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LACTATE ANALYSIS IN HUMAN HUMOUR VITREOUS AS BIOMARKER IN THE ESTIMATION OF POST-MORTEM INTERVAL

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Lactate analysis in human humour vitreous as biomarker in the estimation of post-mortem interval

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ABSTRACT

Forensic Sciences frequently require the time since death, or Post Mortem Interval (PMI) evaluation. This parameter can be investigated by studying the biochemical changes occurring in the corpse after death. Humour Vitreous (HV) is preferred as matrix since it allows a protected environment from microbial action and is easy to collect. Post mortem HV lactate level has been evaluated mainly in relation to glucose metabolism disease concerning the cause of death, nevertheless some investigation observed HV lactate increase with PMI. Thus, the aim of this work was to evaluate the existence of a correlation between the vitreous lactate concentration and PMI. The novelty of present research is also the selected analytical technique, capillary zone electrophoresis (CZE), that has been investigated to carry out these measurements. The HV as biological matrices for forensic investigation confirms suitability and lactate analysis with reproducibility, specificity and sensitivity was successfully assessed. HV samples of known PMI were analysed and lactate concentrations matched the values reported in literature and demonstrated increase with PMI with a linear trend. Despite this work is only a preliminary method validation and would need further optimization, CZE demonstrated to be an optimal technique for this analysis, anyhow the presence of factors influencing the results emerged and earliest personal and literature measurements demonstrated that these aspects represent future investigation topics.

1. INTRODUCTION

1.1 THESIS MASTERPLAN

The aim of this work is the study of the potential of capillary zone electrophoresis for the development of a method to detect lactate in vitreous humour, in order to evaluate the correlation of its concentrations with the time since death and to provide a useful tool to forensic science for inferring the time since death.

1.2 POST MORTEM INTERVAL AND ITS DETERMINATION

Legal medicine often requires the determination of the time since death, or post mortem interval (PMI). This parameter is defined as the "amount of time elapsed since the death of the decedent". This investigation is scientifically carried out by physical and biochemical methods, both based on the observations of the post mortem changes occurring in the corpse (Mathur and Agrawal, 2011). In fact, after decease, the blood circulation ceases, with the progressing death of all the body tissues (Thierauf, Musshoff and Madea, 2009). This takes places though a shift to anaerobic metabolism and other metabolic events, physical processes, biochemical reactions and autolysis that lead to body degradation (Thierauf, Musshoff and Madea, 2009; Mathur and Agrawal, 2011; Swain *et al.*, 2015). Breakdown products formed by these events accumulate in the water and bacteria released from cells disruption initiate putrefaction processes of corpse (Hayman *et al.*, 2016). Macromolecules are destroyed to their smaller constituents: proteins in aminoacids by enzymatic processes or carbohydrates into low molecular weight organic molecules (such as glucose) by microorganisms action, and so on (Hayman *et al.*, 2016). These decomposition products can serve as biological markers of time since death (Hayman *et al.*, 2016). Since these events take place in a time-ordered manner their observation can be helpful for the determination of the time since death (Mathur and Agrawal, 2011; Swain *et al.*, 2015).

The **conventional methods**, named physical methods, mainly rely on subjective descriptions and empiric observations of post-mortem changes. They can be classified by the occurring process (Madea, 2005):

I) Purely physical process, such as algor mortis (the gradual decrease of body temperature), hypostatis (Madea, 2005) and the gradual colour change simply observed on the cadavers that is defined livor mortis (Mathur and Agrawal, 2011).

II) Metabolic processes, which correspond to the supravital reactions occurring after death (Madea, 2005); these can be monitored through the muscular response to electric impulses (Mathur and Agrawal, 2011).

III) rigor mortis, that is corpse stiffening mainly concerning muscles, is also caused by physio-chemical alterations (Madea, 2005; Mathur and Agrawal, 2011). This parameter can be evaluated by observing joints flexibility and the muscle volumes (Mathur and Agrawal, 2011).

IV) Enzymatic reactions by means of bacteria and enzymes lead to putrefaction of tissues into gases, salts and liquids. The physical methods involved evaluates colour changes, gas evolutions and liquefaction (Madea, 2005; Mathur and Agrawal, 2011).

Physical methods are strongly affected by the environment (temperature, climate), external conditions, contaminations and other factors (pre-existing pathologies, drug assumptions, body habits), thus lead to a high error in PMI determination (Mathur and Agrawal, 2011). The need for more accurate data have prompted the research towards methods that provide numerical data, confidence limits, and that can be described through mathematics (Madea, 2005). For this reason, biochemical techniques have become the most studied and used methods in last years.

Biochemical methods employ biochemical techniques in order to investigate the phatophysiological changes occurring in the body immediately after death (Mathur and Agrawal, 2011). Differently from the physical methods, the accomplished measurements can be described with mathematical models and statistics, as it is necessary for a reproducible quantification (Madea, 2005; Henssge and Madea, 2007). They are based on the chemical and biochemical processes activated after death, as a consequence of autolysis, membrane breakdown phenomena, metabolic changes, protein degradation (Madea, 2005). These processes appear in different post-mortem intervals, follow chemical and physical principles and can be statistically described (Madea, 2005). Moreover, each factor changes with an own rate (Mathur and Agrawal, 2011). Furthermore, they have gained attention in clinical and forensic toxicology too, in order to assess both natural and drug induced causes of death. In fact, they can be used to determine the drug responsible of death when endogenous substances are used as poisoning (Belsey and Flanagan, 2016). The results showed that these techniques are more precise than conventional methods and generally they are less influenced by age, sex and environmental temperature, thus less prone to errors (Mathur and Agrawal, 2011; Swain *et al.*, 2015). However, a certain degree of variability between subjects is present (Hayman *et al.*, 2016); of course, eventual influencing factor have to be accounted for in the mathematical models (Henssge and Madea, 2007). As an example, these factors could be related to the agonal period or the cause of death, which affect the biochemistry. A typical shortcoming concern sample availability: in fact, it is often impossible to determine certain parameters in living organisms, making difficult to define reference ranges (Belsey and Flanagan, 2016) and set standardized procedures (Mathur and Agrawal, 2011; Belsey and Flanagan, 2016; Kokavec *et al.*, 2016). Moreover, it could be difficult to collect enough sample (Hayman *et al.*, 2016), or to obtain a certain matrix at all; for these reasons, the parallel analysis of more matrices should be recommended (Belsey and Flanagan, 2016).

