................................... 30  
  2.4. HPLC-ESI-Q-ToF ........................................................................................................................ 32  
  2.5. Analytical procedure .................................................................................................................... 35  
  2.6. Data processing ............................................................................................................................ 36  
**Results** .............................................................................................................................................. 37  
**Chapter 3 Optimization of the method** ............................................................................................... 38  
  3.1. 9-chloromethyl anthracene ........................................................................................................ 38  
  3.2. Reactivity of 9-CMA with lactate ............................................................................................. 40  
  3.3. Optimization of the analytical procedure .................................................................................. 43  
    3.3.1. Extraction ........................................................................................................................... 43  
    3.3.2. Reaction yield ..................................................................................................................... 46
3.3.3. Effect of reaction time ......................... 47
3.3.4. Optimization of the temperature and reaction time using the experimental design . 47
3.3.5. Optimization of 9-CMA excess ............... 51
3.3.6. Doehlert design for the simultaneous optimization of [TEA], [TBAB] and [9-CMA] .............................................. 52
3.4. Optimization of the chromatographic conditions … 58
  3.4.1. HPLC column .................................. 58
  3.4.2. Mobile phase composition ..................... 61
  3.4.3. Fluorescence and absorbance detection ...... 63
3.5. Simultaneous determination of lactate and creatinine ................................................. 64
  3.5.1. Chromatographic determination of creatinine.................................................. 65
  3.5.2. Optimization of the chromatographic conditions for the simultaneous determination of lactate and creatinine .... 67
3.6. Analytical figures of merit .......................... 70
  3.6.1. Linearity, repeatability and detection limit ... 70
  3.6.2. Selectivity and stability .......................... 71

Chapter 4 Characterization of the derivatization products by HPLC-ESI-q-ToF ............................... 72
  4.1. Proof of the lactate derivative formation ........ 73
  4.2. Characterization of the structures by MS interpretation ................................................. 77
  4.3. The lactate derivative characterization .............. 79

Chapter 5 Application to urine samples ......................... 82
  5.1. Improvements of the method for the application in urine samples ................................. 85
  5.2. Dilution of the samples and matrix effect ......... 86
  5.3. Analytical figures of merit .......................... 90
  5.4. Urinary lactate before and after training .......... 91
  5.5. Urinary lactate and lactemia ........................ 92

Chapter 6 Application to saliva samples ................. 93
  6.1. Application of the method to saliva samples ....... 95
    6.1.1. Dilution of the samples and matrix effect .... 95
    6.1.2. Analytical figures of merit ...................... 96
    6.1.3. Salivary lactate during training ................ 96

Conclusions .................................................................. 99
Abbreviations ................................................................ 101
Bibliography ............................................................... 102
Abstract

The determination of lactate in matrices as urine and saliva is challenging due to the sample complexity and the low concentration levels to be determined. This study reports the development of a novel method to determine lactate in biological matrices (urine and saliva) using a pre-column derivatization procedure with 9-chloromethyl anthracene (9-CMA) and liquid chromatography (RP-HPLC) with UV detection at 365 nm and fluorescence detection at 410 nm ($\lambda_{ex} = 365$ nm).

The formation of the lactate derivative and by-products was confirmed by LC-ESI-Q-ToF. The calibration curve was linear in the investigated range 0.09-15 mM and the limit of detection was 0.03 mM for both fluorescence detection and for UV detection. Intra-day and inter-day repeatability were less than 5 and 6 %, respectively.

The calibration curve in urine samples was linear in the investigated range 0.09-15 mM. Intra-day repeatability was less than 4% and 5% for fluorescence and UV detection, respectively. Intra-day recoveries were found to be between 92 and 105 % and between 98 and 106% for fluorescence and UV detection, respectively.

The calibration curve in saliva samples was linear in the investigated range 0.09-15 mM. Intra-day repeatability was less than 2 and 3% for fluorescence and UV detection, respectively. Intra-day recoveries were found to be between 101 and 108 % and between 104 and 109% for fluorescence and UV detection, respectively.

The optimized method for the lactate determination was successfully applied to urine and saliva samples of healthy volunteers before, after and during training and of patients from an intensive care unit.

The method was further optimized for the analysis of urine samples in order to provide the simultaneous determination of lactate and creatinine.

Hence lactate and creatinine are determined in one chromatographic run, representing a great advantage in terms of time of analysis and amount of sample required. This is the first analytical method proposed in the literature able to provide the simultaneous determination of lactate and creatinine.
Introduction and aim of the study

Lactic acid (2-hydroxypropanoic acid) is a product of anaerobic glycolysis resulting from pyruvate by the enzyme lactate dehydrogenase (LDH). The lactic acid can be found in blood and biological fluids of human beings and animals.

The measurement of lactate in biological fluids is an important tool in sport medicine to monitor the maximum performance level of athletes\cite{1-3}.

Lactic acid is indeed the final product of the anaerobic glycolysis and during intense exercise its concentration increases because of the switch of muscle cells to anaerobic metabolism. Once a certain level of lactate is reached, exhaustion occurs and there is a rapid decline in exercise capacity.

Lactic acidosis is also found in all diseases involving inadequate intake of oxygen to tissue (hypoxia) and this is a spy of organ failure and dysfunction: acute congestive heart failure, renal or hepatic failure, respiratory failure, severe pulmonary diseases, pulmonary edema, severe anemia, diabetes not under control and a number of rare inherited metabolic and mitochondrial diseases (forms of muscular dystrophy, ASL, etc.)\cite{4, 5}.

Hyperlactemia is typical of patients with severe sepsis or septic shock, it can be secondary to the anaerobic metabolism due to the hypoperfusion and it has a prognostic value.

In all these conditions the repeated measurements of lactate at intervals is of fundamental importance to keep under control the conditions of the patients also in response to a therapy.

The determination of lactate is usually performed in blood or plasma and based on enzymatic methods which are expensive and loss in sensitivity and specificity.

In the last years the measurement of metabolites in media other than blood is becoming very significant because of the demand of non-invasive analysis. Such measurements are very important to avoid physical and mental stress, risk of infection and the presence of medical staff. Matrices as urine and saliva are very useful either to control daily parameters in hemophiliacs, neonates and elder patient, either to monitor the training of athletes\cite{6, 7}. Indeed, the determination of lactic acid in complex matrices as urine and saliva is challenging. The concentration of urinary and salivary lactate it’s quite small (about 0.1-0.3 mM) and the sample has to be diluted to avoid matrix effects. Furthermore, the direct detection of lactate is unspecific because it is based on the intrinsic absorbance at
210 nm. Thus, a new derivatization agent is required to assure selectivity and sensitivity and to control matrix effect. Moreover, a combined analytical procedure able to simultaneously determine the urinary creatinine could allow the comparison of lactate levels between different samples. In clinical laboratories, urinary creatinine is used as a standardization tool for the quantitative evaluation of urinary metabolites, in order to avoid the interfering influence of the physiological differences in the dilution level of urine.

The main aim of this study was to develop a novel, straightforward derivatization method for the determination of lactate by RP-HPLC with UV and fluorescence detection and to optimize an analytical procedure for the simultaneous determination of lactate and creatinine in urine samples.

9-chloromethyl anthracene was chosen as the fluorescence reagent, which has never been previously used for obtaining a lactate derivative. 9-CMA can react with lactate with high selectivity, without requiring extraction procedures from the aqueous solution, in a very short time (the reaction reaches 70% completion in 30 min). Furthermore, the reaction is catalyzed by tetrabutyl-ammonium bromide (TBAB) instead of pyridine or other hazardous substances, resulting in a safer analytical procedure.

The overall work may be summarized as follows:

- optimization of the derivatization procedure with 9-CMA and comparison with the classical derivatization reagent used for lactate determination;
- demonstration of the formation of the lactate derivative and by-products by HPLC-ESI-Q-ToF;
- optimization of the chromatographic conditions (best column, column temperature and mobile phase) to guarantee a good resolution and a short time of analysis;
- application of the optimized method to urine and saliva samples of healthy volunteers before, after and during training and of patients from an intensive care unit;
- further optimization of the method to simultaneously determine lactate and creatinine in urine samples.
CHAPTER 1

State of the art

1.1. Biochemistry of lactic acid

Lactic acid (2-hydroxypropanoic acid) is a product of anaerobic glicolyisis resulting from pyruvate by the enzyme lactate dehydrogenase (LDH). The lactic acid can be found in blood and biological fluids of human beings and animals.

Glycolysis (Figure 1.1) is the metabolic pathway that converts glucose to pyruvate; the free energy released in the process is used to form the high-energy compounds ATP and NADH.

Fig. 1.1: Scheme of the glycolysis pathway[^8]
The pyruvate formed by glycolysis is further metabolized via one of the three catabolic routes (Figure 1.2).

![Diagram of catabolic fates of pyruvate](image)

**Fig. 1.2:** three possible catabolic fates of the pyruvate formed in glycolysis

In aerobic conditions, glycolysis is only the first stage in the complete degradation of glucose. Pyruvate is oxidized to yield the acetyl group of the acetyl-coenzyme A; the acetyl group is then oxidized completely to CO$_2$ by the citric acid cycle. The electrons from these oxidations are passed to O$_2$ through a chain of carriers in the mitochondrion, to form H$_2$O. The energy from the electron transfer reactions drives the synthesis of ATP in the mitochondrion. In some plant tissues and in certain invertebrates, protists, and microorganisms such as brewer’s yeast, pyruvate is converted under hypoxic or anaerobic conditions into ethanol and CO$_2$ by the alcoholic fermentation.

When vigorously contracting, skeletal muscles work under low oxygen conditions (hypoxia), pyruvate is reduced to lactate via the lactic acid fermentation. Under these conditions NADH can’t be oxidized to NAD$^+$, which is necessary for the oxidation of pyruvate. Hence pyruvate is reduced to lactate, accepting electrons from NADH and thereby regenerating the NAD$^+$ necessary for glycolysis to continue. The reaction is catalyzed by LDH to form the L isomer of lactic acid at pH 7.

The conversion of glucose to lactate does not involve a net change in the oxidation state of carbon (in glucose and lactic acid the H:C ratio is the same) or in the concentration of NAD$^+$ and NADH. The two molecules of NADH formed during the glycolysis are used to
reduce the two molecules of pyruvate. Therefore the lactic fermentation is a process in which an extraction of energy (ATP) occurs, without consuming oxygen.

The lactate produced by active skeletal muscles can be recycled, it is carried in the blood to the liver, where it is converted to glucose by gluconeogenesis during the recovery from strenuous muscular activity (Cori cycle). The produced glucose is then supplied to the muscles through the bloodstream where it can undergo to further glycolysis reactions. If the muscle activity has stopped, the glucose is used to replenish the supplies of glycogen. *Figure 1.3* shows the Cori Cycle.

![Diagram of the Cori Cycle]

*Fig. 1.3: Cori cycle[^8]*

A healthy adult man normally produces about 120 g of lactic acid a day. Among these, 40 g (33%) are produced by tissues characterized by an exclusively anaerobic metabolism (retina and blood red cells). The remaining 80 g (67%) are produced by other tissues (most of all muscle) on the basis of the actual oxygen availability.

Thus, the determination of lactate concentration level is a good approach to evaluate the balance between the oxygen demand of tissues and its use, and it is useful in the study of animal and cellular physiology[^8,^9].
1.2. Lactic acid in sports

In muscle, during exertion, pyruvate derived from glucose and glycogen is reduced to lactate, which is reoxidized and partially reconverted into glucose during rest (Cori cycle). The concentration of blood lactate is usually 1–2 mmol/L at rest, but can rise to over 20 mmol/L during intense exertion because of the switch of muscle cells to an anaerobic metabolism.

The classical explanation of lactate production was that it increases to provide supplementary anaerobically derived energy. However, currently, the predominant view is that the role of lactate formation as an energy source is of minor importance. Instead, it has been suggested that the increased lactate production are metabolic adaptations, which primarily serve to activate the aerobic ATP production[10]. Furthermore, the lactic acid is a strong stimulus to the secretion of anabolic hormones such as GH and testosterone. For this reason high intensity workout combined with short rest periods contributes to gain muscle mass.

However, once a certain level of lactate concentration is reached, exhaustion occurs and there is a rapid decline in exercise capacity. Thus, in sport medicine lactate concentration is used to monitor the maximum performance level of athletes[11].

The maximal lactate steady state (MLST) is considered to be the upper limit of heavy intensity domain and represent the higher exercise intensity that can be maintained over time without continuous lactate accumulation (the rate of lactate production equals the rate of lactate elimination)[12]. Since the lactate concentration is strictly correlated with the oxygen demand, the MLST can be evaluated by determining the blood lactate concentration, by measuring the heart rates at different loads (Conconi test) or by measuring the ventilator parameters. Generally in untrained athletes the MLST coincides with the 55% of the VO$_{2\text{max}}$ (maximum oxygen consumption), while in well-trained athletes the MLST is reached just at the 85% of VO$_{2\text{max}}$.

The measurement of ventilator parameter provide accurate results but a very expensive instrumentation is required (hemogas analyzer). The Conconi test is the most used method to assess the anaerobic threshold of athletes but it’s not suitable for untrained athletes since it is a maximal test. Furthermore, the interpretation of the results is very complex. The determination of blood lactate is performed by enzymatic biosensors collecting the blood from a finger or from the earlobe. This method is invasive and the analysis can be
affected by the presence of sweat (in which the lactate concentration is over an order of magnitude higher than the blood concentration).

*Figure 1.4* shows typical lactate values over a 30 minutes period for different levels of exercise.

![Lactate - Steady States](image)

*Fig: 1.4:* blood lactate trends for six different levels of exercise.

Each line represents a slightly harder effort (from blue to red to green to purple to teal to brown). For the first five effort levels the lactate elimination capability is equal to the lactate production: a steady state is reached. For the sixth level the lactate production is higher than the elimination rate and the lactate level continues to rise (no steady state).

On this basis, it is important to determine the amount of lactate in biological fluids (sweat, serum, plasma, saliva, urine, liquor, amniotic liquid...) using simple, cheap, fast, precise and accurate methods.
1.3. Lactic acid in Clinics

The increase of lactic acid (lactic acidosis) is a pathological state diagnosed when the serum concentration of lactate is persistently over 4 mmol/L (normal lactate concentration is 2 mmol/L or lower) and can occur in two conditions:

- Type A: tissue hypoxia (oxygen deficiency) and hypoperfusion (decreased blood flow through an organ);
- Type B: high rate of anaerobic glycolysis (no clinical evidence of hypoperfusion)

In type A lactic acidosis the aerobic pathway, i.e. the oxidation metabolic pathway of pyruvate to carbon dioxide and water, is blocked for several reasons. In particular, oxygen deficits (tissue hypoxia) are the most common and often refractory causes of lactic acidosis, including pulmonary problems (low PO$_2$), circulatory problems (poor delivery of O$_2$), and hemoglobin problems (low O$_2$-carrying capacity, for various reasons). This occurs in cardiovascular diseases (ischemia, hypoxemia, anemia) and in other diseases. Patients with hypotension either due to septic, hypovolemic or cardiogenic shock, may develop lactic acidosis due to poor perfusion of skeletal muscles and overproduction of lactic acid. The degree of lactic acidosis correlates well with the duration and severity of shock and is a prognostic factor in the survival of the patients.

In type B lactic acidosis the anaerobic glycolitic pathway proceeds at high rate, it causes low ATP concentration levels and the pyruvic acid produced accumulates and is reduced to lactic acid by LDH. This occurs during intense exertion and in all conditions of decoupling of oxidative phosphorilation, giving accumulation of lactate. For example this occurs in the deficiency of pyruvate dehydrogenase enzyme or in B1 vitamin deficiency, in which ATP is produced from fat metabolism, or when the conversion of lactate to glucose is slow because of liver or renal diseases or genetic or drug-induced defects in gluconeogenesis. Type B lactic acidosis is associated also with acquired diseases (like diabetes mellitus, hepatic failure, septicemia, post cardiopulmonary bypass, renal failure, etc.) with some metabolites, drugs and toxins (like acetaminophen, biguanides, cocaine, epinephrine, norepinephrine, etc.) and with inborn errors of the mitochondrial respiratory chain (glucose-6-phosphate dehydrogenase deficiency, fructose-1,6-diphosphatase deficiency, pyruvate carboxylase deficiency, etc.)$^{[4, 10, 13-15]}$. 

$^{[4, 10, 13-15]}$
Metabolic acidosis can also develop during pharmacological treatments, due to the exposition to chemical agents and in oncologic patients because cancer cells produce more lactate than normal cells even in aerobic conditions\textsuperscript{[16, 17]}.

Lactate measurements on the intensive care units have been widely correlated with the mortality of the patients (\textit{Figure 1.5})\textsuperscript{[18-20]}.

\textbf{Fig. 1.5:} correlation within mortality rate and lactate levels in patients affected by septic shock\textsuperscript{[21]}

There are not specific clinical symptoms of lactic acidosis. Lactic acidosis should be suspected in all critically ill patients who are hypovolumic, hypoxic, in septic or cardiogenic shock or if unexplained high anion gap metabolic acidosis is present.

The major clues leading to the diagnosis of lactic acidosis includes: increased anion gap metabolic acidosis, increased level of serum lactic acid, significant acidemia (arterial pH < 7.35) and decrease in plasma bicarbonate. The arterial blood gas analysis is performed to keep under control these, and others, important vital parameters of the patients.

The most important therapy in management of lactic acidosis is the correction of underlying causes. For example in hypovolumic or cardiogenic shock, restoration of perfusion and adequate tissue oxygenation will reverse lactic acidosis, while in septic shock an antibiotic treatment or a surgical drainage are required\textsuperscript{[22]}.

Although much of the world scientific literature is focused on the measurement of blood lactate, the non-invasive measurement of lactate in urine and saliva has a great potential in the home care of the elderly people and in the follow-up of chronic diseases.
1.4. Lactic acid in food, cosmetics and pharmaceuticals

Lactic acid is important not only in clinical diagnosis and sport medicine but also in others application like food quality control, cosmetics preparation, pharmaceutical technologies, detergents, etc.

In food industry lactate measurements are important for the control of fermentation of wine, cider, beer and milk products since it influences their flavor, stability and quality and it’s strictly correlated with their freshness\(^{[23-25]}\). Even in vegetables, fruits and juices lactic acid level is related to stability, storage quality and in some cases it can be a marker for fermentation by lactic acid bacteria\(^{[26-28]}\).

In recent years lactic acid has become very useful in cosmetic products. In fact α-hydroxy acids (as lactic, citric, glycolic, tartaric acids, etc.) are used in cosmetic products as exfoliants, moisturizers and emollient to correct skin disorders, to increase skin hydration and to improve some visible effects of ageing by reducing wrinkles and by stimulating cell renewal\(^{[29]}\).

Lactate salts, especially ammonium lactate, are used in many pharmaceutical preparations to treat severe dry skin or to mitigate the irritating effects of topical corticosteroids\(^{[30]}\). Since lactic acid and its salts are readily accepted by the body, lactate is an ideal vehicle for introducing therapeutic minerals or drugs. Polymers and copolymers of lactic acid, as poly(DL-lactic acid) and poly(lactic-co-glycolic acid), has been extensively used for making systems of delivering therapeutic substances including contraceptive steroids, anti-cancer drugs, anti-malarial agents, peptide hormones, etc\(^{[31-33]}\).

1.5. Determination of lactic acid

Therefore, lactate measurement is an important tool in many research areas. In literature there are lots of different assays able to determine lactic acid because of the heterogeneity of matrices where it has to be determinate. All these matrices are very complex and different from each other so they require different pretreatment of sample and different detection methods.
The main classes of techniques employed to determine lactic acid in complex matrices are:

- Colorimetric methods
- Enzymatic methods
- Biosensors
- Separation methods

1.5.1. Colorimetric methods

The first attempts to measure racemic lactate were colorimetric assays. These methods involved the conversion of lactate to acetaldehyde which is then determined as such or colorimetrically after reacting with some color-producing substances\[^{34,35}\].

One of the firsts method consisted in the oxidation of lactic acid by KMnO\(_4\) in the presence of MnSO\(_4\); the resulting acetaldehyde was aerated out of the solution, absorbed in bisulfite, and then determined by the Clausen titration method\[^{36}\].

Otherwise acetaldehyde could be derivatized with \(p\)-hydroxyphenol in the presence of copper ions and the resulting color change determined spectrophotometrically\[^{37}\].

1.5.2. Enzymatic methods

Enzymatic method is the most employed to determine lactic acid in biological fluids. The assay is based on the formation of pyruvate by oxidation of L-lactate with NAD\(^+\) (Reaction 1.1) by a reaction catalyzed by lactate dehydrogenase (LDH). The same reaction can be used either to determine D-lactate using D-lactate dehydrogenase instead of LDH.

1.1) \[ L\text{-lactate} + NAD^+ \rightarrow \text{pyruvate} + NADH \]

*Reaction 1.1*: enzymatic oxidation of L-lactate to pyruvate by LDH

The concentration of NADH can be determined measuring absorbance at 340 nm and it is directly proportional to the concentration of lactate in the sample\[^{38}\]. To achieve better
detection limit, NADH can be quantified by fluorimetric detection (excitation at 340 nm, emission at 460 nm)\(^{39}\) or by bacterial bioluminescent enzymes as NADH/FMN oxidoreductase and luciferase\(^{40}\).

The conversion of lactate can be improved by removing pyruvate from the reaction medium because the equilibrium is in favor of lactate. The more efficient way to trap pyruvate is its conversion to alanine by reacting with glutamate in a reaction catalyzed by alanine-2-oxoglutarate aminotransferase enzyme (ALT)\(^{41}\).

Another enzymatic assay is based on the oxidation of lactate to pyruvate with lactate oxidase (LOX) according to Reaction 1.2.

\[
1.2) \quad L\text{-lactate} + O_2 \rightarrow \text{pyruvate} + H_2O_2
\]

*Reaction 1.2*: enzymatic oxidation of L-lactate to pyruvate by LOX

In order to determine lactate the reaction is usually coupled to another one (ancillary reaction) in which the hydrogen peroxide formed gives a chemiluminescent oxidation reaction with reagents such as lucigenin or luminal, or oxidizes a spectroscopically active organic dye. Alternatively the hydrogen peroxide can be monitored with an enzyme electrode. Quantitation of lactate can also be achieved monitoring the fluorescence of lactate oxidase: the fluorescence of the enzyme during the reaction it’s proportional to the lactate concentration\(^{42}\).

### 1.5.3. Biosensors

The use of biosensors in clinical diagnosis has become very important in the last years because of the many advantages offered besides classical analytical methods. The use of an appropriate biosensor to monitor a specific analyte represents an important gain in terms of time and costs because the classical procedure of analysis is avoided and a continuous control of the metabolite is guaranteed.

The main requirements for an efficient biosensor are: sensitivity, specificity and ability to detect the disease with no false negative\(^{6, 30}\).
A biosensor is an analytical device incorporating a biological, or biologically derived material, intimately associated or integrated within a physiochemical transducer. The aim is to provide an electronic, or optical, response which is proportional to the concentration of the analyte. The most used sensing materials are enzymes due to their specificity and also because the role of enzymes in clinical diagnosis has been known for several years. The attachment of enzymes to an insoluble matrix is an essential step in the development of biosensors because they have poor stability in solutions. Numerous techniques of immobilization have been developed to enhance enzyme stability and to enable their reuse: covalent linkage, physical adsorption, cross-linking, encapsulation and entrapment (Figure 1.6). The matrix or support to be chosen for immobilization depends on the nature of the biomolecule and on the method of immobilization.[43]

Biosensors are classified on the type of signal transduction which can be: optical, electrochemical, electrical, magnetic or thermometric. The most used are electrochemical biosensor because they can be easily miniaturized due to the small distance required between biological material and transducer. Then they can operate even in muddy medium (as biological fluids) and they have shorter response time and lower detection limits besides optical biosensors[44].

Biosensors have been widely used for determination of lactate because of the short time required for the response. The main type of biosensors are amperometric and based on lactate-oxidase (LOx) or lactate-dehydrogenase (LDH). In amperometric biosensors the
current resulting from the electrochemical oxidation or reduction of an electroactive species is measured and it’s directly correlated to the bulk concentration of the analyte\textsuperscript{[45]}. Amperometric measurements are usually performed by maintaining a constant potential at a working electrode, generally by using an Ag/AgCl or saturated calomel electrodes. LOx-based amperometric biosensors are the most widely used configuration and allow the determination of $\text{H}_2\text{O}_2$ at the working electrode according to the enzymatic reaction (Reaction 1.2). The electrochemical measurement of hydrogen peroxide involves high positive potentials causing strong interferences by many easily oxidizable species like ascorbic acid\textsuperscript{[46]}.

When LDH is used as enzyme for lactate detection instead of LOx, an additional co-factor as NAD$^+$ is necessary and NADH is the species to be detected at the electrode. Although this configuration requires high positive potential either, LDH is less used than LOx because many inconvenient occurs. There is an additional immobilization step required because of the presence of the co-factor. Moreover the reaction takes place through radical intermediates giving lack of electrode stability. Then these biosensors were found to be less sensitive because of the low oxidizing potential of NAD$^+$ (-500mV vs. SCE at pH 7.0) which leads to small reaction yields.

In spite of these inconveniences there are some strategies to eliminate interferences and to improve sensitivity. One example of interference elimination is the conversion of the ascorbate into an electrochemically inert species by using ascorbate oxidase\textsuperscript{[47]}.

To increase the sensitivity multi-enzyme configurations are proposed and based on the recycling of the substrate of the reaction leading to signal amplification\textsuperscript{[48]}. In fact the problem of the unfavorable reaction equilibrium concerning reactions with LDH can be solved by coupling a second enzyme to the first. Examples are the transformation of pyruvate to glutamate by glutamic-pyruvic transaminase or of pyruvate to L-alanine by alanine aminotransferase. The product of the oxidation of lactate is subtracted and so the reaction is forced to its product side resulting in higher sensitivity\textsuperscript{[49, 50]}. In biosensors also lactate dehydrogenase cytochrome has been used to detect lactate. This enzyme is independent of oxygen and external cofactor, representing an important advantage during immobilization. However, lactate dehydrogenase cytocrome suffers from some disadvantages as well. In fact D-lactate is a competitive inhibitor of the catalyzed lactate oxidation and pyruvate is a competitive inhibitor of the oxidized enzyme at low concentration. Furthermore, this enzyme is inactive in acidic media so a good buffering system is required. Finally lactate dehydrogenase cytocrome is readily saturated.
by lactate ($K_{\text{max}} = 1.2 \text{ mM}$); therefore a dilution step is required for the determination of blood lactate since its concentration lies in the range of 1 mM$^{[51]}$.

1.5.4. Separation methods

There are several chromatographic methods which can be used for selective and sensitive determination of lactic acid in complex matrices:

- Gas chromatography-mass spectrometry (GC-MS)
- Capillary Electrophoresis (CE)
- Ion-Exchange Chromatography (IEC)
- Liquid chromatography-tandem mass spectrometry (LC-MS/MS)
- High performance liquid chromatography-diode array/fluorescence detectors (HPLC-DAD/FD)

1.5.4.1. GC-MS

Determination of lactic acid by GC/MS requires a derivatization step in order to increase its volatility by modifying the carboxylic and hydroxylic groups. This technique has been applied to biological matrices as blood, plasma and urine. The sample pretreatment consists in a deproteinization step and an extraction step of the acid, generally by ethyl-acetate. The most used derivatization procedure consists in the conversion of the organic acid into a silyl-ester and ether by using $t$-butyl-dimethyl-silyl-cloride$^{[52]}$ and N,O-bis-(trimethylsilyl)-trifluoro-acetamide (BSTFA)$^{[53]}$, although the last method was found to be very irreproducible.

Separation of lactic acid enantiomers can be achieved by using a chiral derivatization reagent on an achiral stationary phase$^{[35]}$. These derivatives include mainly esters, such as (-)-menthoxy carbonyl-methyl esters, acetyl/(-)-menthyl esters and trifluoroacetyl or trimethylsilyl/(+)-3-methyl-2-butyl ester$^{[54,55]}$. 
1.5.4.2. CE

Capillary electrophoresis can provide a simple and automated method to detect lactate in many kinds of matrices like food, beverage, cosmetics and also biological matrices. The small volume of sample required for the injection and the reduced sample pretreatment are the most important advantages of CE for body fluids analysis\textsuperscript{[56]}. Determination of lactate is generally achieved in short time with an UV detector, monitoring the absorbance at 200 nm\textsuperscript{[57]}. Separation of lactic acid enantiomers can be achieved using chiral selectors in the separation buffer as 2-hydroxypropyl-\(\beta\)-cyclodextrin (2-HP-\(\beta\)-CD)\textsuperscript{[58]}.

1.5.4.3. IEC

Ion-exchange chromatography is an instrumental technique used for the separation and determination of organic and inorganic anions and cations and their determination by a conductivity detector. IEC is particularly useful for the determination of anions from organic acids, the existence of which depends on the pH. This technique is often applied to food samples for the quality control of beverages as fruit juices and wines\textsuperscript{[59, 60]}.

1.5.4.4. HPLC-DAD/ FD

The isolation and quantification of lactic acid, as many others organic acids, in complex matrices is challenging because of the presence of lots of endogenous substance which may interfere with the detection of the analytes of interest. The direct detection of lactic acid is indeed based on the intrinsic absorbance of the carboxylic group at 210 nm\textsuperscript{[61]}. There are only few reports about direct detection of lactic acid, which require difficult and time consuming extraction procedure\textsuperscript{[62]}. Lactate can be easily determined by HPLC-DAD without any pretreatment in sweat samples because the lactate concentration is high enough to allow a high dilution of the sample, eliminating any interference\textsuperscript{[11]}. Thus, the determination of lactic acid in raw matrices requires highly sensitive and selective detection methods.
The most advantageous strategy to improve the determination of organic acids by HPLC-DAD/FD is the chemical derivatization of the carboxylic group with a suitable chromophore or fluorophore in order to enhance the UV-Vis absorption or to give fluorescence properties.

The most used labels for carboxylic acid are fluorophores because the obtained derivatives can be monitored by both fluorescence and UV-Vis detectors.

The fluorescence (FL) derivatization methods fall into two categories due to the reaction timing before and after the chromatographic separation: off-line pre-column and on-line post-column. The pre-column derivatization is used more frequently than the post-column for sensitive detection by HPLC. In fact post-column derivatization suffers from some disadvantages as time and temperature restriction on reaction conditions, interdependence between mobile phase and the reaction medium, risk of band-broadening and loss of resolution due to post column mixing[63].

The FL tagging involves the covalent binding formation between the reactive functional group of the FL reagent and the analyte. The most used reagents for FL tagging are listed in Table 1.1.

### Table 1.1: reagents for FL tagging

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Functional reactive group</th>
<th>Matrix</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkyl halides</td>
<td>R-X</td>
<td>river water, serum, blood, brain tissue</td>
<td>[64-68]</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>R-CONHNH₂</td>
<td>serum</td>
<td>[69, 70]</td>
</tr>
<tr>
<td>Amine</td>
<td>R-NH₂</td>
<td>blood, plasma, serum</td>
<td>[71-73]</td>
</tr>
<tr>
<td>Alcohols</td>
<td>R-OH</td>
<td>plasma</td>
<td>[74]</td>
</tr>
<tr>
<td>Sulfonate</td>
<td>R-SO₃CF₃</td>
<td>mouse brain</td>
<td>[75]</td>
</tr>
<tr>
<td>Anthracene derivatives</td>
<td>Ar-Y (Y = Cl, Br, NH₂, OH, CH=N⁺=N⁺)</td>
<td>serum, plasma, soil, mud, slurry, fruit juice, rat pleural cells</td>
<td>[76-80]</td>
</tr>
</tbody>
</table>

Chiral reagents which have an asymmetric center in the structure have been developed for the resolution of enantiomers of carboxylic acids. This is based upon diastereomer formation with the reagents before HPLC separation[81, 82].
There are only few works published on the determination of lactic acid by HPLC-DAD/FD using the FL tagging. The most used FL reagents are: (+)-1-(9-fluorenyl)-ethyl-chloroformate ((+)-FLEC)[54], 4-(N,N-dimethylamino sulfonyl)-7-piperazino-2,1,3-benzoxadiazole (DBD-PZ)[83], 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ)[84, 85], (2S)-2-amino-3-methyl-1-[4-(7-nitro-benzo-2,1,3-oxadiazol-4-yl)-piperazin-1-yl]-butan-1-one (NBD-PZ-Val)[86], α-bromo-acetophenone (α-BAP)[87], 2,4’-dibromo-acetophenone (2-DBAP)[88], O-(4-nitrobenzyl)-N,N’-diisopropylisourea (PNBDI)[89, 90].