However, since some parameters are subjected to a huge amount of inter individual differences, animal models could be useful to obtain steady results, because animals are influenced by fewer variables: this favours easier results interpretation and the validation of a method (Donaldson and Lamont, 2013). Finally, most of these techniques are expensive and require advanced technologies and highly qualified personnel (Tagliaro *et al.*, 2001; Mathur and Agrawal, 2011). For these reasons, among the investigated possibilities, only a few of them is routinely applied in forensic analysis.

1.3 PARAMETERS AND MATRICES INVESTIGATED BY BIOCHEMICAL METHODS

A variety of parameters and matrices have been analysed. Some have proved to be useful to determine the cause of death, others have been studied only to assess the existence of a correlation with the time of death, but most of the parameters have been investigated for both purposes. Some examples are reported in table 1.

Table 1: examples of some parameters investigated and the associated matrixes in diagnosis of cause of death.

interpretation of altered levels/cause of death	*Parameter*	*matrix*	*Ref work*
investigated for PMI determination	Na	serum	Mathur and Agrawal, 2011
investigated for PMI determination	Nitrogen	bird muscle	Mathur and Agrawal, 2011
investigated for PMI determination	creatinine	bird muscle	Mathur and Agrawal, 2011
myocardial infarction; drug induced cardiac diseases	cardiac troponin, creatin kinase, calcium ions	blood and pericardial fluid	Mathur and Agrawal, 2011, Belsey and Flanagan, 2016
Myocardial damage	Troponin	Perycardial fluid, blood	Mathur and Agrawal, 2011, Belsey and Flanagan, 2017
investigated for PMI determination	K	HV, blood, serum	Mathur and Agrawal, 2011, Belsey and Flanagan, 2018
Anaphylaxis	chymase, histamine, diamine oxidase	blood	Belsey and Flanagan, 2016
death caused by alcohol or diabetic diseases	Acetone, β-hydroxybutyrate	blood, urine, vitreous, CSF	Belsey and Flanagan, 2016
diabetic ketoacidosis (type I diabete)	acetone	blood, HV, urine	Belsey and Flanagan, 2016
type II diabete	glucose	HV	Belsey and Flanagan, 2016
indicates dehydration or salt water drawning	Na	HV	Belsey and Flanagan, 2016
related to poor renal function	creatinine	HV	Belsey and Flanagan, 2016
Hypothermia	adrenaline vs noradrenaline ratio	urine	Belsey and Flanagan, 2016
liver failure	ammonia	VH	Belsey and Flanagan, 2016
alcohol abuse	CDT	blood, VH	Belsey and Flanagan, 2016
EtOH ingestion	Ethyl glucoronide, Ethyl sulphate	HV, urine	Belsey and Flanagan, 2016
Hyperthermia	Myoglobin	Blood, urine	Belsey and Flanagan, 2016
diabetic ketoacidosis	Fructosamine	HV	Belsey and Flanagan, 2016
lactic acidaemia (if very high)	L-lactate	HV	Belsey and Flanagan, 2016
investigated for PMI determination	calcium	VH	Belsey and Flanagan, 2016

As regards the determination of the **cause of death,** many of them are related to one or more biomarkers that change after death. The detection and characterization of these biomarkers in the tissue of interest can help the investigation. However, in some cases also a correlation with PMI has been reported (Skopp, 2004; Belsey and Flanagan, 2016).

The precise cause of death has been found difficult to obtain from the evaluation of electrolytes as parameters. The reason is due to the interferences deriving from the events occurring during the survival periods and in the early post mortem interval (Maeda, Michiue and Ishikawa, 2016).

As regards the **PMI determination**, the correlation with the time of death has been investigated on different matrices, and since putrefaction and degradation phenomena involve all the tissues, organs and cells, this has been accomplished by monitoring the variations in each matrix (Mathur and Agrawal, 2011). Gastric contents, organ tissues, bone marrow, muscles, teeth etc. are examples of matrices that can be investigated (Skopp, 2004). These degradation phenomena involve protein decomposition in amino acids and the consequent accumulation of metabolites and decomposition by-products, which have been studied in different tissues and organs, such as kidney, liver, brain, muscles and heart (Mathur and Agrawal, 2011; Hayman *et al.*, 2016). Similarly, muscular degeneration has been investigated by measuring non-protein nitrogen, aspartic amino transferase, creatinine (Mathur and Agrawal, 2011). Morphology and functionality of cells change after death until losing their availability. This was detected in bone marrow by staining technique, giving an uncertain correlation with the time since death (Hayman *et al.*, 2016). Cellular degradation has also been studied in leukocytes and neutrophils (Mathur and Agrawal, 2011). Antigens and enzymes are subjected to changes after death over longer period respects to other parameters and they can also be monitored with specific principles. Antigens undergo structural variations related to PMI that are reflected in a negative response to immunoreactions (Mathur and Agrawal, 2011). Enzymes activity have been studied in liver and showed PMI correlation over a period of days (Hayman *et al.*, 2016).

1.4 BODY FLUIDS

The most extensively studied biosamples for PMI determination are blood, CSF, humour vitreous (HV), bile and urine (Belsey and Flanagan, 2016). In fact, also in these matrices, metabolite profiles reflect the local environmental changes occurring after death as a consequence of altered cellular activity in anaerobic condition (Thierauf, Musshoff and Madea, 2009). Electrolytes, bicarbonate and other molecules (lactate) have been studied too. They are particularly important because of their concentration regulation mechanism: the amount of these substances varies in a narrow range while the organism is alive and in normal conditions (Maeda, Michiue and Ishikawa, 2016).

However, the variability of post mortem phenomena is affected by microbial proliferation which is highly unpredictable. Thus, some analytes, especially in certain matrices, are not suitable for PMI estimation (Hayman *et al.*, 2016). The biological fluids contained in closed compartments are the best choice among body fluids for PMI evaluation studies (Hayman *et al.*, 2016). For above reported reasons, to date post-mortem, biochemistry investigation is focused mainly on matrices such as cerebrospinal fluid (CSF), synovial fluid (Coe, 1993; Belsey and Flanagan, 2016; Hayman *et al.*, 2016), pericardial fluid (Coe, 1993; Swain *et al.*, 2015; Hayman *et al.*, 2016) and vitreous humour (HV): all these matrices are located in closed compartments. The main features of the first three matrices are summarized below, while HV will be extensively described in the following paragraph.