*Table 1.2* summarizes the detection limits and the application fields of the techniques cited above.

*Table 1.2*: detection limits and application matrices of the derivatization technique to determine lactate

<table>
<thead>
<tr>
<th>FL reagent</th>
<th>Matrix</th>
<th>LOD (pmol)</th>
<th>Linear range (mM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-FLEC</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>[54]</td>
</tr>
<tr>
<td>DBD-PZ</td>
<td>Rat urine</td>
<td>0.300</td>
<td>0.01-20</td>
<td>[83]</td>
</tr>
<tr>
<td>NBD-PZ</td>
<td>Human serum</td>
<td>0.050</td>
<td>1-10</td>
<td>[84]</td>
</tr>
<tr>
<td>NBD-PZ-Val</td>
<td>Human plasma</td>
<td>750</td>
<td>0.5-4</td>
<td>[86]</td>
</tr>
<tr>
<td>α-BAP</td>
<td>Human plasma</td>
<td>36</td>
<td>0.18-6</td>
<td>[87]</td>
</tr>
<tr>
<td>2-DBAP</td>
<td>Body tissues</td>
<td>0.034</td>
<td>/</td>
<td>[88]</td>
</tr>
<tr>
<td>PNBDI</td>
<td>Table olives, Fruit juices</td>
<td>1.5</td>
<td>0.001-0.011</td>
<td>[90]</td>
</tr>
</tbody>
</table>

In all these cases the derivatization procedure requires the extraction of lactate from the sample and long reaction times, resulting in difficult and time consuming methods. Furthermore toxic substances as pyridine are often required to catalyze the reaction between lactate and the FL reagent.

Thus there is a need of a new, simple and fast derivatization method to determine lactate in biological fluids.

1.5.4.5. LC-MS/MS

Liquid chromatography-tandem mass spectrometry is a useful technique that provides high sensitivity and selectivity because of the identification of analytes by their masses and by their specific fragments.
Hence LC-MS/MS would be the technique of choice for the determination of lactate in complex matrices because to avoid several limits typical of HPLC-DAD/FD methods. However, the low molecular weight of lactate makes difficult its determination by LC-MS and especially by LC-MS/MS, where a fragmentation of the parent ion is required. There is only one work about the direct determination of lactate in urine samples by LC-MS, without the derivatization step, but a difficult and time consuming pretreatment of the samples is used\cite{91}. In order to overcome this problem a derivatization step, before the analysis by LC-MS/MS, would be helpful to provide an increase of the molecular mass of the analyte. The use of a chiral derivatization reagent as (+)-O,O’-diacetyl-L-tartaric anhydride (DATAN) provides the separation and determination of lactic acid enantiomers by UPLC-MS/MS\cite{92}.

### 1.6. Creatinine

#### 1.6.1. Biochemistry of Creatinine

Creatinine is a metabolic waste product resulting from the non-enzymatic conversion of creatine and phosphocreatine, which are fundamental molecules for the energy transfer in muscle\cite{93}. Over 90\% of bodily creatine is present in the muscle, with the remainder being in the plasma and brain\cite{94}. Creatine is synthesized in the body using glycine, arginine (kidney) and methionine (liver) and the main dietary source of creatine is red meat.

Creatine is converted to phosphocreatine in muscle in a reversible reaction with adenosine triphosphate (ATP), facilitate by creatine kinase. When muscle contractions deplete the immediate supply of ATP, the phosphocreatine can rephosphorylate ADP to replenish the supply of ATP. Creatine supplementation has been demonstrated to be effective in increasing peak force and peak power during short-duration, high-intensity activity, but to have no effect on endurance\cite{95}. 


In muscle tissues creatinine is formed by cyclization and dehydration of creatine and phosphocreatine (Reaction 1.3)\(^{[96]}\).

\[
\begin{align*}
\text{Reaction 1.3: formation of creatinine by creatine cyclization}
\end{align*}
\]

Creatinine is excreted by kidneys into the urine and, under normal conditions its excretion is relatively constant. Approximately 2% of the body’s creatine is converted into creatinine every day. Hence the amount of creatinine produced is proportional to the muscle mass of individual.

1.6.2. Urinary creatinine relevance

Quantification of urinary creatinine is an important tool in clinical diagnosis for several reasons. Patients with muscle wasting disease such as muscular dystrophy exhibit reduced concentrations of muscle creatine and decreased excretion of creatinine\(^{[97]}\). Urinary creatinine in newborn infants and neonates is also monitored\(^{[96]}\).

Creatinine levels are also correlated with renal function because it is excreted by kidneys. Therefore quantification of urinary creatinine is useful to monitor patients with liver cirrhosis\(^{[98, 99]}\) and renal insufficiency\(^{[100]}\).

In clinical laboratories, urinary creatinine is used as a standardization tool for the quantitative evaluation of urinary concentrations of drugs, xenobiotics and metabolites, in order to avoid the interfering influence of the physiological differences in urine water content\(^{[93]}\). Thus, particularly in forensic toxicology, it is a fairly common practice to report drug or drug metabolite concentrations in urine as the ratio drug mass/mg of urinary creatinine. Moreover, determination of creatinine in urine samples can be used to identify possible adulteration of the specimen, by dilution or substitution with different liquids\(^{[101]}\).
The urinary creatinine concentration in the normal population ranges from 2.5 to 23 mM depending on age, gender and diet\cite{93}.
In this work the determination of urinary creatinine is useful to allow the comparison of lactate levels between different samples.

### 1.6.3. Determination of urinary creatinine

Determination on urinary creatinine can be achieved by several immunoassays or enzymatic methods\cite{102-104}, capillary electrophoresis\cite{105, 106} and liquid chromatography\cite{107-109}.

The clinical assay for detection of creatinine is based on the Jaffé reaction. This is a colorimetric method used to quantify creatinine levels in blood and urine samples\cite{96}.
Creatinine is combined with an alkaline picrate solution to form an orange-red complex (according to Reaction 1.4\cite{110}).

\[
\text{Reaction 1.4: Jaffé reaction between creatinine and picrate}
\]

Spectrophotometric detection of the complex is achieved reading the absorbance at 485 nm after 45 or 60 and 180 seconds. In fact the use of the absorbance increments between the two time periods provides higher precision.

A dilution of the urine sample in distilled water (1:50) is the only pretreatment required.
There are many interferences which may affect creatinine detection; the most important are urea, glucose, lactose, uric acid, acetone, ammonia, creatine and albumin\cite{111}. However these compounds do not interfere with creatinine determination up to a concentration of 10-200 times that of creatinine and urine has fairly low concentrations for most of these compounds\cite{112}.

This method is time-consuming and often overestimates the creatinine concentration due to the interference of endogenous and exogenous pseudo-creatinine chromogens\cite{113}.
CHAPTER 2

Experimental

2.1. Chemicals

L-Lactic acid (L-6402, purity 98%), creatinine anhydrous (C4255, purity > 99.0%), acetate (51791), butyrate (08089) and propionate (51716) (1000 mg/L in H2O), 9-chloromethylanthracene (196517, purity > 98%) and tetra-n-butyrammonium bromide (426288, purity > 98%) were purchased from Sigma-Aldrich-Fluka (Milan, Italy), pyruvate sodium salt (8593589) from Merk and triethanolamine (purity > 99%) from Ashland Chemical Italiana. Stock solutions of L-lactic acid, creatinine and pyruvate were prepared in ultra-pure water obtained by a Milli-Q system (Purerlab Pro + Purelab Classic, Millipore, USA). Stock solution of 9-chloromethyl anthracene, tetra-n-butyrammonium bromide and triethanolamine were prepared in acetonitrile (LC-MS Chromasolv, 34967, purity > 99.9%, Sigma-Aldrich-Fluka). Phosphate buffer solution (PBS) at pH 2.5 was prepared from monobasic monohydrate sodium phosphate (BDH Laboratory Supplies, Poole, England) and phosphoric acid (345245, Sigma-Aldrich-Fluka).

Methanol for RP-HPLC was purchased from Carlo Erba (Rodano, MI, Italy).

2.1.1. Standard solutions

Lactate standard solutions were prepared by diluting the stock solution in Milli-Q water. Six standard solutions were employed for the calibration curves and three of them were
used as low, medium and high quality control: 0.05 mM, 0.15 mM (LQC), 0.5 mM (MQC), 1.5 mM, 5 mM (HQC) and 15 mM. Low, medium and high quality controls were used to evaluate the intra-day and inter-day repeatability and recoveries. Creatinine standard solutions were prepared by diluting the stock solution in Milli-Q water. Eight standard solutions were employed for the calibration curves and three of them were used as low, medium and high quality control: 0.05 mM, 0.15 mM, 0.5 mM, 1.5 mM (LQC), 5 mM (MQC), 15 mM (HQC), 50 mM and 150 mM. Low, medium and high quality controls were used to evaluate the intra-day and inter-day repeatability.

2.2. Biological samples

All the biological samples (urine and saliva) were provided by healthy volunteers and by patients from the intensive care unit of Cisanello Hospital (Pisa) after their consensus. Urine samples from healthy volunteers were collected before and after training into a 10 mL Eppendorf tube, centrifuged at 5000 rpm for 10 minutes (Eppendorf Centrifuge 5804R) and divided into aliquots of 1 mL. Urine samples from the patients of the intensive care unit were collected by an urometer for a period of time of one hour and placed in 1 mL Eppendorf tubes. Saliva samples were collected by STARSTED Salivette® for Cortisol Testing. All the samples were frozen at -20°C until analysis and were found to be stable for more than 1 year. Urine and saliva samples were made to thaw at room temperature and centrifuged at 14000 and 3000 rpm for 10 minutes respectively. The supernatant was pipetted into a 1 mL Eppendorf tube and vortexed (Velp Scientifica) for 30 seconds.
2.3. HPLC-DAD/FD

An HPLC gradient pump (P4000, ThermoFinnigan) was coupled with a vacuum membrane degasser (SCM1000, ThermoFinnigan), an AS3000 autosampler (ThermoFinnigan), a UV6000 diode array detector and a FL3000 fluorescence detector (ThermoFinnigan). Separations of carboxylic acid derivatives were carried out using a reversed-phase HPLC column GEMINI C18 (250 mm x 4.6 mm, 5 μm, Phenomenex), equipped with a guard cartridge (KJ0-4282, Phenomenex) with the column temperature set at 40°C. The mobile phase with 65% ACN and 35% water was used and the flow rate was set constant at 0.8 mL/min. The chromatographic run was complete in 32 minutes, including a rinsing of the column in 100% acetonitrile and the re-equilibrating step (Method 1). An injection volume of 20 μL was used for each sample. The detection of lactate was performed in absorbance at 365 nm and in fluorescence setting the excitation and emission wavelengths at 365 and 410 nm, respectively.

For the simultaneous determination of lactate and creatinine an isocratic elution in 20% ACN and 80% water was used before the gradient. The chromatographic run was complete in 42 minutes (Method 2) and the detection of creatinine was performed at 234 nm.

The direct determination of lactate (to estimate the derivatization yield) was carried out using a reversed-phase HPLC column Synergi-Hydro RP-C18 (250mm x 4.6mm, 4μm, Phenomenex), equipped with a guard cartridge (KJ0-4282, Phenomenex) with the column temperature set at 30°C. The mobile phase with 99% 0.05 M PBS (pH = 2.5) and 1% methanol was used and the flow rate was set constant at 0.8 mL/min. The chromatographic run was complete in 30 minutes, including a rinsing of the column in 100% methanol and the re-equilibration step (Method 3). The injection volume was 20 μL and detection was performed at 210 nm[1]. The lactate calibration curve was linear in the investigated range 0.01-30 mM and the detection limit and the quantification limit were 0.0001 and 0.0004 mM respectively (calculated as 3 and 10 times the standard deviation of the blank).

Table 2.1 summarizes the three elution methods employed.
Table 2.1: the three elution methods employed for the determination of: A) lactate derivative, B) lactate derivative and creatinine, C) lactic acid

A) Elution method 1

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% ACN</th>
<th>% Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>10</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>11</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>32</td>
<td>65</td>
<td>35</td>
</tr>
</tbody>
</table>

B) Elution method 2

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% ACN</th>
<th>% Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>16</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>17</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>27</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>42</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

C) Elution method 3

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% PBS</th>
<th>% Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>19</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>30</td>
<td>99</td>
<td>1</td>
</tr>
</tbody>
</table>
2.4. HPLC-ESI-Q-ToF

Analyses were carried out using a 1200 Infinity HPLC (Agilent Technologies, USA), coupled by a Jet Stream ESI interface (Agilent) with a Quadrupole-Time of Flight tandem mass spectrometer 6530 Infinity Q-ToF detector (Agilent Technologies). Column temperature was 40 °C.

The ESI operating conditions were: drying gas (N₂, purity >98%): 350 °C and 10 L/min; nebulizer gas 35 psig; sheath gas (N₂, purity >98%): 375 °C and 11 L/min. Collision gas for MS/MS analysis was nitrogen (purity 99.999%).

High resolution MS spectra were acquired in positive mode in the range 100–1700 m/z, while tandem MS/MS spectra were acquired in the range 85-600 m/z. The data were collected with a MS scan rate of 1.46 spectra/s. The mass axis was calibrated using the Agilent tuning mix HP0321 (Agilent Technologies) in acetonitrile.

Mass spectrometer control and data acquisition were performed with MassHunter® Workstation Software (B.04.00).

Two different chromatographic methods were used for different columns.

- **Method 4**

  Separations of carboxylic acid derivatives and creatinine were carried out using a reversed-phase HPLC column GEMINI C18 column (250 mm x 4.6 mm, 5 µm, Phenomenex), equipped with a guard cartridge (KJ0-4282, Phenomenex).

  The injection volume was 4 μL and the needle wash injection type was used. The flow rate was 0.4 mL/min.

  *Table 2.2 A), B) and C)* shows the elution method used, the time acquisition segments and the scan source parameters, respectively.
Table 2.2: (A) The elution method used for the determination of lactate derivative and creatinine; (B) time acquisition segments; (C) the scan source parameters for time segment #2 and #4

A) Elution method

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% ACN</th>
<th>% Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>32</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>33</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>53</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>54</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>74</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

(B) Time Segment

<table>
<thead>
<tr>
<th>Time Segment</th>
<th>Start Time (min)</th>
<th>Diverter Valve State</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>Waste</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>MS</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>Waste</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>MS</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>Waste</td>
</tr>
</tbody>
</table>

C)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (V)</th>
<th>Time Segment #2</th>
<th>Value (V)</th>
<th>Time Segment #4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary voltage</td>
<td>4500</td>
<td></td>
<td>3500</td>
<td></td>
</tr>
<tr>
<td>Nozzle voltage</td>
<td>1000</td>
<td></td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>Fragmentor</td>
<td>150</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Skimmer 1</td>
<td>65</td>
<td></td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Octopole RF</td>
<td>750</td>
<td></td>
<td>750</td>
<td></td>
</tr>
</tbody>
</table>

- Method 5

Separations of carboxylic acid derivatives and creatinine were carried out using a reversed-phase Zorbax Extend C18 column (2.1 mm x 50 mm, 1.8 µm) with a Zorbax Extend C18 guard column (2.1 mm x 12.5 mm, 5 µm). The injection volume was 1 µL and the needle wash injection type was used. The flow rate was 0.3 mL/min.
Table 2.3 A), B) and C) shows the elution method used, the time acquisition segments and the scan source parameters, respectively.