CSF. Cerebrospinal fluid is a transcellular fluid that contains substances filtrated from serum and brain tissue (Maeda, Michiue and Ishikawa, 2016). From the few studies performed on CSF it appears that potassium concentration rises after death, while sodium falls down and these phenomena are correlated with the time after death (Hayman *et al.*, 2016). Chloride and calcium change after death in a similar trend that in blood (Maeda, Michiue and Ishikawa, 2016).

Synovial fluid. Another closed matrix that demonstrated to be useful for PMI determination is synovial fluid (Hayman *et al.*, 2016), albeit it has been scarcely investigated. Its knowledge mainly concerns alcohol and drug detection and the investigation of cause of death (Madea, 2005). From the studies reported by Hayman *et al.*, it appears that only potassium in synovial fluid shows a correlation with time since death even if of less extent as compared to HV (Hayman *et al.*, 2016). A drawback of this matrix is its high viscosity (Hayman *et al.*, 2016).

Pericardial fluid. Pericardial fluid hasn't shown promising results as regard electrolytes concentration correlation with PMI. It appears that sodium and chloride undergo a moderate decrease, while potassium and magnesium increase, but the correlation is considered not sufficiently strong (Hayman *et al.*, 2016).

1.5 HUMOUR VITREOUS

1.5.1 HV description

Humour vitreous (HV) is the transcellular fluid filling the eye cavity (Kokavec *et al.*, 2016), it is settled in the posterior part of the bulb (Boulagnon *et al.*, 2011) and in the space between lens and retina (Kleinberg *et al.*, 2011; Swindle-Reilly, Reilly and Ravi, 2016).

HV is hydrophilic (Jashnani, Kale and Rupani, 2010; Swindle-Reilly, Reilly and Ravi, 2016), viscous (Thierauf, Musshoff and Madea, 2009; Kokavec *et al.*, 2016; Swindle-Reilly, Reilly and Ravi, 2016), and

highly transparent (Kleinberg *et al.*, 2011). The central part is gelatinous and is composed by a suspension of collagen fibrils (fibrous component), in hyaluronic acid medium (mucinous medium), (Boulagnon *et al.*, 2011; Kleinberg *et al.*, 2011; Swindle-Reilly, Reilly and Ravi, 2016). This physical assembly of rigid collagen network and interfibrillary hyaluronic acid makes it similar to hydrogels (Swindle-Reilly, Reilly and Ravi, 2016), in which a polymer forms an insoluble network; hyaluronic acid avoids the collapse of the collagen structure (Swindle-Reilly, Reilly and Ravi, 2016) and this gel-like structure supports the anatomic swelling of eye (Kokavec *et al.*, 2016). HV has viscoelastic properties, in particular a high storage shear modulus that makes it capable to absorb trauma (Swindle-Reilly, Reilly and Ravi, 2016); collagen provides elasticity to the structure (Swindle-Reilly, Reilly and Ravi, 2016). The high viscosity near the retina is due to an increased hyaluronic acid density and has the roles of retina protection and water content regulation acting as osmotic control (Swindle-Reilly, Reilly and Ravi, 2016). With age, the gel-structure undergoes degradation and becomes more liquid (Swindle-Reilly, Reilly and Ravi, 2016). HV has also a protective function and can serve as a metabolic source for adjacent tissues (Kleinberg *et al.*, 2011).

HV water content is around 95-99% (Thierauf, Musshoff and Madea, 2009; Kleinberg *et al.*, 2011; Swindle-Reilly, Reilly and Ravi, 2016) and it is not vascularized (Swindle-Reilly, Reilly and Ravi, 2016). The main vitreous component is hyaluronic acid (Kleinberg *et al.*, 2011); it also contains soluble proteins (from 200 to 1400 ug/ml) (Kleinberg *et al.*, 2011), mainly albumin and transferrin, and collagen (300 μg/ml) (Thierauf, Musshoff and Madea, 2009; Kleinberg *et al.*, 2011); other important constituents are chondroitin sulphate, heparin sulphate, ascorbic acid, amino acids, fatty acids and prostaglandins (Kleinberg *et al.*, 2011). The content of cells and enzymes is very low in normal conditions (Boulagnon *et al.*, 2011) as some macrophages (Albert, Pruett and Craft, 1986), while pigments, astrocytes, melanocytes, condensed collagen filaments have been found in retinal pigmentosis disease (Albert, Pruett and Craft, 1986). Enzymatic activity of the vitreous is supported by glucose (Kleinberg *et al.*, 2011), while lactic acid is the main metabolite in HV (Kleinberg *et al.*, 2011). It also contains electrolytes, mainly sodium, potassium, chloride, calcium and magnesium (Maeda, Michiue and Ishikawa, 2016); finally, urea and creatinine are also present (Jashnani, Kale and Rupani, 2010; Kokavec *et al.*, 2016; Maeda, Michiue and Ishikawa, 2016). Many of these substances have a composition gradient along the HV with a major concentration in the anterior part (Kleinberg *et al.*, 2011). The denser portion closest to the retina is called vitreous cortex (Kleinberg *et al.*, 2011) and its function is to protect the retina (Swindle-Reilly, Reilly and Ravi, 2016). Vitreous and retina are separated by a membrane (the vitreous interface) constituted by collagen fibrils (Kleinberg *et al.*, 2011). This plays a fundamental role in the regulation of substances diffusion through the retina and between the structures of the eye, thus maintaining constant the concentrations of glucose, oxygen and ascorbic acid (Mitchell *et al.*, 2013; Kokavec *et al.*, 2016). A

constant movement of ions through the vitreous can also influence the metabolism of the contiguous tissues (Kleinberg *et al.*, 2011).

1.5.2 Post-mortem changes in HV

After death, breakdown of cell membranes and termination of substances transport processes occur in HV (Boulagnon *et al.*, 2011). As a consequence, many biochemical processes take place. Putrefaction leads to the proliferation of anaerobic bacteria, which produce organic acids such as lactate, acetoacetate and acetone (Boulagnon *et al.*, 2011). In addition, loss of water from the eye cause changes in the concentrations of substances present in HV, with an increased viscosity (Belsey and Flanagan, 2016; Materazzi *et al.*, 2017). Moreover, metabolic activities in tissues go on for a certain period after death until autolysis takes place, allowing ions to pass across the membrane compartments (Swain *et al.*, 2015). The variations of substances concentration deriving from these phenomena often present correlation with PMI and can be described mathematically (Swain *et al.*, 2015). As a consequence, HV is nowadays the most used matrix to determine PMI (Tagliaro *et al.*, 1999; Mathur and Agrawal, 2011; Chandrakanth *et al.*, 2013; Belsey and Flanagan, 2016). In fact, HV is in a topographically isolated and well protected compartment, and this minimizes contamination risks and microbes diffusion from other compartments (Boulagnon *et al.*, 2011; Hess, Musshoff and Madea, 2011; Belsey and Flanagan, 2016), moreover it does not contain cells (Gagajewski *et al.*, 2004). A further advantage is that this matrix is not affected by glycolysis (Boulagnon *et al.*, 2011).

A significant drawback of HV is that it is not available from living subjects, so that ante-mortem concentrations values are poorly known (Gagajewski *et al.*, 2004; Mitchell *et al.*, 2013) and, as a consequence, it is difficult to define reference ranges (Belsey and Flanagan, 2016). However, it is known that in living organisms, low molecular substances present equilibrium between their concentrations in blood and HV (Gagajewski *et al.*, 2004; Mitchell *et al.*, 2013; Belsey and Flanagan, 2016), especially electrolytes (Chandrakanth *et al.*, 2013). Conversely, the investigation of electrolytes in HV allows additional data to strengthen blood analysis (Maeda, Michiue and Ishikawa, 2016). Helpfully, HV appears unvaried in the early post-mortem period (Boulagnon *et al.*, 2011). Another relevant issue is the time window in which the correlation between time of death and the measured HV component is considered reliable (Chandrakanth *et al.*, 2013). Most of the studies relating some parameter to PMI have been conducted on a time frame of some hours, up to few days (Jashnani, Kale and Rupani, 2010; Mihailovic *et al.*, 2011; Chandrakanth *et al.*, 2013; Donaldson and Lamont, 2013; Swain *et al.*, 2015; Hayman *et al.*, 2016). Rarely, long periods have been considered (Materazzi *et al.*, 2017). The early work of Lange *et al.* as well as Boulagnon al. (Boulagnon *et al.*, 2011), even stated that the precision of PMI estimation was around 24 h for potassium (Lange, Swearer and Sturner, 1994), but that approximate results can be obtained up

to 72 h for glucose (Boulagnon *et al.*, 2011). Potassium correlation with PMI was found linear mostly for the first 20 h post mortem, while Madea (Madea, 2005) found a good correlation up to 130 h.

The presence of ante-mortem pathologic conditions is usually suggested by observing consistent deviations from the most common range of detected values of most substances (Coe and Apple, 1985). Some substances can diffuse from serum after death (Maeda, Michiue and Ishikawa, 2016) and in presence of certain pathologies, also some cells enter HV compartment through retinal blood vessel or from optic nerve (Albert, Pruett and Craft, 1986). These substances are useful as biomarkers for the determination of ante-mortem biochemical or electrolytes abnormalities (Coe and Apple, 1985; Gagajewski *et al.*, 2004; Maeda, Michiue and Ishikawa, 2016). For the same reasons, HV is a matrix useful in forensic toxicology for post-mortem drug detection and alcohol analysis (Skopp, 2004). In this matrix, drugs concentrations are closer to the ones in blood and increase after death probably due to the dehydration process (Skopp, 2004). Other substances of forensic interest analysed in HV are ethanol, glucose, creatinine and urea (Harper, 1990).

A potential turning point in characterization of HV ante mortem composition should be the study of HV obtained from retinal surgery patients (Kokavec *et al.*, 2016). These studies could be an important reference for both the PMI and ocular diseases detection investigation (Kokavec *et al.*, 2016). Early studies of this kind have also confirmed the correlations between solutes concentrations in HV and blood, i.e. the differences in substances concentrations between healthy and diabetic subjects (Kokavec *et al.*, 2016). However, it is important to assess the kind of pathology or injury, because in case of necessitated enucleation, the patient would be no more considered in normal conditions (Gagajewski *et al.*, 2004).

1.5.3 HV in post mortem analysis

Sampling

A good point of HV use as matrix for post mortem forensic investigations is its easy sampling. In fact, thanks to its large volume (around 4-5 mL, (Jashnani, Kale and Rupani, 2010) and at least 1 mL in infants (Coe, 1993), it can be simply collected from the corpse by aspiration from the eyes. Only rarely this matrix could not be available, such as in presence of vitreous diseases, of trauma or immersion in water or in very young children (Belsey and Flanagan, 2016). Collection should be standardized; a common procedure was suggested by Coe (Coe, 1993) concerning potassium, the main analysed parameter, although the same procedure may presumably be valid for different analytes. This practice recommended to insert a n.20 gauge needle in the central part of the globe (Coe, 1993) and the liquid should be gently aspired by keeping attention to avoid contamination of retinal fragments (Hayman *et*

al., 2016) and cells incorporation that may affect solute concentration (Coe, 1993; Mitchell *et al.*, 2013; Belsey and Flanagan, 2016). Because of the HV component gradient, all the liquid should be collected (Coe, 1993; Hayman *et al.*, 2016). In the early post mortem period HV is colourless and transparent while as decomposition begins, it becomes opaque and brownish (Coe, 1993; Boulagnon *et al.*, 2011). However, more recently microsampling of 50 µL from both eyes demonstrated good reproducibility, avoiding the eventual release of substances from cells tissues that could happen due to pressure stress if suction is not appropriately performed (Tagliaro *et al.*, 2001). Moreover, no differences was found between pellet and supernatant of centrifuged samples (Zilg *et al.*, 2009).

Storage

To know the sample stability and the preservation modes for the analysis is important. In order to study the time course of physical/physiological events occurring in the body after death, HV aliquots should be taken at different times (Mihailovic *et al.*, 2011) but this is in contrast with the need to take the entire liquid to minimize the concentration inhomogeneity (Jashnani, Kale and Rupani, 2010). Donaldson *et al.* demonstrated that blood stored in tubes are less subjected to the influence of substances and microbes present in the corpse (Donaldson and Lamont, 2013); this may signify that post-mortem changes occur slower in tubes also for HV, even if in reduced extent due in corpse to the more isolation of this compartment. After sampling, HV is usually stored in freezer at -20°C (Mihailovic *et al.*, 2011; Kokavec *et al.*, 2016; Materazzi *et al.*, 2017) or +4°C (Jashnani, Kale and Rupani, 2010; Materazzi *et al.*, 2017). The stability of samples when refrigerated or frozen have been evaluated leading to contradictory opinions (Boulagnon *et al.*, 2011). Studies considering the storage effects on various analytes showed that refrigeration at +4° lead to sodium, potassium and chloride increase, but the authors didn't investigated the reasons of their finding (Gagajewski *et al.*, 2004); conversely, other studies found that this didn't change the results on HV potassium concentration (Jashnani, Kale and Rupani, 2010). However, Gagajewski *et al.* recommend storing the HV samples at -20°C (Gagajewski *et al.*, 2004). Freezing/thawing cycles have no influence on water content (Materazzi *et al.*, 2017) but some studies found that this lead to an alteration of substances concentration (Gagajewski *et al.*, 2004; Jashnani, Kale and Rupani, 2010); however, eyes differences in analytes concentrations were not affected by this kind of sample handling procedure (Gagajewski *et al.*, 2004). Lactate stability has not been widely investigated. It was reported that the correlation between lactate and PMI is better for samples stored at +4°C than at room temperature, due to enzyme activities effects at higher temperature (Mihailovic *et al.*, 2011). In addition, both lactic acid and glucose demonstrated stability storing HV at +4°C for 12 days (Peclet, Picotte and Jobin, 1994).