Table 2.3: (A) The elution method used for the determination of lactate derivative and creatinine; (B) time acquisition segments; (C) the scan source parameters for time segment #1 and #3

A)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% ACN</th>
<th>% Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>1</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>3.4</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>7.5</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th>Time Segment</th>
<th>Start Time (min)</th>
<th>Diverter Valve State</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>MS</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>Waste</td>
</tr>
<tr>
<td>3</td>
<td>5.5</td>
<td>MS</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>Waste</td>
</tr>
</tbody>
</table>

C)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time Segment #1 Value (V)</th>
<th>Time Segment #3 Value (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary voltage</td>
<td>4000</td>
<td>3500</td>
</tr>
<tr>
<td>Nozzle voltage</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Fragmentor</td>
<td>150</td>
<td>70</td>
</tr>
<tr>
<td>Skimmer 1</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>Octopole RF</td>
<td>750</td>
<td>750</td>
</tr>
</tbody>
</table>
2.5. Analytical procedure

The final analytical procedure optimized in this work is summarized in Figure 2.1.

---

**Fig. 2.1:** scheme of the final optimized analytical procedure

---

Lactate standard solutions and biological samples were thaw at room temperature. Biological samples were centrifuged at 14000 rpm for 10 minutes. Briefly, 10 μL of sample were added to the reaction vial with 20 μL of 5% TEA, 90 μL of 90 mM TBA, 370 μL of 10 mM 9-CMA and 10 μL of ACN. The reaction solution was incubated at 70°C for 30 minutes in a thermostatic water-bath in the dark to facilitate derivatization. The reaction solution was cooled at room temperature and diluted 1:10 in mobile phase prior injection in HPLC. For the analysis with HPLC-ESI-Q-ToF all the samples were filtered through a PTFE syringe filter (0.45 μm, GRACE).
2.6. Data processing

ChromQuest™ 4.2 Chromatography Data System was used to carry out HPLC-DAD/FD control, data acquisition and data analysis.

MassHunter® Workstation Software (B.04.00) was used to carry out mass spectrometer control, data acquisition and data analysis of the analysis in HPLC-ESI-Q-ToF.

Excel 2007 by Microsoft Corporation and OriginLab Origin 8.6® Corporation were used for the statistical treatment of the data.
Results
CHAPTER 3

Optimization of the method

3.1. 9-chloromethyl anthracene

9-chloromethyl anthracene (Figure 3.1) was chosen as the derivatization reagent:

![9-chloromethyl anthracene](image)

*Fig. 3.1: 9-chloromethyl anthracene*

9-chloromethyl anthracene (9-CMA) is an anthracene derivative useful for the derivatization of carboxylic acids, phenols, thiophenols and mercaptans.

The physical-chemical properties of 9-CMA are summarized in Table 3.1:\textsuperscript{[114]}

*Table 3.1: 9-CMA properties*

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical formula</td>
<td>C\textsubscript{15}H\textsubscript{11}Cl</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>226.70</td>
</tr>
<tr>
<td>Melting point</td>
<td>138-140 °C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>402 °C at 760 mmHg</td>
</tr>
<tr>
<td>Appearance</td>
<td>yellow powder</td>
</tr>
<tr>
<td>Sensitive</td>
<td>Light sensitive</td>
</tr>
<tr>
<td>Chemical stability</td>
<td>4 °C at dark for 1 month</td>
</tr>
<tr>
<td>CAS number</td>
<td>24463-19-2</td>
</tr>
<tr>
<td>Hazard Codes</td>
<td>Xi</td>
</tr>
<tr>
<td>Risk Statements</td>
<td>36/37/38</td>
</tr>
<tr>
<td>Safety Statements</td>
<td>26-36</td>
</tr>
</tbody>
</table>
9-CMA is a fluorescent compound with a strong absorption at 256 nm. Figure 3.2 shows the UV-Vis absorption spectrum (A) and the fluorescence spectrum ($\lambda_{\text{ex}}$: 256nm) (B) of a 9-CMA solution in ACN.

Fig. 3.2: UV-Vis absorption spectrum (A) and the fluorescence spectrum ($\lambda_{\text{ex}}$: 256nm) (B) of a 9-CMA solution in ACN
9-CMA has been used for the derivatization of long chain carboxylic acids\textsuperscript{[115]}, as fatty and bile acids, and for some organic acids (i.e. formic, acetic, proprionic, butyric, pentanoic and benzoic acids) in food samples\textsuperscript{[76]}. Carboxylic acids react with 9-CMA to form fluorescent esters according to \textit{Reaction 3.1}:

\begin{equation}
3.1) \quad R-COO^{-} + 9-CMA \quad \xrightarrow{TBA/ACN, \Delta} \quad \text{Ester}
\end{equation}

\textit{Reaction 3.1: reaction of carboxylic acids with 9-CMA}

The reaction is a nucleophilic substitution that proceeds in aprotic solvents (as acetonitrile or cicloexane) to avoid the protonation of carboxylates and because of the low solubility of 9-CMA in protic solvents. The reaction must be also catalyzed by a phase-transfer catalyst as tetra-\textit{n}-butylammonium bromide or hydroxide to form an ion pair with the carboxylate to make it soluble in the aprotic solvent. In order to improve the derivatization yield the reaction solution must be heated for a desired time. The obtained esters have the same spectroscopic properties of 9-CMA and so can be detected easier and with higher sensitivity than the correspondent carboxylic acids\textsuperscript{[115]}.

\subsection*{3.2. Reactivity of 9-CMA with lactate}

The method was first applied to standard solutions of lactic acid (in water) to check if the analyte reacts with 9-CMA. Xie et al.\textsuperscript{[76]} employed 9-CMA to derivatize several carboxylic acids with the procedure described in \textit{Figure 3.3}. 
As lactic acid is quite different from the carboxylic acids previously derivatized because of the presence of a hydroxilic group in $\alpha$ to the carboxylic group, we first verified its reactivity in the same conditions. This was not granted because in basic media the formation of an intramolecular H-bond in lactate molecule occurs (Figure 3.4) leading to a stable 5-terms cycle that may makes difficult for lactate to react.

\[ \text{H}_3\text{C} \]
\[ \begin{array}{c}
  \text{O} \\
  \text{O} \\
  \text{O} \\
  \text{H} \\
\end{array} \]

**Fig. 3.4:** intramolecular H-bond
Standard solutions of lactic acid, prepared by dilution from the stock solution, were extracted and derivatized as explained in Figure 3.3. Briefly, an aliquot of the lactate standard solution was diluted 1:1 with 2 M HCl. The analyte was extracted in ethyl-acetate and vortexed for 3 minutes. The procedure was repeated three times and the supernatant solvent was collected. The organic layer was evaporated to dryness in a vial under a stream of nitrogen at 40 °C after adding 20 μL of triethanolamine solution (5% in acetone) in order to obtain the lactate. 450 μL of 20 mM TBAB in ACN were added to the residue and successively 50 μL of 10 mM 9-CMA solution in ACN. The use of the phase reagent transfer (TBAB) is fundamental to form an ion-pair with the carboxylate making it soluble in ACN. The reaction solution was kept at 75°C for 50 minutes in a thermostatic water-bath in the dark to facilitate the derivatization. The reaction solution was cooled at room temperature and diluted 1:10 in the mobile phase prior to the injection in HPLC.

Figure 3.5 shows the fluorescence chromatogram at 410 nm (λ_ex = 256 nm) of 0.5 mM and a 2 mM lactate standard solutions extracted and derivatized as explained above.

**Fig. 3.5:** Fluorescence chromatogram at 410 nm (λ_ex = 256 nm) of 0.5 mM (red line) and a 2 mM (black line) lactate standard solutions extracted and derivatized with 9-CMA\textsuperscript{[76]}.

Chromatographic conditions: Synergi-Hydro RP-C18 (250x4.6mm, 5μm, Phenomenex), isocratic elution with 64% ACN, 36% Milli-Q water
The anthrylmethyl ester derivative of lactate elutes at 6.5 min well separated from the peak of the 9-CMA excess (tR = 5.4 min). These results show that lactic acid can react with 9-CMA and elutes in a very short time.

### 3.3. Optimization of the analytical procedure

Once proved that lactic acid reacts with 9-CMA the derivatization procedure was optimized.

#### 3.3.1. Extraction

The extraction of the analyte is a crucial step of the analytical procedure proposed by Xie et al. This requires the elimination of water from the reaction media and a great part of endogenous compounds which may be found in the raw matrices analyzed. Hence, the extraction of analytes should lead to a higher selectivity of the method and to a control of the matrix effect.

However, in the specific case of lactate, the extraction suffers of several disadvantages. Lactate is very soluble in water and it is difficult to find an adequate extraction solvent. Several solvents were assayed to extract lactic acid from an acidic aqueous solution and ethyl-acetate was found to be the best solvent but with a 20% extraction yield. Furthermore, the extraction is time consuming and it is desirable to limit the sample handling in order to reduce the risk of infection for the operator.

For all these reasons we tried the derivatization of lactate by adding an aliquot of the aqueous lactate standard solution to the reaction vial with TEA, TBAB and 9-CMA in ACN (10 µL in 500 µL final volume). Figure 3.6 shows the fluorescence chromatogram at 410 nm of 1 mM standard solution of lactate derivatized as reported above without the extraction procedure and a corresponding blank solution obtained by applying the same derivatization procedure to 10 µL of ultrapure water.
Fig. 3.6: Fluorescence chromatogram at 410 nm ($\lambda_{ex} = 365$ nm) of 1 mM standard solution of lactate derivatized with 9-CMA without the extraction procedure (blank= red line; 1 mM lactate= black line).
INLET: zooming between 6 and 9 min. Chromatographic conditions: Synergi-Hydro RP-C18 (250x4.6mm, 5μm, Phenomenex), isocratic elution with 64% ACN, 36% Milli-Q water

According to the chromatogram lactate reacts with 9-CMA even in the presence of traces of water. However, two peaks ($t_R$ 5.7 and 6.5 min) appear when a lactate standard solution is derivatized without extraction: the peak at 6.5 min has the retention time of the derivatized lactate (see Figure 3.5). The peak at 5.7 min corresponds likely to a by-product of the reaction.

The blank chromatogram evidences also the presence of a small peak eluting at the lactate derivative retention time. This peak does not affect the determination of lactate because it has been minimized in the optimization step (see Paragraph 3.3.3.).

A further experiment in order to study the origin of the by-product peak was performed by applying the same derivatization procedure to a lactate standard solution prepared in ACN. Figure 3.7 shows the fluorescence chromatograms at 410 nm of 1 mM standard solution of lactate derivatized from an aqueous (red line) and ACN (black line) standard solution.
Consider the different chromatographic conditions employed for this experiment, we confirmed that the peak at 20.05 min (at 6.5 min in Figure 3.6) is due to lactate derivative and the peak at 18.03 (at 5.7 in Figure 3.6) is due to the by-product. Probably the peak at 5.7 min corresponds to a by-product of a decomposition reaction of the lactate derivative. In fact, the lactate derivative is an ester which may hydrolyze, during the heating time, to 9-hydroxymethyl anthracene (9-OHMA) and lactate (Reaction 3.2) in the basic reaction medium.

\[
\text{Lactate derivative (t_R = 20.05 min)} \quad \text{Lactate derivative (t_R = 18.03 min)}
\]

\[
\text{9-CMA (t_R = 14.25 min)} \quad \text{9-CMA (t_R = 18.03 min)}
\]

\[
\text{3.2) } \quad \text{Reaction 3.2: possible hydrolysis of the lactate derivative}
\]
This hypothesis was confirmed by the analysis with HPLC-ESI-Q-ToF (see Chapter 4). The experiment also showed that the area of the lactate derivative peak is double when lactic acid is dissolved in ACN instead of water.

### 3.3.2. Yield of the reaction

The reaction yield was investigated by determining the free lactate in the reaction vial by using Method 3 (see Paragraph 2.4)[1]. The derivatized lactate standard solutions were diluted 1:10 in the mobile phase and injected in the HPLC. Table 2.2 summarizes the concentrations of free lactate found in five different derivatization experiments performed at five different concentration levels of lactate and the corresponding reaction yields.

**Table 2.2:** Yield of the derivatization reaction of lactate with 9-CMA for five different concentration levels of lactate

<table>
<thead>
<tr>
<th>[lactate]/mM</th>
<th>Reaction yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>70</td>
</tr>
<tr>
<td>0.03</td>
<td>72</td>
</tr>
<tr>
<td>0.01</td>
<td>82</td>
</tr>
<tr>
<td>0.003</td>
<td>88</td>
</tr>
<tr>
<td>0.001</td>
<td>76</td>
</tr>
</tbody>
</table>

The yields ranged between 70 and 88%. However, when lactic acid was dissolved in ACN instead of aqueous medium, the free lactate was one half and the yields ranged among 85 and 97%. This confirms the result presented above (see Figure 3.7, Paragraph 3.3.1.)

In summary: i) the lactate reacts with 9-CMA also in the presence of trace amount of water; ii) the reaction yield in the presence of trace of water is 80% instead of 95% in ACN, thus the extraction step can be by-passed.
3.3.3. Effect of reaction time

The effect of reaction time was studied at 75 °C (Xie, et al.[76]) to determine the minimum time required to reach a plateau. Figure 3.8 shows the peak area of lactate derivative in the fluorescence chromatogram ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 410$ nm) as a function of time.

![Figure 3.8: effect of reaction time at 75 °C for a 0.05 mM lactate standard solutions derivatized with 0.1 mM 9-CMA (injected concentrations)](image)

The reaction reaches a plateau after about 2.5 hours. Lactate and 9-CMA can react also at room temperature but the plateau is reached after about 20 hours.

3.3.4. Optimization of the temperature and reaction time using the experimental design

The reaction yield can be affected by several parameters: temperature, reaction time, 9-CMA excess, TBAB concentration, TEA concentration.

As the reaction temperature plays an important role in the reaction yield first we performed a central composite design with two factors (temperature and time) at five levels (Figure 3.9).
**Fig. 3.9:** experimental design with two factors (temperature and time) at five levels for the optimization of the reaction yield of lactate with 9-CMA

*Table 3.3* shows the concentrations of the reagents employed, on the basis of Xie et al.\cite{76}.

*Table 3.3:* concentrations of the reagents employed, on the basis of Xie et al.\cite{76}

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[lactate]</td>
<td>0.05 mM</td>
</tr>
<tr>
<td>9-CMA excess</td>
<td>2:1</td>
</tr>
<tr>
<td>[TBAB]</td>
<td>16.2 mM</td>
</tr>
<tr>
<td>[TEA]</td>
<td>10.8 mM</td>
</tr>
</tbody>
</table>

All the thirteen experiments were performed on 0.05 mM lactate standard solutions (injected concentration) and on a blank solution. *Figure 3.10 A* and *B*) shows the response surfaces obtained by plotting the area of the lactate and blank peaks of fluorescence chromatograms ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 410$ nm) as a function of reaction time and temperature. The response surfaces were obtained with Origin by converting the raw data into a 100x100 matrix using a Random (Renka Cline) gridding method. Response surfaces were calculated not only for the lactate derivative but for the unknown peak of the blank that has the same retention time of the lactate derivative (*Figure 3.6, Paragraph 3.3.1*). The unknown peak of the blank increases as the 9-CMA excess increases and it is
not negligible for low lactate concentrations. Thus, the response surface of the blank solution is very important in order to minimize this peak.
Fig. 3.10: response surfaces obtained from the experimental design (factors: temperature and heating time) of a 0.5 mM lactate standard solution (A) and of blank solution (B). The reagent concentrations were: 1 mM 9-CMA, 16.2 mM TBAB, 10.8 mM TEA. Chromatographic conditions: Synergi-Hydro RP-C18 (250x4.6mm, 5μm, Phenomenex), isocratic elution with 64% ACN, 36% Milli-Q water.
The results show that the signal increases as the time and temperature increase. However, for temperatures higher than 80 °C many peaks appear in the chromatogram indicating the massive formation of by-products/degradation products. In fact, at 90°C the signal decreases as the reaction time increases.

We designated 70 °C and 1.5 h reaction time as the best derivatization conditions, which provide the maximum signal and minimize the risk of degradation.

However, in these conditions (70 °C for 1.5 h) the unknown peak of the blank solution reached a maximum as well (Figure 3.10 (B)). Hence, we selected 70 °C for 30 min as the best derivatization conditions to get only about 7% less of lactate derivative peak and 22% less of the blank peak. Furthermore a shorter reaction time provides a faster procedure.