Treatment

Due to its viscosity, HV may require pre-treatment before the analysis (Coe, 1993; Madea, 2005; Thierauf, Musshoff and Madea, 2009; Hayman *et al.*, 2016). This can be performed with simple laboratory procedures such as centrifugation (Coe, 1993; Mihailovic *et al.*, 2011; Hayman *et al.*, 2016), heating, deep-freezing, ultrasonic bath, filtering or hyaluronidase addition (Madea, 2005; Thierauf, Musshoff and Madea, 2009) or other not expensive routine techniques (Belsey and Flanagan, 2016). Thierauf found better results with ultrasonic bath with respect to centrifugation, and also observed an improvement of both precision and stability with diluted samples (Thierauf, Musshoff and Madea, 2009). Enzymatic pre-treatment revealed to be a method that assures reproducibility (Madea, 2005). Addition of sodium fluoride should help the sample conservation without affecting the analysis (Zilg *et al.*, 2009), mainly preserving from glycolysis and enzymes activity.

1.5.4 Parameters evaluated in HV for PMI determination

The most studied parameter in HV has been potassium (Lange, Swearer and Sturner, 1994; Mathur and Agrawal, 2011) that has been found to increase after death (Mathur and Agrawal, 2011; Chandrakanth *et al.*, 2013; Belsey and Flanagan, 2016); this rapid change hampers the estimation of normal (reference) values after death (Coe and Apple, 1985). Despite the suggestions of some authors to replace potassium with other reference parameters (Madea, 2005; Mitchell *et al.*, 2013), up to now it appears that most of them have scarce role in estimating PMI (Jashnani, Kale and Rupani, 2010).

Urea, glucose, creatinine, chlorine, inorganic phosphate, calcium, sodium lactic acid, pyruvic acid, ascorbic acid, non-protein nitrogen, sodium, and chloride have been studied in HV (Hayman *et al.*, 2016). Electrolytes concentration in HV were found to be not correlated with personal factors such as age and sex, and no statistically significant differences were found between left and right eye (Chandrakanth *et al.*, 2013). Earliest studies showed that lactate, pyruvic acid and ascorbic acid were not useful in determining time since death (Coe, 1993). Calcium (Jashnani, Kale and Rupani, 2010; Swain *et al.*, 2015) creatinine, and urea were reported to be stable after death. Sodium normal range in the early post mortem period was estimated to be 130-155 meq/L (Coe and Apple, 1985). Also, it was found to be constant up to 50 hours after death (Thierauf, Musshoff and Madea, 2009; Jashnani, Kale and Rupani, 2010), but in other studies it appeared to decrease like chloride at rates of up to 1 mmol L-1h-1 (Chandrakanth *et al.*, 2013; Belsey and Flanagan, 2016).

Hypoxanthine was also reported to be related with the time of death (Materazzi *et al.*, 2017). Hypoxanthine is a purine derivative that occurs as an intermediate in the degradation of adenosine monophosphate (Hayman *et al.*, 2016). Its concentration increases after death (Madea, 2005) due to the inhibition of xanthine oxidase enzyme (Hayman *et al.*, 2016) and the rise is temperature dependent (Madea, 2005). In HV its correlation with PMI was found to be less strong than potassium, due to the different origin

of these substances (Hayman *et al.*, 2016). This also suggests that a better correlation with PMI is present for substances that undergo a diffusion process after death in a compartment, while substances that are produced by enzymatic degradation and exist as by-products are subjected to many factors/reactions, thus yielding greater error (Madea, 2005; Hayman *et al.*, 2016).

Glucose has been widely studied as a diagnostic marker of diseases related to disorders of glucose metabolism as the cause of death by means of biochemical methods (Palmiere, 2015). Indeed, glucose amount in HV is strongly increased or decreased in diabetic (200 mg/dl) (Coe, 1993) and hypoglycemic patients (Gagajewski *et al.*, 2004), respectively.

HV is preferred to blood as sampling matrix for glucose for a more accurate post-mortem diagnosis of hypoglycemia and diabetic coma (Coe, 1993). One reason is due to the rapid metabolization process of blood glucose into lactate that occurs within 8 h (Hess, Musshoff and Madea, 2011), making very difficult the determination of its original concentration (Hess, Musshoff and Madea, 2011); moreover, HV is less influenced by high glucose levels during agony in particular conditions (De Letter and Piette, 1998). Another important motivation is its not homogeneous distribution in the post mortem blood: in fact, glucose concentration is greater in the right side of the hearth and in inferior vena cava; then, it diffuses into adjacent vessels and gradually decreases (Coe, 1993). This uneven distribution from venous and arterial blood could affect the results of glucose values in the diagnostic process. Vitreous glucose varies irregularly during time (Coe and Apple, 1985); in fact it decreases only during the first 24 h post mortem as it is probably consumed by the action of retinal cells and hyalocytes (Zilg *et al.*, 2009). Afterwards, intraocular diffusion take place and consequently, equilibration between intra and extra cellular space will take place (Zilg *et al.*, 2009). Glucose concentration in HV is correlated to the serum glucose values both ante mortem and post-mortem (Hess, Musshoff and Madea, 2011). Differently from most of the other substances, it has been shown that glucose is uniformly distributed in the vitreous (Zilg *et al.*, 2009). However, glycolysis is strongly temperature-dependent (Hess, Musshoff and Madea, 2011).