### 3.3.5. Optimization of 9-CMA excess

The concentration of 9-CMA is critical for the derivatization reaction and the effect of 9-CMA excess on the derivatization yield was studied.

A 0.5 mM lactate standard solution was derivatized for 30 min at 70 °C with 9-CMA by varying the 9-CMA/lactate ratio in the range of 1-10. Figure 3.11 shows the trend of the area of lactate derivative peak in the fluorescence chromatogram ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 410$ nm) as a function of 9-CMA/lactate ratio.

![Fig. 3.11: trend of the area of lactate derivative peak in the fluorescence chromatogram ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 410$ nm) as a function of 9-CMA/lactate ratio](image-url)
The signal reached a maximum for 9-CMA/lactate ratio higher than 5.
For our experiments we chose the maximum concentration of 9-CMA compatible with the solubility of 9-CMA in ACN (saturated stock solution) in order to get the maximum dynamic linear range in the lactate determination, which corresponded to 7.4 mM 9-CMA in the reaction medium.

### 3.3.6. Doehlert design for the simultaneous optimization of [TEA], [TBAB] and [9-CMA]

The Doehlert designs can be used in optimization processes because they offer a uniform distribution of points over the whole experimental region. In the case of three-variables designs, a cuboctahedron (a cube with eight vertices symmetrically truncated producing eight equilateral triangles whose edges are equal to those of the remaining squares) is produced geometrically (Figure 3.12).

![Fig. 3.12](image.png)

*Fig. 3.12: 3D view of the experimental domain in the three variables Doehlert design, the dots represent the experiments required[^116]*
The Doehlert designs require the same number of experiments, $N$, related to the number of variables under study, $k$, as do Box-Behnken designs ($N = k^2 + k + 1$) and a lower number than ones used in central composite designs ($N = 2k^2 + 2k + 1$). Furthermore, in both central composite and Box-Behnken designs the number of levels, $l$, for each variable is always the same (for $k = 3$, $l = 5$ in a central composite design or $l = 3$ in a Box-Behnken design), while in the Doehlert design the first variable is studied at five levels, the second one at seven levels and the third one at three levels. This allows us to select the order of the variables, studying the most critical ones at five and seven levels, and considering only three levels for the less critical ones\textsuperscript{[116-118]}. We used the Doehlert design in order to study simultaneously the effect of the concentration of TEA, TBAB and 9-CMA on the derivatization reaction of a 0.4 mM lactate standard solution. 9-CMA and TBAB play an important role on the reaction as explained above. The TEA concentration is an important parameter as well, since the analysis carried out using the HPLC-ESI-Q-ToF showed that TEA reacts with 9-CMA (see Chapter 4).

The number of experiments required is thirteen, but fifteen experiments were carried out in order to assure the validity of the model by analyzing in triplicate the center point of the experimental domain. For each experiment the lactate derivative peak area in the fluorescence chromatogram ($\lambda_{\text{ex}} = 365 \text{ nm}, \lambda_{\text{em}} = 410 \text{ nm}$) was considered as response. Table 3.4 reports the fifteen experiments carried out with the corresponding response expressed as area of the lactate derivative peak in the fluorescence chromatogram ($\lambda_{\text{ex}} = 365 \text{ nm}, \lambda_{\text{em}} = 410 \text{ nm}$) and Figure 3.13 shows the experimental domain.
Table 3.4: experiments performed for the Doehlert design (the coded values are reported in parentheses)

<table>
<thead>
<tr>
<th>Exp. N°</th>
<th>$x_1$ (TEA) mM</th>
<th>$x_2$ (TBAB) mM</th>
<th>$x_3$ (9-CMA) mM</th>
<th>Peak area 410 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.4 (0)</td>
<td>8.8 (0)</td>
<td>4.1 (0)</td>
<td>651466</td>
</tr>
<tr>
<td>2</td>
<td>5.4 (0)</td>
<td>3.7 (-0.577)</td>
<td>0.8 (-0.816)</td>
<td>272436</td>
</tr>
<tr>
<td>3</td>
<td>10.8 (1)</td>
<td>8.8 (0)</td>
<td>4.1 (0)</td>
<td>757142</td>
</tr>
<tr>
<td>4</td>
<td>5.4 (0)</td>
<td>13.9 (0.289)</td>
<td>7.4 (0.816)</td>
<td>870157</td>
</tr>
<tr>
<td>5</td>
<td>0 (-1)</td>
<td>8.8 (0)</td>
<td>4.1 (0)</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2.7 (-0.5)</td>
<td>1.2 (-0.866)</td>
<td>4.1 (0)</td>
<td>218665</td>
</tr>
<tr>
<td>7</td>
<td>8.1 (0.5)</td>
<td>1.2 (-0.866)</td>
<td>4.1 (0)</td>
<td>363961</td>
</tr>
<tr>
<td>8</td>
<td>8.1 (0.5)</td>
<td>6.3 (-0.289)</td>
<td>7.4 (0.816)</td>
<td>734358</td>
</tr>
<tr>
<td>9</td>
<td>2.7 (-0.5)</td>
<td>6.3 (-0.289)</td>
<td>7.4 (0.816)</td>
<td>444089</td>
</tr>
<tr>
<td>10</td>
<td>2.7 (-0.5)</td>
<td>11.3 (0.289)</td>
<td>0.8 (-0.816)</td>
<td>331878</td>
</tr>
<tr>
<td>11</td>
<td>8.1 (0.5)</td>
<td>11.3 (0.289)</td>
<td>0.8 (-0.816)</td>
<td>463244</td>
</tr>
<tr>
<td>12</td>
<td>8.1 (0.5)</td>
<td>16.4 (0.866)</td>
<td>4.1 (0)</td>
<td>910655</td>
</tr>
<tr>
<td>13</td>
<td>2.7 (-0.5)</td>
<td>16.4 (0.866)</td>
<td>4.1 (0)</td>
<td>664949</td>
</tr>
<tr>
<td>14</td>
<td>5.4 (0)</td>
<td>8.8 (0)</td>
<td>4.1 (0)</td>
<td>661162</td>
</tr>
<tr>
<td>15</td>
<td>5.4 (0)</td>
<td>8.8 (0)</td>
<td>4.1 (0)</td>
<td>664355</td>
</tr>
</tbody>
</table>

Figure 3.13: 3D view of the experimental domain
By applying multiple regression analysis on the experimental data, the following second order polynomial equation was obtained:

\[
R = 658994 + 317548 \cdot [TEA] + 273604 \cdot [TBAB] + 200489 \cdot [9 - CMA] \\
- 280423 \cdot [TEA]^2 - 65778 \cdot [TBAB]^2 - 123037 \cdot [9 - CMA]^2 \\
+ 59793 \cdot [TEA] \cdot [TBAB] + 117899 \cdot [TEA] \cdot [9 - CMA] + 34252 \\
\cdot [TBAB] \cdot [9 - CMA]
\]

The response function contains three linear, three quadratic, and three interaction terms and one block term.

The model was validated by comparing the predicted value in the central point of the experimental design with the experimental value, which resulted not significantly different at 95% confidence.

The significance of the obtained coefficient was determined by Student’s t-test: the larger the magnitude of the calculated t-value, the more significant is the corresponding coefficient\(^{119,120}\).

Table 3.5 summarizes the calculated coefficients of the model and the corresponding t-values.

**Table 3.5**: calculated coefficients of the model and the corresponding t-values; the terms refer to the coded concentrations (mM) of the variables

<table>
<thead>
<tr>
<th>Term</th>
<th>Coefficient Value</th>
<th>t-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>(b_0 = 6.6 \times 10^5)</td>
<td>12.6</td>
</tr>
<tr>
<td>[TEA]</td>
<td>(b_1 = 2.9 \times 10^7)</td>
<td>6.40</td>
</tr>
<tr>
<td>[TBAB]</td>
<td>(b_2 = 2.7 \times 10^7)</td>
<td>6.02</td>
</tr>
<tr>
<td>[9-CMA]</td>
<td>(b_3 = 2.0 \times 10^7)</td>
<td>4.41</td>
</tr>
<tr>
<td>[TEA][TEA]</td>
<td>(b_{11} = -2.8 \times 10^5)</td>
<td>-3.39</td>
</tr>
<tr>
<td>[TBAB][TBAB]</td>
<td>(b_{22} = -7 \times 10^4)</td>
<td>-0.79</td>
</tr>
<tr>
<td>[9-CMA][9-CMA]</td>
<td>(b_{33} = -1.2 \times 10^5)</td>
<td>-1.56</td>
</tr>
<tr>
<td>[TEA][TBAB]</td>
<td>(b_{12} = 6 \times 10^4)</td>
<td>0.55</td>
</tr>
<tr>
<td>[TEA][9-CMA]</td>
<td>(b_{13} = 1.2 \times 10^5)</td>
<td>1.01</td>
</tr>
<tr>
<td>[TBAB][9-CMA]</td>
<td>(b_{23} = 3 \times 10^5)</td>
<td>0.29</td>
</tr>
</tbody>
</table>
The values show that the more significant terms are the constant, the three linear and the first quadratic terms; while the less significant are the interaction terms between TEA and TBAB and between TBAB and 9-CMA.

Figure 3.14 shows the correlation plot between the predicted and the experimental values ($R^2 = 0.972$).

![Correlation plot between predicted and experimental values](image)

**Fig. 3.14:** correlation plot between the experimental and the predicted values

Figure 3.15 A), B) and C) shows the response surfaces obtained by plotting the area of the lactate peaks of the fluorescence chromatograms ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 410$ nm) as a function of TEA and 9-CMA concentration, TBAB and 9-CMA concentration and TEA and TBAB concentration, respectively.

The response surfaces were obtained with Origin by converting the raw data into a 100x100 matrix using a Random (Renka Cline) gridding method.
Fig. 3.15: response surfaces obtained from the experimental design (factors: TEA, TBAB and 9-CMA concentration) of a 0.04 mM lactate standard solution (injected concentration) as a function of (A) TEA and 9-CMA concentrations, (B) TBAB and 9-CMA concentrations and (C) TEA and TBAB concentrations. Chromatographic conditions: Gemini C18 (250x4.6mm, 5μm, Phenomenex), isocratic elution with 65% ACN, 35% water.
The results indicate that the signal increases as the TBAB and 9-CMA concentration increase. The TBAB concentration affects the reaction between lactate and 9-CMA and the maximum signal is achieved when the highest TBAB concentration is used. The optimal TEA concentration was found to be between 4.5 and 7.5 mM: higher TEA concentrations produce a 10% decrease of the signal because TEA reacts with 9-CMA (see Chapter 4).

3.4. Optimization of the chromatographic conditions

Once optimized the reaction conditions for lactate, the chromatographic and detection conditions were also optimized.

3.4.1. HPLC column

Four different HPLC columns were tested in order to achieve the best separation of the lactate derivative with respect to the 9-CMA excess:

- **Synergi-Hydro RP-C18 (250 x 4.6mm, 5μm), Phenomenex**
  Polar end-capped C18 phase, extreme hydrophobic and slight polar selectivity

- **Poroshell 120 EC-C18 (100 x 4.6mm, 2.7μm), Agilent**
  End-capped C18 stationary phase chemically bonded to the porous shell of the 1.7 μm silica support, maximum deactivation of the silica surface

- **MOS-2 Hypersil (250 x 2.1mm, 5μm), Thermo Scientific**
  Not end-capped C8 phase, similar selectivity to C18 but less retentive

- **Gemini C18 (250 x 4.6mm, 5μm), Phenomenex**
  Non polar end-capped C18 phase, rugger than Synergi-Hydro, extended lifetime under extreme pH conditions.
Table 3.6 summarizes the characteristics of the columns employed for the determination of lactate\(^{[121-124]}\).

**Table 3.6:** characteristics of the columns employed for the determination of lactate. Isocratic elution with 50% ACN-50% water, column temperature set at 30 °C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Synergi-Hydro</th>
<th>Poroshell</th>
<th>MOS-2-Hypersil</th>
<th>Gemini</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size</td>
<td>5 μm</td>
<td>2.7 μm</td>
<td>5 μm</td>
<td>5 μm</td>
</tr>
<tr>
<td>Pore size</td>
<td>100 Å</td>
<td>120 Å</td>
<td>120 Å</td>
<td>110 Å</td>
</tr>
<tr>
<td>Carbon load</td>
<td>19%</td>
<td>8%</td>
<td>6.5%</td>
<td>6.5%</td>
</tr>
<tr>
<td>pH Range</td>
<td>1.5-7.5</td>
<td>2-8</td>
<td>2-8</td>
<td>2-8</td>
</tr>
<tr>
<td>(t_R) lactate(^a)</td>
<td>8.7 ± 0.5</td>
<td>7.0 ± 0.2</td>
<td>4.8 ± 0.1</td>
<td>20.05 ± 0.09</td>
</tr>
<tr>
<td>Theoretical plates(^b)</td>
<td>14268</td>
<td>10244</td>
<td>10124</td>
<td>13806</td>
</tr>
<tr>
<td>Retention factor(^c)</td>
<td>2.71</td>
<td>4.08</td>
<td>4.09</td>
<td>7.77</td>
</tr>
</tbody>
</table>

\(^a\) expressed as average ± confidence range at 95% (n = 6)

\(^b\) calculated by \(N = 5.54 \times (t_R/\omega_{1/2})^2\) where \(\omega_{1/2}\) is the width at half height of the peak

\(^c\) calculated as \(K = (t_R-t_0)/t_0\)

Figure 3.16 shows the typical absorbance chromatograms at 365 nm of 0.01 mM lactate standard solution (injected concentration) in the four tested RP columns.

\(A\)
Chapter 3

Optimization of the method

B)

Lactate derivative (t_R = 7.0 min)

CMA (t_R = 3.9 min)

CMA (t_R = 4.0 min)

Lactate derivative (t_R = 7.0 min)

Lactate derivative (t_R = 4.8 min)

9-CMA (t_R = 4 min)

9-CMA (t_R = 3.9 min)
Gemini RP column gave the best chromatographic, reproducible separation of lactate derivative with respect to the 9-CMA peaks.

### 3.4.2. Mobile phase composition

The mobile phase composition in Gemini RP column was optimized by changing the percentage of ACN from 40 to 65% in order to separate the lactate derivative peak from the interfering peak present in the blank solution. *Figure 3.17* shows the fluorescence chromatograms ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 410$ nm) of two representative isocratic elution conditions obtained with 45 and 65% ACN.
The optimal condition was achieved using 65\% of ACN. This mobile phase composition provided a good separation between the two peaks and a very short time of analysis (lactate derivative $t_R = 8.73$ min).

Fig. 3.17: fluorescence chromatograms at 410 nm ($\lambda_{ex} = 365$ nm) of 0.01 mM lactate standard solution (injected concentration) derivatized with 9-CMA using 45\% (A) and 65\% (B) ACN in the mobile phase.
Furthermore, these experiments showed the dependence of the fluorescence quantum yield on the composition of mobile phase. A significant quenching of fluorescence was observed as the concentration of water increases, according to Korte et al.\cite{115}. Hence the using of 65% ACN in the mobile phase gave also an enhancement of the fluorescence response of about 30%.

### 3.4.3. Fluorescence and absorbance detection

The influence of the excitation wavelength on the fluorescence detection of the lactate derivative was investigated. As the UV spectrum of 9-CMA has two characteristic absorptions at 256 and 365 nm (Figure 3.2 (A), Paragraph 3.1), both these wavelengths can be used as excitation frequencies. The excitation at 256 nm leads to a higher sensitivity, but it is a unspecific wavelength because many compounds in biological matrices absorb at this wavelength. Figure 3.18 shows a comparison of the fluorescence chromatograms at 410 nm of derivatized lactate standard solutions obtained by exciting at 256 nm and at 365 nm.

![Fluorescence chromatograms](image)

**Fig. 3.18:** fluorescence chromatograms at 410 nm ($\lambda_{ex} = 365$ nm) of lactate standard solutions derivatized with 9-CMA, obtained exciting at 256 nm (black line, 0.02 mM lactate injected concentration) and at 365 nm (red line, 0.2 mM lactate injected concentration). Chromatographic conditions: Synergi-Hydro RP-C18 (250x4.6mm, 5μm, Phenomenex), isocratic elution with 64% ACN, 36% Milli-Q water
The excitation at 365 nm was selected in order to guarantee the best selectivity. The determination of lactate can be achieved even with the Diode Array Detector (DAD) monitoring the absorbance at 365 nm. 