Glucose is present in blood of healthy subjects at a concentration around 5.8 mmol/L (Karlovsek, 2004), remaining in the range 3.8-7.7 mmol/L even after a meal (Hess, Musshoff and Madea, 2011). After death and termination of cardiac functions, anaerobic glycolysis naturally goes on through metabolization from survival cells (Karlovsek, 2004; Palmiere, 2015), until fading from all body fluids (Hess, Musshoff and Madea, 2011). In detail, in the glycolysis pathway one glucose molecule decrease with the parallel production of two lactate molecules (Palmiere, 2015). The results of post mortem glucose studies showed the existence of a parallel lactate increase. In presence of glucose disorders, lactate reaches levels around 450-680 mg/dl in blood (Hess, Musshoff and Madea, 2011) (corresponding to

25-38 mM) and a significant quantity have also been found for several days after death in HV (Boulagnon *et al.*, 2011). These finding prompted further investigations about lactate relation with PMI, as will be deeply discussed in this thesis, in order to evaluate lactate usefulness in PMI determination (Mihailovic *et al.*, 2011; Belsey and Flanagan, 2016).

1.5.5 Factors involved in HV analysis for PMI determination

Despite the advantages of HV, discrepancies are present in literature in the post mortem trends of the parameters analysed. The reasons rely on both internal and external factors. The internal factors are the age of the individual, the duration of the terminal episode and the presence or absence of nitrogen retention (Hayman *et al.*, 2016).There are also external factors, such as the temperature of environment during the PMI and, most importantly, the temperature of the body. The above mentioned issues concerning the sampling technique, sample treatment procedure (the necessity to avoid retinal fragments…) (Hayman *et al.*, 2016) and the different precision with time (best precision is hypothesized within the first 24 hours after death (Lange, Swearer and Sturner, 1994) are cause of variability. Furthermore, the employed analysis technique, different statistical approaches (Chandrakanth *et al.*, 2013) and data analysis methods have been shown to largely affect results. Serum is less affected by these aspects, maybe because of the different water content of the two media that can affect electrolytes concentration (Coe and Apple, 1985). Moreover, the methods employed for HV study have generally not been validated for this matrix, while this occurred for plasma, serum and urine (Madea, 2005; Thierauf, Musshoff and Madea, 2009); this generally hampers the accuracy of analysis (Thierauf, Musshoff and Madea, 2009). All the factors (external and internal) that could be a potential cause of variability between samples should be taken into account in setting up the analysis model (Henssge and Madea, 2007), while they are not considered in many of the regression curves (Madea, 2005; Henssge and Madea, 2007). In this regards, it should be mentioned that statistically significant differences have been found depending on the length of the agony period (De Letter and Piette, 1998).

The evaluation of potassium correlation with PMI in HV is a peculiar example of the multitude of factors that influence the results. Earlier studies resulted in different regression curves with different slopes (Jashnani, Kale and Rupani, 2010) or even deviation from linearity. In fact, loess curve (Lange, Swearer and Sturner, 1994), and logarithmic curve (Hayman *et al.*, 2016) were extrapolated from the correlation between potassium concentration and PMI. In order to obtain homogeneous results, the above mentioned internal factors were taken into account. As a consequence, samples of unknown cause of death, or affected by particular diseases, coloured or scarce samples were excluded; urea concentration was used as an indication of chronic ante-mortem diseases (Mitchell *et al.*, 2013). Moreover, in order to obtain more precise results, potassium was used as the independent variable, for example

evaluating sodium/potassium ratio (Jashnani, Kale and Rupani, 2010). Finally, great differences have been found by using different experimental methods such as ion selective electrode or flame photometry or colorimetric techniques (Coe, 1993; Madea, 2005; Jashnani, Kale and Rupani, 2010).

All the reported issues concerning HV analysis with the specific example of potassium have to be considered for the set-up of new PMI determination method considering other HV analytes. With this purpose, vitreous lactate has been deeply investigated in this work. In fact, previous studies about post mortem diabetes diagnosis, where lactate and glucose were combined as biomarkers, lead to the hypothesis that also lactate is somehow correlated with the time of death. However, up to now, the few investigations accomplished on lactate correlation with PMI lead to incoherent results. In present work, CZE was used as it appeared the most appropriate analytical tool suitable for biological matrices in low amount and to allow the precision and reproducibility required to overcome the discrepancies.

2. LACTIC ACID

2.1 LACTIC ACID BIOCHEMISTRY AND ITS METABOLIC SIGNIFICANCE

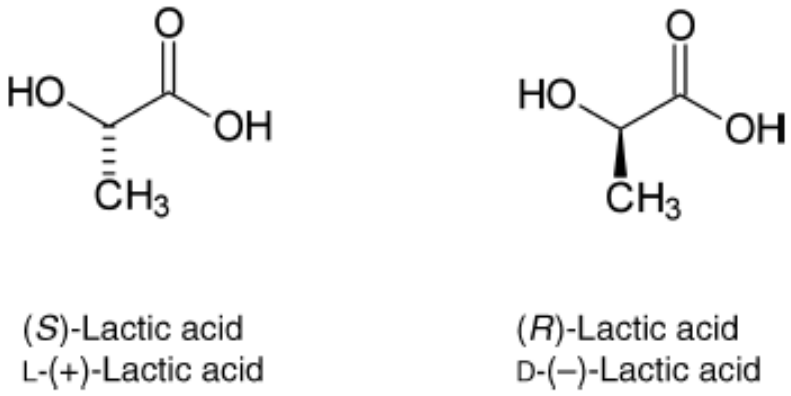


Figure 1: illustration of the two lactic acid enantiomeric forms

Lactic acid (2-hydroxypropanoic acid) is the organic acid with a shorter chain and three carbon atoms that belong to a carboxylic terminal group, an acidic terminal group and a central alcoholic group (Hayman *et al.*, 2016). It exists in two enantiomeric forms (Saavedra and Barbas, 2002; Hayman *et al.*, 2016), as represented in Fig. 1, with distinctive characteristics. In mammals, lactic acid is mostly present in the L-form (Tan *et al.*, 2005). Lactic acid normally exists in organisms as a carbohydrate metabolite (Saavedra and Barbas, 2002; Valenza *et al.*, 2005). Metabolic pathways are different depending on the L or D enantiomers (Saavedra and Barbas, 2002). The presence of a hydroxyl group and an acidic functional group can easily lead to esterification reactions of lactic acid monomers into a dimer named lactoyl lactic acid and higher oligomers, (Sharman, 1997). A dimeric cyclic form termed dilactide (Hayman *et al.*, 2016) can also be present but in small percentage, because its formation requires a catalyst to occur with a high yield (Sharman, 1997). As these substances are present in equilibrium solutions of lactic acid (Hayman *et al.*, 2016), also commercial preparations of lactic acid contain dimer and trimer besides the monomer (Sharman, 1997); pure lactic acid can be obtained only by means of crystallization (Hayman *et al.*, 2016). Conversely, free lactic acid can be obtained by poly(lactic-acid) by adding an excess of NaOH (Sharman, 1997).