*Table 3.7* summarizes the optimal operating conditions for the derivatization and chromatographic determination of lactate.

*Table 3.7*: summary of the optimal operating conditions for the derivatization and chromatographic determination of lactate

<table>
<thead>
<tr>
<th>Derivatization conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction temperature</td>
</tr>
<tr>
<td>Reaction time</td>
</tr>
<tr>
<td>[9-CMA]</td>
</tr>
<tr>
<td>[TBAB]</td>
</tr>
<tr>
<td>[TEA]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chromatographic and detection conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC RP-Column</td>
</tr>
<tr>
<td>Mobile phase</td>
</tr>
<tr>
<td>DAD</td>
</tr>
<tr>
<td>FD</td>
</tr>
</tbody>
</table>

### 3.5. Simultaneous determination of lactate and creatinine

In the case of urine samples the determination of lactate requires a normalization of lactate level with respect to the amount of urinary creatinine in order to compare the results from different samples. Clinical determination of urinary creatinine, as explained in Paragraph 1.5.3., is in general performed by the colorimetric determination of creatinine (*Jaffé method*). This represents, in fact, a great inconvenience in terms of costs and time of the analysis.

Thus, we optimized the simultaneous determination of both lactate and creatinine in our experimental conditions.
3.5.1. Chromatographic determination of creatinine

Several authors proposed the simultaneous determination of creatinine and other analytes in urine samples by HPLC-DAD\textsuperscript{[108, 109, 125, 126]}. Creatinine has, indeed, a characteristic absorption at 234 nm (\textit{Figure 3.19}) and elutes in a RP column typically with high percentages of water in the mobile phase\textsuperscript{[109, 126]}.

\textit{Fig. 3.19}: UV-Vis spectrum of creatinine

\textit{Figure 3.20} shows a representative absorbance chromatogram at 234 nm of 0.3 mM creatinine aqueous solution analyzed by our system with an isocratic elution 1\% ACN-99\% water. Creatinine elutes at 5.45 min.
Fig. 3.20: representative absorbance chromatogram at 234 nm of 0.3 mM creatinine aqueous solution. Chromatographic conditions: Gemini C18 (250x4.6mm, 5μm, Phenomenex), isocratic elution with 1% ACN-99% water.

Figure 3.21 shows the calibration plot of creatinine analyzed in the above described conditions, which is linear over 4 order of magnitude (10⁻⁴ – 10⁻¹ mM).

Fig. 3.21: creatinine calibration plot obtained by plotting the chromatographic peak area as a function of creatinine concentration.
3.5.2. Optimization of the chromatographic conditions for the simultaneous determination of lactate and creatinine

A standard solution of creatinine was added to the reaction vial in order to check if our derivatization procedure affects the creatinine detection.

In our optimized conditions (65% ACN, see Table 3.5, Paragraph 3.4.4) creatinine elutes just after the dead volume of the column and coelutes with an anthracene derivative peak. Thus, ACN percentage was reduced to 20% to get a good separation between the two peaks (Figure 3.22).

![Absorbance chromatogram at 234 nm of a 0.02 mM creatinine standard solution (injected concentration) after the lactate derivatization procedure. Chromatographic conditions: Gemini C18 (250x4.6mm, 5μm, Phenomenex), isocratic elution with 20% ACN -80% water](image)

**Fig. 3.22:** absorbance chromatogram at 234 nm of a 0.02 mM creatinine standard solution (injected concentration) after the lactate derivatization procedure. Chromatographic conditions: Gemini C18 (250x4.6mm, 5μm, Phenomenex), isocratic elution with 20% ACN -80% water

Thus, the optimized chromatographic conditions reported in Table 3.7 (Paragraph 3.4.4) were modified in order to obtain the simultaneous determination of creatinine and lactate derivative, as reported in Table 3.8. This method was adopted for the analysis of urine samples.
Table 3.8: summary of the optimal operating conditions for the derivatization and the simultaneous chromatographic determination of lactate and creatinine

<table>
<thead>
<tr>
<th>Derivatization conditions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction temperature</td>
<td>70 °C</td>
</tr>
<tr>
<td>Reaction time</td>
<td>0.5 h</td>
</tr>
<tr>
<td>[9-CMA]</td>
<td>7.4 mM</td>
</tr>
<tr>
<td>[TBAB]</td>
<td>16.2 mM</td>
</tr>
<tr>
<td>[TEA]</td>
<td>10.8 mM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chromatographic and detection conditions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC RP-Column</td>
<td>Gemini</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>gradient from 20 % to 65% ACN</td>
</tr>
<tr>
<td>DAD</td>
<td>365 nm (lactate derivative)</td>
</tr>
<tr>
<td></td>
<td>234 nm (creatinine)</td>
</tr>
<tr>
<td>FD</td>
<td>$\lambda_{em} = 410$ nm, $\lambda_{ex} = 365$ nm</td>
</tr>
</tbody>
</table>

Figure 3.23 shows the calibration curve of creatinine obtained by adding the calibration solutions to a reaction medium containing all the reagents employed and adopting the same procedure for the lactate derivatization.

Fig. 3.23: creatinine calibration plot obtained by plotting the chromatographic peak area as a function of creatinine concentration

The slope of the calibration curve was \((4.1 \pm 0.3) \cdot 10^7\) \((R^2=0.999)\), corresponding to 89% of the slope of calibration curve obtained in water.
Detection and quantification limits were 0.00009 and 0.0003 mM respectively, calculated as 3 and 10 times the standard deviation of the blank divided for the calibration curve slope.
Table 3.9 summarizes the intra-day and inter-day repeatability evaluated at three different concentration levels of creatinine (see Paragraph 2.1.1).

Table 3.9: intra-day and inter-day repeatability for three concentration levels of creatinine

<table>
<thead>
<tr>
<th>Concentration level/mM</th>
<th>Intra-day&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Inter-day&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQC (0.0003)</td>
<td>7%</td>
<td>8%</td>
</tr>
<tr>
<td>MQC (0.001)</td>
<td>2%</td>
<td>7%</td>
</tr>
<tr>
<td>HQC (0.01)</td>
<td>1%</td>
<td>6%</td>
</tr>
</tbody>
</table>

<sup>a</sup>expressed as RSD%, n = 3 (intra-day), n = 9 (inter-day)
3.6. Analytical figures of merit

3.6.1. Linearity, repeatability and detection limits

Calibration was performed on the lactate derivative. Standard solutions covering the concentration ranges 0.0001-0.03 mM (injected concentrations) were employed as calibration samples. These solutions were prepared by diluting the corresponding stock solution with Milli-Q water. A new calibration was carried out monthly when a new stock solution of 9-CMA was prepared. The calibration curves were reproducible over 5 months giving a slope of \((3.3 \pm 0.1) \times 10^7\) \((R^2 = 1.000)\). A control standard solution (MQC) was analyzed in each set of experiments in order to correct the concentrations of the samples analyzed. The quality control standard solutions (see Paragraph 2.1.1) were analyzed in triplicate every day for three days in order to evaluate the intra-day and inter-day repeatability. Calibration curves for the lactate derivative were obtained by linear regression analysis of the peak area plotted against the nominal analyte concentration.

*Figure 3.24* shows the calibration curve of lactate.

*Table 3.10* shows the method validation parameters for the determination of lactate.

---

*Fig. 3.24:* lactate calibration plot obtained by plotting the peak area of the fluorescence chromatogram \((\lambda_{\text{ex}} = 365 \text{ nm}, \lambda_{\text{em}} = 410 \text{ nm})\) as a function of lactate concentration.
Table 3.10: method validation parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>410 nm</td>
</tr>
<tr>
<td><strong>Linearity</strong></td>
<td></td>
</tr>
<tr>
<td>Range (mM)</td>
<td>0.0002-0.03</td>
</tr>
<tr>
<td>Slope(^a)</td>
<td>((3.3\pm0.1)\cdot10^7)</td>
</tr>
<tr>
<td>(R^2)</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Intra-day repeatability(^b)</strong></td>
<td></td>
</tr>
<tr>
<td>LQC (0.0003 mM)</td>
<td>5%</td>
</tr>
<tr>
<td>MQC (0.001 mM)</td>
<td>2%</td>
</tr>
<tr>
<td>HQC (0.01 mM)</td>
<td>3%</td>
</tr>
<tr>
<td><strong>Inter-day repeatability(^b)</strong></td>
<td></td>
</tr>
<tr>
<td>LQC (0.0003 mM)</td>
<td>5%</td>
</tr>
<tr>
<td>MQC (0.001 mM)</td>
<td>6%</td>
</tr>
<tr>
<td>HQC (0.01 mM)</td>
<td>5%</td>
</tr>
<tr>
<td><strong>LOD (mM)</strong>(^c)</td>
<td>0.00005</td>
</tr>
<tr>
<td><strong>LOQ (mM)</strong>(^d)</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

\(^a\) 95% confidence interval (n = 7)
\(^b\) expressed as RSD%, n = 3 (intra-day), n = 9 (inter-day)
\(^c\) calculated as 3 times the standard deviation of the blank divided by the calibration curve slope
\(^d\) calculated as 10 times the standard deviation of the blank divided by the calibration curve slope

3.6.2. Selectivity and stability

The selectivity of the method was assessed by derivatizing several organic acids that can be found in the biological matrices analyzed, i.e. proprionic, acetic, butyric and pyruvic acids. Only the pyruvate derivative formation was observed but it does not represent an interference for the determination of lactate because it elutes before the 9-CMA peak.

The lactate derivative was found to be stable for 24 hours at 4 °C. Fifteen days after preparation we observed an increase of the peak area by about 50% likely because of the formation of interfering compounds.
Chapter 4

Characterization of the derivatization products by HPLC-ESI-Q-ToF

LC-MS/MS is a useful technique that provides information about the molecular weight and the structure of analytes.

In this work we employed an HPLC-ESI-Q-ToF instrument in order to confirm the formation of the lactate derivative (by the reaction between lactate and 9-CMA) and to investigate on the by-products of the reaction.

The electrospray ionization (ESI) is a soft ionization technique that occurs on a spray, which is produced by forcing the eluent flow from the liquid chromatographic system to pass through a capillary. An electric field (2.5-4 kV) is applied to the capillary and it causes ionization. Figure 4.1 shows the ESI ionization process.

![Fig. 4.1: schematic representation of the ESI ionization process](image-url)
The main feature of the ESI spectrum is the presence of protonated ([M+H]$^+$, in positive mode) or deprotonated ([M-H], in negative mode) molecules, ionic adducts ([M+Na]$^+$ and [M+K]$^+$ in positive mode, and [M+HCOO]$^-$ in negative mode) and multiply charged ions. The MS/MS experiment provides the fragmentation (by the collision with nitrogen in the collision cell) of a specific ion (selected with the first quadrupole). The Time of Flight analyzer provides the scanning of the fragments. 

*Figure 4.2* shows the mass spectrometry system.

**4.1. Proof of the lactate derivative formation**

The first analyses were carried out employing the same HPLC column used for the analysis by HPLC-DAD/FD (Gemini C18) in order to assure the selectivity of the method. The elution method was suitably modified since the maximum flow rate of the eluent, required for an adequate ionization, is 0.4 mL/min (see Table 2.2 A), Paragraph 2.4). The same flow rate was employed in the HPLC-DAD/FD analysis of 0.002 mM lactate standard solution (injected concentration) and a blank solution in order to identify uniquely the lactate peak. The time acquisition segments and the scan source parameters are summarized in Table 2.3 B) and C), Paragraph 2.4.
Figure 4.3 A) and B) shows a representative TIC and absorbance (365 nm) chromatograms of a lactate standard solution derivatized with 9-CMA and analyzed by HPLC-ESI-Q-ToF and HPLC-DAD/FD, respectively. The chromatographic profile was the same using the two systems, showing the two peaks at $t_R = 29.54$ and $t_R = 30.88$ min, characteristic of aqueous lactate standard solution derivatized with 9-CMA. The *Find by Formula* command of the software attributes the peak at 30.88 min to the lactate derivative (85% match). Figure 4.4 A) and B) shows the mass spectrum of the peak at 30.88 min and the peak attribution to the lactate derivative, respectively.

**A)**

**B)**

*Fig. 4.3:* chromatographic profile of a lactate standard solution and a blank derivatized with 9-CMA and analyzed by HPLC-ESI-Q-ToF (A) and HPLC-FD (B). INLET: zoomin between 25 and 37 min
The main m/z in the peak spectrum is 191.086, which corresponds to the anthrylmethyl cation resulting from the loss of lactate\(^{[127]}\). Moreover, it is possible to identify the adduct ions of the lactate derivative with K\(^+\) (m/z 319.074), with Na\(^+\) (m/z 303.100) and with H\(^+\) (m/z 281.122). Although the software attributed the mass 281.122 to the lactate derivative, this mass belongs to the isotopic cluster of the mass 280.122, which corresponds to the exact mass of the [M]\(^+\) ion (see Paragraph 4.3 in this chapter for further discussion).

The comparison between the extract-ion chromatograms at 191 m/z of a lactate standard solution and a blank derivatized with 9-CMA, allowed us to unambiguously identify the lactate derivative and the 9-OHMA (Figure 4.5). The 9-OHMA is not formed if lactate is not present in the reaction medium.
Chapter 4  
Characterization of the derivatization products by HPLC-ESI-Q-ToF

Fig. 4.5: extract-ion chromatograms at 191 m/z of a 0.005 mM lactate standard solution (injected concentration) and of a blank. In these conditions the creatinine elutes at 5.39 min. Figure 4.6 A) and B) shows the extract-ion chromatogram at 114 m/z and the creatinine mass spectrum.

A)

B)

Fig. 4.6: extract-ion chromatogram at 114 m/z (A) and creatinine mass spectrum (B)
4.2. Characterization of the structures by MS interpretation

The method was transferred to another HPLC column (Zorbax Extend C18 column, 2.1 mm x 50 mm, 1.8 µm) with a Zorbax Extend C18 guard column (2.1 mm x 12.5 mm, 5 µm), in order to complete the chromatographic run in 10 min. Although the Zorbax column is shorter, the reduced diameter and particle size allowed us to obtain a resolution comparable with that achieved with the previous set up. Injection volumes were reduced accordingly.

The mass spectrometer conditions were optimized in order to find the best ionization conditions. The fragmentation of the anthracene derivatives leads to unspecific fragments (m/z 191.086), which do not provide any information about the structure of the parent ion. Thus, the fragmentor and capillary voltages were chosen in order to produce the highest abundance of the parent ions and a reduced fragmentation, resulting in a higher intensity of the analytes diagnostic masses ([M+Na]+ and [M+K]+).

The interpretation of the mass spectra was based on the determination of the exact mass and thus of the raw formula of the analytes and on the interpretation of the fragmentation patterns.

In particular, the peak at 4.44 min was attributed to 9-TEA-MA (Figure 4.7): m/z 340.191 corresponds to the [M+H]+ pseudomolecular ion of 9-TEA-MA. The peak at 191.086 m/z is clearly visible in the spectrum.

![mass spectrum of 9-TEA-MA](image)

**Fig. 4.7:** mass spectrum of 9-TEA-MA (t<sub>r</sub> = 4.44 min)
Characterization of the derivatization products by HPLC-ESI-Q-ToF

Furthermore, the mass spectrum of the compound eluting at 5.46 min (Figure 4.8) shows a peak at m/z 231.078 and 247.058, corresponding the [M+Na]⁺ and [M+K]⁺ adducts of 9-OHMA, respectively. The anthrylmethyl fragment, m/z 191.086 m/z, is also clearly visible. Interestingly, the pseudo-molecular ion [M+H]⁺ is not present in the mass spectrum of 9-OHMA. Similarly to the lactate derivative, a peak corresponding to the exact mass of [M]⁺ is present.

![Figure 4.8: mass spectrum of 9-OHMA (t_R = 5.46)](image)

The unambiguous identification of the 9-CMA peak was not possible due to its complete fragmentation in the source.

Table 4.1 summarizes the peaks attribution on the basis of the mass spectra obtained in the optimized conditions (see Table 2.3, Paragraph 2.4.).

Table 4.1: peaks attribution on the basis of the mass spectra obtained in the optimized conditions

<table>
<thead>
<tr>
<th>t_R (min)</th>
<th>Compound</th>
<th>MW</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>Creatinine</td>
<td>113</td>
<td>136.057 [M+Na]⁺, 114.066 [M+H]⁺</td>
</tr>
</tbody>
</table>
In conclusion, it was possible to positively identify the lactate derivative, and to assess the presence of the 9-OHMA. Moreover, a product of the reaction between TEA and 9-CMA was determined. This suggests that the concentration of the TEA is critical for the overall reaction of derivatization.

The comparison between a standard solution of lactate and a blank proved that 9-OHMA is not formed if the analyte is absent. Thus, 9-OHMA is the final product of the hydrolysis of lactate ester.

4.3. The lactate derivative characterization

The lactate derivative was characterized by target MS/MS experiments on the main masses of its mass spectrum. In particular, the nature of the ions attributed to [M+H]^+ and [M]^++ was investigated.

The fragmentor potential was set at 70 V, instead of 100 V, in order to yield the maximum abundance of the pseudo-molecular ions, and the mass range was set at 85 – 600 m/z. Figure 4.9 shows the mass spectrum of the lactate derivative acquired in full scan.

**Fig. 4.9:** mass spectrum of the lactate derivative obtained by setting the fragmentor potential at 70 V

The intensity of the m/z 303.084, 319.074, 583.208 and 281.109 were found to be higher than the previous experiments. As expected, a lower fragmentor potential induced a softer fragmentation of the lactate derivative, resulting in a higher intensity of the diagnostic masses and in the absence of the fragment 191.086.
As reported above, the m/z 303.084 and 319.074 correspond to the [M+Na]$^+$ and [M+K]$^+$ adducts, respectively. The attribution of the other m/z peaks was based on MS/MS experiments. *Figure 4.10 A), B) and C)* shows the target MS/MS spectra of the m/z 583.2, 280.1 and 281.1, respectively.

*Fig. 4.10:* target MS/MS spectra of m/z 583.2 (A), 280.1 (B) and 281.1 (C)
The MS/MS spectrum of the m/z 583.2 (Figure 4.10 A) confirmed that this ion corresponds to the dimer of the Na⁺ adduct of the lactate derivative due to the presence of the m/z 303.097 ([M+Na]+ of the lactate derivative) and 191.084 (anthrylmethyl cation).

The MS/MS spectrum of the m/z 280.1 (Figure 4.10 B) only contains the ion corresponding to the anthrylmethyl cation (m/z 191.084). It is important to notice that only one isotope of the anthrylmethyl cation is detected. On the contrary, the MS/MS spectrum of the m/z 281.1 (Figure 4.10 C) shows both the isotopes at 191.084 and 192.085 of the anthrylmethyl cation, confirming that this ion belongs to the isotopic cluster of m/z 280.108.

Thus, the mass spectrum of lactate derivative does not show the ion corresponding to [M+H]⁺. Only a m/z corresponding to [M]⁺ was identified.

The mobile phase was modified by adding 1% of formic acid in order to increase the protonation of the molecules during the ionization in the ESI source.

An increase of the intensity of all the masses in the mass spectrum of the lactate derivative was observed. Thus, another MS/MS experiment on the m/z 280.1 was carried out.

*Figure 4.11* shows the corresponding MS/MS spectrum.

![Fig. 4.11: target MS/MS spectrum of m/z 280.1](image)

In the MS/MS spectrum the presence of the m/z 91 (corresponding to protonated lactic acid) confirms that the m/z 280.109 contains the lactate. It has to be stressed that for m/z lower than 100, the resolution of the mass spectrometer is lower than for higher masses. This result confirms our attribution of the m/z 280.109 to the [M]⁺ of the lactate derivative[127].
CHAPTER 5

Application to urine samples

Urine contains more than 95% water and its physical properties and chemical composition are highly variable because they depend on diet, metabolism and body mass. Tables 5.1, 5.2 and 5.3 summarizes the amounts of the most abundant compounds (inorganic, nitrogen and non-nitrogen compounds respectively) excreted in the urine of healthy people per day (the average urinary flow is 690-2690 mL/d)\(^{128}\).

Table 5.1: concentrations of the most abundant inorganic compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride</td>
<td>80-270</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Phosphate</td>
<td>26-65</td>
<td>mmol/d</td>
</tr>
<tr>
<td>S total</td>
<td>39-47</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Thiocyanate</td>
<td>0-0.10</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Fluoride</td>
<td>0.047-0.153</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Bromide</td>
<td>0.020-0.084</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Potassium</td>
<td>40-100</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Sodium</td>
<td>80-560</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Calcium</td>
<td>&lt; 10</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Magnesium</td>
<td>2.5-8.3</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Iron</td>
<td>0.0009-0.0063</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Copper</td>
<td>0.0002-0.0018</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.0021-0.0182</td>
<td>mmol/d</td>
</tr>
</tbody>
</table>
Table 5.2: concentrations of the most abundant nitrogen compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>343</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Creatine</td>
<td>0.14-2.06</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.35-8.8</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Uric acid</td>
<td>3.14</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Methyl-guanidine</td>
<td>0.008-0.040</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Guanidine acetic acid</td>
<td>0.235</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Ammonia</td>
<td>42</td>
<td>mmol/d</td>
</tr>
<tr>
<td>α-amino nitrogen</td>
<td>30</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Carnitine</td>
<td>0.358</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>0.003</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Piperidine</td>
<td>0.055-0.083</td>
<td>mmol/d</td>
</tr>
<tr>
<td>p-tiramine</td>
<td>0.0036-0.012</td>
<td>mmol/d</td>
</tr>
</tbody>
</table>

Table 5.3: concentrations of the most abundant non-nitrogen compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0-1</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.01-0.05</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Glucoronic acid</td>
<td>2.22</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.04-0.73</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.11-3.7</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Isobutyric acid</td>
<td>9-122</td>
<td>mmol/mol</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>0.24-0.80</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>0.2-0.5</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.1-1.7</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>0.06-0.13</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.21-6.35</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>1-660</td>
<td>μmol/L</td>
</tr>
<tr>
<td>Acetone</td>
<td>3-43</td>
<td>μmol/L</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.085-0.140</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Phelyacetic acid</td>
<td>0.46-2.7</td>
<td>mmol/d</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.162</td>
<td>mmol/L</td>
</tr>
</tbody>
</table>
The optimized method was applied to the determination of lactate in urine samples. In particular, two kinds of samples have been analysed:

1. Urine from healthy volunteers before and after a training session;
2. Urine from patients from the intensive care unit of Cisanello Hospital (Pisa).

The use of urine samples to monitor the athletes during training allows to find the average lactate concentration accumulated during the training, because the samples were collected before and after the training. The comparison of the lactate content before and after training provides information about the endurance leading to find the best and personalized type of training for each athlete. The increase in lactate concentrations depends, indeed, on the type of training but also on the physical conditions and endurance of the athlete. For example a strong training is required to produce an increase in lactate concentration in well-trained athletes, while a soft training is enough for an untrained athletes.

Since a comparison of two samples (before and after training) is required, the dilution of the urine samples plays a crucial role in these type of analysis. In fact the urinary concentration of metabolites is strictly dependent on the amount of water consumed by the subject. Hence the absolute concentration values are meaningless.

Our method, which allows the simultaneous determination of lactate and creatinine, overcomes this problem because it allows the normalization of the absolute lactate concentration with respect to the urinary creatinine content.

The excretion rate of lactate in urine has been poorly studied and reported in only two published papers. Liljestrand and Wilson have made the most complete study of urinary lactate excretion\textsuperscript{[129]}. They obtained samples of urine every 10 minutes from their subjects and constructed a complete curve for rates of excretion during recovery. They found that the urinary lactate reaches the maximum value 10 min after the completion of the exercise and disappears after 50 min. For this reason the urine samples analyzed in this work were collected by 30 min the end of the exercise.

The study of the urine samples of the patients from the intensive care unit was carried out to find an eventual correlation between the urinary lactate concentration and the lactemia in order to understand if the urinary lactate concentration can be employed as a prognostic marker of a respiratory failure.
The physical conditions of these patients are constantly monitored in terms of \( pO_2 \), \( pCO_2 \), [\( Na^+ \)], [\( K^+ \)], glicemia, lactemia, etc. and is performed every hour on the arterial blood. Urine samples were collected for 1 h after the measure of lactemia. The normalization of the lactate concentration for the creatinine content was found to be useless because in many cases the renal function of these patients was compromised. For this reason the lactemia values were compared with the absolute concentration values of the urinary lactate. However, the comparison of the absolute lactate concentration between the different samples is meaningful because these patients were bedridden and the daily water intake is almost constant.

### 5.1. Improvements of the method for the application in urine samples

A further optimization of the method was required for its application to urine samples. This biological matrix is very complex due to the presence of many endogenous compounds which may affect the selectivity.  

*Figure 5.1* shows the fluorescence chromatogram (\( \lambda_{ex} = 365 \) nm, \( \lambda_{em} = 410 \) nm) of a representative urine sample obtained in our optimized conditions (*Table 3.8, Paragraph 3.5.2.*) where it results that an interfering compound elutes at 16.20 min, close to the lactate derivative (\( t_R = 16.01 \) min).
To overcome this problem we tried to increase the column temperature to improve the chromatographic resolution. The best chromatographic resolution was achieved by thermostating the column at 40°C.

5.2. Dilution of the samples and matrix effect

The presence of proteins may affect the HPLC analysis because they can precipitate in the column. In general, the protein content in urine of healthy people is very low, but it increases in case of infection or other pathologies. In order to overcome this problem a deproteinization step or a high dilution of the samples is required. We decided to dilute the samples not only to preserve the column but also to reduce the matrix effect and to increase the 9-CMA excess available for the lactate derivatization.

Figure 5.2 shows a representative fluorescence chromatogram of a post training urine sample (A), and a 3D absorbance chromatogram of the same urine sample (B).
Fig. 5.2: (A) fluorescence chromatogram at 410 nm ($\lambda_{ex} = 365$ nm) of a post training urine sample derivatized with 9-CMA.; (B) 3D absorbance chromatogram of the same urine sample.

Chromatographic conditions: Gemini C18 (250x4.6mm, 5μm, Phenomenex), elution method 1 (see Table 2.1 B), Paragraphe 2.3), column temperature 40 °C.

Figure 5.2 (B) shows numerous peaks with the characteristic UV-Vis absorption spectrum of 9-CMA, indicating that 9-CMA reacts with many endogenous compounds.

The analytical addition method was applied to urine samples to study the matrix effect. Furthermore, the effect of 9-CMA concentration was also evaluated.
Two levels of urine dilution were used and for each dilution level, 3 different concentrations of 9-CMA were employed. In each case the analytical addition method was carried out. Table 5.4 summarizes the slope values of the calibration curves found in each experiment obtained by plotting the area of lactate derivative in the fluorescence chromatogram ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 410$ nm) as a function of lactate concentration added.

**Table 5.4:** slope values found in the analytical addition methods

<table>
<thead>
<tr>
<th>[9-CMA]/mM</th>
<th>slope</th>
<th>[9-CMA]/mM</th>
<th>slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>5.7 E+05</td>
<td>0.4</td>
<td>2.8 E+06</td>
</tr>
<tr>
<td>4</td>
<td>5.7 E+06</td>
<td>3</td>
<td>1.0 E+07</td>
</tr>
<tr>
<td>6.4</td>
<td>6.8 E+06</td>
<td>7.4</td>
<td>1.3 E+07</td>
</tr>
</tbody>
</table>

*Figure 5.3* shows the trends of the slope reported above as a function of 9-CMA concentration. The dotted line indicates the slope value of the external calibration curve.

The slopes increase as 9-CMA concentration increases and for the highest dilution level. However a significant matrix effect on both the dilution levels was observed. This matrix effect does not depend on the 9-CMA excess because for both the dilution levels a *plateau* is reached for 9-CMA concentration higher than 4 and 3 mM for 1:10 and 1:25 dilution levels, respectively.
The slope of the analytical addition methods carried out on urine samples diluted 50 times with the maximum 9-CMA concentration (7.4 mM), was 70% of the external calibration curve slope ($2.3 \times 10^7$ vs. $3.3 \times 10^7$ for fluorescence detection and $3.1 \times 10^7$ vs. $4.5 \times 10^7$ for UV detection). Higher dilution does not provide a significant increase in the analytical addition method curve slope. Thus, urine samples were diluted 50 times.

The matrix effect was studied on 4 different urine samples from healthy volunteers containing different creatinine concentration, which reflects a different dilution level. *Table 5.5* shows the slopes of the calibration curves obtained from the analytical addition method carried out on the four different samples.

*Table 5.5*: slopes of the calibration curves obtained from the analytical addition method carried out on four different samples containing different creatinine concentrations

<table>
<thead>
<tr>
<th>Sample</th>
<th>Slope (410 nm)</th>
<th>Slope (365 nm)</th>
<th>Creatinine (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$(2.17 \pm 0.03) \times 10^7$</td>
<td>$(3.14 \pm 0.03) \times 10^7$</td>
<td>8.5</td>
</tr>
<tr>
<td>2</td>
<td>$(2.28 \pm 0.05) \times 10^7$</td>
<td>$(3.30 \pm 0.04) \times 10^7$</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>$(2.34 \pm 0.04) \times 10^7$</td>
<td>$(3.06 \pm 0.06) \times 10^7$</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>$(2.27 \pm 0.04) \times 10^7$</td>
<td>$(3.21 \pm 0.05) \times 10^7$</td>
<td>11.5</td>
</tr>
</tbody>
</table>

All the slope values are expressed as the average ± the standard deviation (n = 2)

Although the t-test has a low reliability because of the low number of replicates, the slope values were not significantly different (t-test, 95% confidence, n=2). Hence the matrix effect, considering the 1:50 dilution of the samples, is independent of the creatinine concentration level.

The analytical addition method was also carried out on a pooled urine sample (obtained by mixing ten different urine samples). The slope values obtained were ($(2.31 \pm 0.03) \times 10^7$ for fluorescence detection and $(3.20 \pm 0.04) \times 10^7$ for UV detection) not significantly different from the average values calculated from data of *Table 5.5*. The 95% confidence interval (n = 5) was $(2.3 \pm 0.1) \times 10^7$ for the fluorescence detection and $(3.2 \pm 0.1) \times 10^7$ for the UV detection.
The matrix effect on the determination of creatinine was also investigated, carrying out the analytical addition method on several urine samples. The average analytical addition method slope was found to be \((4.4 \pm 0.4) \cdot 10^7\) that is not significantly different (95% confidence level) from the slope of the external calibration curve, which indicates the absence of any matrix effect. Therefore the creatinine concentration was calculated by using the external calibration curve.

### 5.3. Analytical figures of merit

*Table 5.6* summarizes the fitting parameters of the analytical addition method curves obtained on the pooled urine sample.

*Table 5.6:* Fitting parameters of the analytical addition method curves obtained on the pooled urine sample for fluorescence detection at 410 nm \((\lambda_{ex} = 365 \text{ nm})\) and UV detection at 365 nm

<table>
<thead>
<tr>
<th>Parameter</th>
<th>410 nm</th>
<th>365 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>((2.31 \pm 0.03) \cdot 10^7)</td>
<td>((3.20 \pm 0.04) \cdot 10^7)</td>
</tr>
<tr>
<td>(R^2)</td>
<td>0.999</td>
<td>0.999</td>
</tr>
</tbody>
</table>

All the slope values are expressed as the average ± the standard deviation \((n = 2)\)

Repeatability and recovery were calculated in order to estimate the precision and the accuracy of the method. Intra-day repeatability was calculated as the RSD% of the concentrations of lactate found in the urine sample as such and in the same urine sample with different spikes of a lactate standard solution (LQC, MQC and HQC). Intra-day recoveries were calculated as the percentage ratio of the concentration of lactate in a spiked urine sample after subtraction of the endogenous lactate concentration, with respect to the nominal concentration of lactate added to the sample. *Table 5.7* summarizes the intra-day repeatability and recoveries for three different spike levels in a pooled urine sample.
Table 5.7: intra-day repeatability and recoveries for three different spike levels in a pooled urine sample

<table>
<thead>
<tr>
<th>Lactate spike</th>
<th>410 nm</th>
<th>365 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-day repeatability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4%</td>
<td>5%</td>
</tr>
<tr>
<td>LQC (0.0003 mM)</td>
<td>3%</td>
<td>2%</td>
</tr>
<tr>
<td>MQC (0.001 mM)</td>
<td>1%</td>
<td>4%</td>
</tr>
<tr>
<td>HQC (0.01 mM)</td>
<td>2%</td>
<td>1%</td>
</tr>
<tr>
<td><strong>Intra-day recovery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>LQC (0.0003 mM)</td>
<td>92%</td>
<td>99%</td>
</tr>
<tr>
<td>MQC (0.001 mM)</td>
<td>105%</td>
<td>106%</td>
</tr>
<tr>
<td>HQC (0.01 mM)</td>
<td>94%</td>
<td>98%</td>
</tr>
</tbody>
</table>

5.4. Urinary lactate before and after training

Table 5.8 shows the lactate concentration before and after training for eleven healthy volunteers (the values are also expressed as mmol of lactate per mol of creatinine).