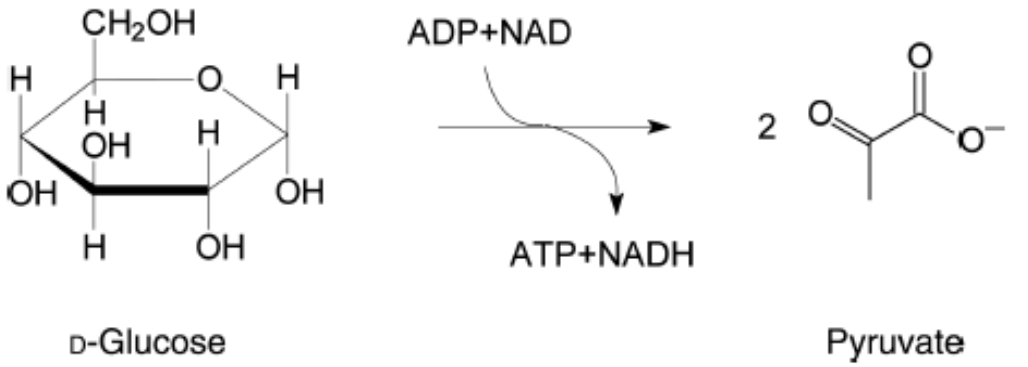
Lactic acid is a dense and viscous substance (Hayman *et al.*, 2016) with a pKa of 3.86 at 25° C (Hayman *et al.*, 2016) and pKa is 3.79 for the L-form (Hayman *et al.*, 2016). It is an acidic substance that can be involved in many reactions (Hayman *et al.*, 2016) thus it is widely present in food, dermatological preparations and in human metabolism. At body pH it is completely dissociated (Kreisberg, 1980).

Lactic acid is produced by carbohydrate fermentation in oxygen absence by microorganisms action (Hayman *et al.*, 2016). Glycolysis produces ATP with pyruvate (Fig. 2a) as product of several enzymatic

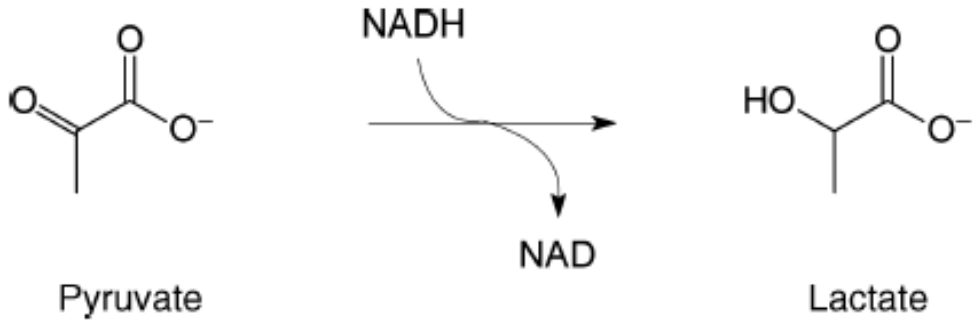
reactions (Mihailovic *et al.*, 2011; Hayman *et al.*, 2016) in muscles, blood cells and liver (Thierauf, Musshoff and Madea, 2009; Pundir, Narwal and Batra, 2016); the chemical energy obtained has many functions in cellular processes (Hayman *et al.*, 2016). Then, pyruvate is reduced under catalytic activity of LDH (lactate dehydrogenase) leading to lactic acid formation (Mihailovic *et al.*, 2011; Pundir, Narwal and Batra, 2016), along with NADH re-oxidation and energy production in the form of ATP (Hayman *et al.*, 2016). Lactic acid can hence be measured by means of enzymatic methods (Fig. 2b) (Tan *et al.*, 2005). The reaction occurring during glycolysis can be summarized by the following equation:

$$Pyruvate + NADH + H^+ = Lactate + NAD^+$$

These reactions are related to the redox state of cells and to mitochondrial functions (Kreisberg, 1980).



a)



b)

Figure 2: scheme of the conversion reaction of glucose to pyruvate (a) and of pyruvate into lactate (b), through ATP formation and NAD/NADH action.

Lactate is continuously produced in cells until all the glucose is converted to ATP with a temperature and pH depending process (optimal temperature of the living body and neutral pH) (Mihailovic *et al.*, 2011). At the same time, it is consumed in the liver at a rate of 320 mmol/L/h (Pundir, Narwal and Batra, 2016) as a driving force for other metabolic processes where it is reconverted to pyruvate (Pundir, Narwal and Batra, 2016), thus having the role of energy shuttle (Valenza *et al.*, 2005). Lactic

acid is also industrially produced in order to obtain polymeric materials with a variety of applications (Hayman *et al.*, 2016).

Lactate is a "waste" product of lactic acid (Kreisberg, 1980). Its higher concentration is indicative of abnormal production of lactic acid and/or lactate consumption (Kreisberg, 1980); conversely, lactate amount decrease as increasing pH.

In hypoxia conditions, different metabolic processes are generated (Valenza *et al.*, 2005). In man, oxygen regulator mechanism occurs, meaning that it is not able to shut down energy consumption in case of oxygen absence, generally leading to cells death (Valenza *et al.*, 2005). Nevertheless, some mechanisms of adaptation to energy demand in hypoxia conditions are possible (Valenza *et al.*, 2005; Pundir, Narwal and Batra, 2016). The most known, named Pasteur effect, is a metabolic rearrangement in which pyruvate is converted to lactate instead of entering the Krebs' cycle (Valenza *et al.*, 2005). In this way, production of energy is possible even without oxygen (Valenza *et al.*, 2005). Thus, L-Lactate form is increased in case of lactic acidosis, hypoxia, shock (Saavedra and Barbas, 2002) and for this reason, it is also used as a marker for hypoxia in tissues (Valenza *et al.*, 2005). The rise of lactate level in blood is very rapid, more than blood pH increase, and for this reason is a good indicator of hypoxia in blood (Maeda, Michiue and Ishikawa, 2016).