Table 5.8: lactate concentration before and after training for eleven healthy volunteers (the values are also expressed as mmol of lactate per mol of creatinine; the 95% confidence interval is reported for each value, n = 3)
It is interesting to observe that the concentration of lactate in urine before training ranges between 0.09-0.18 mM (8-31 mmol/mol) in healthy people. Higher basal values are due to:

- Diabetes (sample S.A.)
- Concentrated urine (sample V.D.P. 1)

The urinary lactate concentration after exercise is very different and depends on the type of training and on the personal endurance (as explained above). The highest values were found in the samples from four subjects (A.B., S.R., P.D., E.B. 1) after intense work out.

### 5.5. Urinary lactate and lactemia

*Table 5.9* summarizes the urinary lactate concentrations (determined by our optimized method, n = 3 replicates) and the lactemia values of six urine samples from patients of the intensive care unit of Cisanello Hospital (Pisa).

*Table 5.9:* urinary lactate concentrations (determined by our optimized method; 95% confidence interval is indicated; n = 3) and the lactemia values of six urine samples from patients of the intensive care unit of Cisanello Hospital (Pisa)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lactemia / mM</th>
<th>Urinary lactate / mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>2.2</td>
<td>0.89 ± 0.02</td>
</tr>
<tr>
<td>NS</td>
<td>4.3</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>SP1</td>
<td>2.1</td>
<td>0.86 ± 0.08</td>
</tr>
<tr>
<td>D</td>
<td>1.8</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>SA</td>
<td>1.5</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>M</td>
<td>0.9</td>
<td>&lt; LOQ</td>
</tr>
</tbody>
</table>

The results indicate that the optimized method allows the monitoring of the urinary lactate concentration. Future studies will be carried out in order to find a correlation between urinary lactate and lactemia.
CHAPTER 6

Application to saliva samples

Saliva is a watery substance located in the mouth of organisms and secreted by salivary glands. The two parotid glands are wrapped around the mandibular ramus and produce saliva to facilitate mastication and swallowing. Most of the stimulated saliva comes from the parotid glands. The two submandibular glands are located beneath the lower jaws, superior the digastrics muscles and produce about the 70% of the saliva in the oral cavity. There are also the sublingual and other minor glands which produce about the 10% of the total saliva. Salivary flow can be stimulated by drugs, as pilocarpine or by mastication. 

*Figure 6.1* shows the salivary glands.

![Salivary Glands Diagram](image)

*Fig. 6.1: parotid (1), submandibular (2) and sublingual (3) glands*

Human saliva is 99.5% water, while the other 0.5% consists of electrolytes, mucus, glycoproteins, enzymes and antibacterial compounds. The salivary concentration of metabolites depends on the type of saliva collected, the way of stimulation, the period of the year and on the person (age, gender, diet, etc.).
Generally the average daily production of saliva is 500-1500 mL. 

*Tables 6.1 and 6.2* summarizes the concentration of the most abundant compounds (inorganic and organic compounds respectively) found in saliva of healthy people\[128\].

*Table 6.1:* concentrations of the most abundant inorganic compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride</td>
<td>6.5-42.9</td>
</tr>
<tr>
<td>Phosphate</td>
<td>1.89-5.53</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>6.7-22.1</td>
</tr>
<tr>
<td>Thiocyanate</td>
<td>0.41-6.55</td>
</tr>
<tr>
<td>Potassium</td>
<td>11-23</td>
</tr>
<tr>
<td>Sodium</td>
<td>5.2-24.4</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.69-2.46</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.065-0.38</td>
</tr>
</tbody>
</table>

*Table 6.2:* concentrations of the most abundant organic compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>2.4-12.5</td>
</tr>
<tr>
<td>Amino acids</td>
<td>0.74</td>
</tr>
<tr>
<td>Choline</td>
<td>0.04-0.3</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.11-0.56</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>0.01-0.07</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0-0.1</td>
</tr>
<tr>
<td>Lipids</td>
<td>20-28 mg/L</td>
</tr>
<tr>
<td>Proteins (total)</td>
<td>1.4-6.4 g/L</td>
</tr>
<tr>
<td>Carbohydrates (total)</td>
<td>154-334 mg/L</td>
</tr>
</tbody>
</table>

The correlation between salivary and blood lactate has been studied in several works as it represents an attractive alternative to the invasive blood analysis. Salivary lactate is possibly formed by passive diffusion from blood and salivary glands\[130\]. Shannon et al. suggested that the salivary lactate increases because of an increase in the concentration of blood lactate, which leads to an increase in the permeability of the blood-saliva barrier\[131\]. However a blood lactate threshold was found (3 mmol/L), below which the changes in blood lactate concentration apparently are not reflected in salivary concentration during a 30-Km race\[132, 133\].
The results of these studies suggest that the salivary lactate has a high correlation with the concentration of blood lactate during exercise of various intensities, duration and muscle groups involved, and it does not depend on temperature and relative air humidity.

### 6.1. Application of the method to saliva samples

The optimized method was successfully applied to saliva samples collected before, during and after training.

The use of saliva samples to monitor the athletes during training is very attractive because the samples can be collected many times during the training and not only before and after the training as for urine samples. This allows to determine the lactate concentration at every step of the training and to find out the switch of cell metabolism from aerobic to anaerobic (aerobic-anaerobic threshold). This parameter, as well as the blood lactate measure during graded incremental exercise tests, or the maximum oxygen consumption and heart rate, may contribute to evaluate aerobic endurance performance capacity and to determine the optimal work load intensities during endurance training.

### 6.1.1. Dilution of the samples and matrix effect

The presence of proteins in saliva samples affects the analysis by HPLC because they can precipitate in the column. Therefore a dilution of the samples is required, as in the case of urine samples.

A pooled saliva sample was diluted 50 times (as urine samples) and the analytical addition method was carried out in order to study the matrix effect.

The analytical addition method slope was found to be $(3.31\pm0.03)\cdot10^7$ ($R^2 = 0.999$), indicating that no matrix effect occurs.
6.1.2. Analytical figures of merit

Repeatability and recovery were calculated in order to estimate the precision and the accuracy of the method. Intra-day repeatability was calculated as the RSD% of the concentrations of lactate found in the saliva sample as such and in the same saliva sample with different spikes of a lactate standard solution (LQC, MQC and HQC). Intra-day recoveries were calculated as the percentage ratio of the concentration of lactate in a spiked saliva sample after subtraction of the endogenous lactate concentration, with respect to the nominal concentration of lactate added to the sample.

Table 6.3 summarizes the intra-day repeatability and recoveries for three different spike levels in a pooled saliva sample.

Table 6.3: intra-day repeatability and recoveries for three different spike levels in a pooled saliva sample

<table>
<thead>
<tr>
<th>Lactate spike</th>
<th>410 nm</th>
<th>365 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-day repeatability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2%</td>
<td>3%</td>
</tr>
<tr>
<td>LQC (0.0003 mM)</td>
<td>2%</td>
<td>2%</td>
</tr>
<tr>
<td>MQC (0.001 mM)</td>
<td>1%</td>
<td>3%</td>
</tr>
<tr>
<td>HQC (0.01 mM)</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td><strong>Intra-day recovery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>LQC (0.0003 mM)</td>
<td>108%</td>
<td>109%</td>
</tr>
<tr>
<td>MQC (0.001 mM)</td>
<td>101%</td>
<td>106%</td>
</tr>
<tr>
<td>HQC (0.01 mM)</td>
<td>102%</td>
<td>104%</td>
</tr>
</tbody>
</table>

6.1.3. Salivary lactate during training

The optimized method was applied to three healthy volunteers to study the trend of the salivary lactate during training.

*Figure 6.2 A), B) and C)* shows the trend of salivary lactate in three different athletes during different training sessions.
Fig. 6.2: trend of the salivary lactate concentration during different training sessions for three different athletes: A) 27 years old girl, B) 45 years old woman, C) 27 years old boy
For the first two athletes the lactate concentration in saliva before-training was found to be lower than the limit of quantification of the method.

For the first athlete (Figure 6.2 A) the salivary lactate concentration reached 0.27 mM after 50 minutes of a cardio training session. This session was followed by a 30 minutes of soft workout session in which the salivary lactate concentration fell under the LOQ. The final session consisted in another 50 minutes cardio session in which the salivary lactate increased to 0.26 mM.

In the athlete of Figure 6.2 B) the lactate in saliva had a trend related to the intensity of the exercise (Watt of cicloergometer).

The athlete of Figure 6.2 C) performed a pyramidal work out with leg extension increasing the weight lifted.

The results indicate that the performance of athletes can be monitored by measuring the salivary lactate during training.
In this work we developed a novel and straightforward method for the determination of lactate in urine and saliva samples by HPLC-DAD/FD. The derivatization of lactate was mandatory to achieve the selectivity and sensibility required for the application of the method to complex matrices.

The novelty of this work is the derivatization of lactate with 9-chloromethyl anthracene (9-CMA) without any extraction procedure, with yields higher than 70 %. 9-CMA was employed in previous works for the derivatization of several organic, fatty and bile acids, but never investigated as derivatization agent for lactate.

First, the derivatization reaction was studied in pure organic medium (ACN) and in the presence of trace of water in the reaction medium. The high derivatization yield (> 70 %) indicated that the extraction of lactate from the aqueous phase could be avoided. We also observed the formation of 9-hydroxymethyl anthracene, resulting from the partial hydrolysis of the lactate derivative in the reaction medium; a side reaction that could be controlled. Thus, the derivatization procedure was optimized. A central composite design was carried out in order to find the optimal temperature and reaction time for the reaction between lactate and 9-CMA. A Doehlert design was carried out to study simultaneously the effect of the concentration of TEA, TBAB and 9-CMA on the derivatization of a lactate standard solution. The derivatization yield increased as the TBAB and 9-CMA concentration increased. The TEA concentration was found to be a critical parameter because TEA reacts with 9-CMA.

The anthracene derivatives were characterized by HPLC-ESI-Q-ToF and the formation of the lactate derivative was proved. The MS and MS/MS spectra also showed the formation of the 9-OHMA and of a 9-TEA-MA adduct.

The chromatographic conditions were optimized in order to find the best column, column temperature and mobile phase able to guarantee a good resolution and a short time of analysis. A further optimization of the elution method was required to get the simultaneous determination of the lactate derivative with creatinine for the application of the method in urine samples.
The detection of the lactate derivative was achieved both in absorbance (365 nm) and in fluorescence ($\lambda_{ex} = 365$ nm; $\lambda_{em} = 410$ nm) and the creatinine was detected at 234 nm. The calibration curve was linear in the investigated range 0.09-15 mM and the limit of detection was 0.03 mM for both fluorescence and UV detection.

The optimized method was successfully applied to urine and saliva samples from healthy volunteers before, after and during training in order to study the endurance performance.

The increase in the lactate concentration in the post training urine samples was found to be related to the type of training, indicating that not only the blood lactate, but also the urinary lactate concentration can be used to estimate the endurance performance of athletes during training. The determination of salivary lactate was found to be very useful because provided a monitoring of the performance not only before and after training but also during training.

The method was also applied to urine samples from patients of an intensive care unit and the results indicated that the optimized method allow the monitoring of the urinary lactate. Currently we found a correlation between the urinary lactate and lactemia on the four samples analyzed ($R^2 = 0.977$). This study is in progress in order to confirm this correlation on a significant number of samples. This correlation would be useful in order to understand if the urinary lactate concentration can be used as a prognostic marker of respiratory failure.

The optimized method was found to be fast, simple and competitive with the other RP-HPLC methods for the determination of lactate. This is also the first method reported in the literature able to guarantee the simultaneous determination of lactate and creatinine.
**Abbreviations**

(+)-**FLEC**: (+)-1-(9-fluorenyl)-ethyl-chloroformate

**2-DBAP**: 2,4’-dibromo-acetophenone

**2-HP-β-CD**: 2-hydroxypropyl-β-cyclodextrin

**9-CMA**: 9-chloromethyl anthracene

**9-OHMA**: 9-hydroxymethyl anthracene

**α-BAP**: α-bromo-acetophenone

**ATP**: adenosine triphosphate

**BE**: base excess

**BSTFA**: N,O-bis-(trimethylsilyl)-trifluoro-acetamide

**CE**: capillary electrophoresis

**DAD**: diode array detector

**DATAN**: (+)-O,O’-diacetyl-L-tartaric anhydride

**DBD-PZ**: -(N,N-dimethylaminosulfonyl)-7-piperazino-2,1,3-benzoxadiazole

**FD**: fluorescence detector

**FL**: fluorescence

**GC/MS**: gas chromatography-mass spectrometry

**Hct**: hematocrit

**HPLC**: high performance liquid chromatography

**HQC**: high quality control

**IEC**: ion-exchange chromatography

**LC-ESI-q-ToF**: liquid chromatography-electronspray ionization-quadrupole-time of flight

**LC-MS/MS**: liquid chromatography-tandem mass spectrometry

**LDH**: lactate dehydrogenase

**LO**: lactate oxidase

**LQC**: low quality control

**MLST**: maximum lactate steady state

**MQC**: medium quality control

**NAD***: nicotinamide adenine dinucleotide

**NBD-PZ**: 4-nitro-7-piperazino-2,1,3-benzoxadiazole

**NBD-PZ-Val**: 2S)-2-amino-3-methyl-1-[4-(7-nitro-benzo-2,1,3-oxadiazol-4-yl)-piperazin-1-yl]-butan-1-one

**PNBDI**: O-(4-nitrobenzyl)-N,N’-diisopropylisourea

**RP**: reversed-phase

**RSD%**: relative standard deviation percent

**TBAB**: tetra-n-butyrammonium bromide

**TEA**: triethanolamine

**tHb**: total hemoglobin

**VO_{2max}**: maximum oxygen consumption
Bibliography


Development of a chromatographic method for the determination of lactate in urine and saliva


Development of a chromatographic method for the determination of lactate in urine and saliva


Development of a chromatographic method for the determination of lactate in urine and saliva


**Ringraziamenti**

Ringrazio Emilia Bramanti per avermi dato la possibilità
di svolgere questo lavoro di tesi,
per la disponibilità e la pazienza
con le quali mi ha seguito in questi nove mesi di tirocinio.

Ringrazio inoltre Massimo Onor, Emanuela Pitzalis e Alessandro D’Ulivo
per il supporto datomi, e tutto il gruppo di ricerca dell’Istituto ICCOM del CNR.

Ringrazio la mia relatrice, la prof.ssa Maria Perla Colombini,
per avermi seguito ancora una volta,
e Ilaria Degano per la sua disponibilità e il suo aiuto.

Un ringraziamento speciale va a tutti i miei parenti,
in particolare i miei genitori, i miei fratelli e le mie sorelle,
i miei nonni, lo zio Angelo e la zia Marina
che mi hanno costantemente incoraggiato e aiutato in questi anni.

Ringrazio tutti i miei amici,
in particolare Sara, Eleonora, Margherita e Paolo
per la loro amicizia e per i bellissimi anni di università passati insieme.

Ringrazio Beatrice, Valentina, Helen, Giulia, Ilaria, Cristina, Stefano, Lucia e Daiane,
per aver reso indimenticabili questi mesi trascorsi al CNR.