The D-form is produced by D-lactate dehydrogenase in presence of intestinal bacteria and it could be found in human physiological fluids in a concentration nearly around the 1% with respect of the main L-form (Tan *et al.*, 2005). The D-form is found to increase in serum diabetic condition, thus it could be a marker of this disease (Tan *et al.*, 2005).

Lactate is produced at 1300 mmol/day by erythrocytes, hepatocytes, skeletal myocytes and skin (Pundir, Narwal and Batra, 2016). It is present in blood at concentration around 1 mM (Valenza *et al.*, 2005; Pundir, Narwal and Batra, 2016) and in plasma (Valenza *et al.*, 2005). Critically ill patients present major values, less than 2 mmol/L (Valenza *et al.*, 2005). Moreover, it has been found to increase after death both in blood, from animal model studies (Donaldson and Lamont, 2013), in CSF (Coe, 1993) and in HV up to 20-30 mM in the first 24 hours (Harper, 1990; Mihailovic *et al.*, 2011).

Lactate determination is important in many fields, such as in serum to diagnose and handle severe diseases, as well as in food and wine industry for quality control (Pundir, Narwal and Batra, 2016).

Table 2 shows lactate values reported in literature in different matrices, indicating the normal ranges or typical levels reached in present of metabolism diseases.

Table 2: Typical values of lactate found in blood, plasma and HV in both healthy peoples and critically ill patients.

[L]	condition	matrix	reference
up to 450-680 mg/dL (corresponding to 50.5-76.3 mM)	glucose metabolism disorders	blood	(Hess, Musshoff and Madea, 2011)
1 mM	life	blood	(Valenza *et al.*, 2005; Pundir, Narwal and Batra, 2016)
< 2 mM	critically ill patients	blood	(Valenza *et al.*, 2005)
0.9-1.7 mM	venous	blood	(Burtis, C.A., Ashwood, 2007)
< 1.3 mM	arterious	blood	(Burtis, C.A., Ashwood, 2007)
0.5-1.3 mM	reference values	blood	(De Palo, 1999)
0.5 - 1.5 mM	normal range	blood or plasma	(Kreisberg, 1980)
20-30 mM	maximum work of short duration	plasma	(Kreisberg, 1980)
12.7 mM	patients with grand mal seizure	plasma	(Kreisberg, 1980)
0.5-2.2 mM	venous	plasma	(Burtis, C.A., Ashwood, 2007)
0.5-1.6 mM	arterious	plasma	(Burtis, C.A., Ashwood, 2007)
5.5-2.2 mM/die	normal range	urine (24 h)	(Burtis, C.A., Ashwood, 2007)
< 2.8 mM	normal range	CSF	(Burtis, C.A., Ashwood, 2007)
< 10 mM	reference values	HV	(Belsey and Flanagan, 2016)
up to 20 mM	after death (in the first 24 h)	HV	(Mihailovic *et al.*, 2011)
3.54-4.40 mM	various conditions	HV	(Kokavec *et al.*, 2016)
> 20.8 mM	diabetic patients	HV	(Karlovsek, 2004)
27.5 mM	after death (mean value; from 0 to 68.5); PMI up to 18 days; mean 4 days	HV	(Mitchell *et al.*, 2013)
13.4 - 50.4 mM	different cause of death	HV	(Harper, 1990)
132.3 mg/dl (corresponding to 14.7 mM)	could be the blank value	HV	(Peclet, Picotte and Jobin, 1994)

2.2 LACTIC ACID IN DEATH

The presence of lactate in HV could be due to the diffusion processes that take place after death between intra and extracellular space (Zilg *et al.*, 2009). Lactic acid produced by the retina is released in the vitreous cavity by the inner retinal neurons (Kokavec *et al.*, 2016). However, it should be investigated if

other mechanisms or other sources should lead to its generation. A lactate repository in the posterior part of HV has been reported (Gagajewski *et al.*, 2004). Moreover, other sources of lactate may contribute to diffusion processes into the HV (Zilg *et al.*, 2009). For these reasons, in the anaerobic environment present after death, lactate concentration increases (Zilg *et al.*, 2009).

Lactate and LDH concentration in the retina were measured through the variation in optical density derived from the enzimatic production of lactic acid from NADH, and a significant concentration was found in the retina of both lactic acid and NADH (Graymore, 1966). On the other hand, LDH was fractionated by means of gel electrophoresis (Graymore, 1966) and five bands were found: the former corresponds to a unit mainly found in muscles tissues, its activity is enhanced in anoxic conditions and produces lactic acid; the last one prevails in heart tissue, characterized by high oxygenation, while the intermediates are hybrid forms (Graymore, 1966). The retina contains muscles type isoenzymes and its lactic acid production is elevated (Graymore, 1966). This suggests that after death, in anaerobic condition, LDH activity increases and consequently the lactic acid concentration. Due to the permeation of substances from retina into HV, lactate rises in HV too. It has to be noted that while LDH has been found to be present in normal condition in retina, cornea, stroma… (Graymore, 1966), it seems to be absent in HV in normal conditions. If this is true, lactate in HV is not influenced by LDH enzymes in vitro, unless a quantity diffused form retina before sampling. As a consequence, there are no external factors in vitro responsible for lactate changes.

The temperature dependence explains the better correlation between PMI and lactate concentration occurring in a body left at room temperature, since the storing at 4°C affects the normal regulation mechanisms of cells (Mihailovic *et al.*, 2011). Moreover, an additional amount of lactic acid produced after death by enzymatic activity contributes to the lactate concentration. For these reasons, the estimation of the correlation between lactate concentration and PMI is reported with less precision after few hours after death (Mihailovic *et al.*, 2011). It may be noted that the diffusion processes may probably lead to a non-homogeneous distribution of lactate concentration in the HV, affecting the sampling procedure (Zilg *et al.*, 2009).

As regards the level of lactate present in HV, some studies were focused on a correlation with the cause of death (Harper, 1990). On the basis of a previous work that found different lactate values depending on the cause of death, Harper employed isotachophoresis, in order to find a reliable method for lactate determination in the same matrix (Harper, 1990). The obtained results demonstrated that many factors other than the specific cause of death may be responsible for highly increased lactate levels. Some of them concern the circumstances of death and have been previously described. Thus, Harper recommended to take into account the patient history before making conclusions about the cause of death.

Anyway, elevated lactate levels could be often indicative of ante mortem glucose metabolism disorders, as will be discussed in the following paragraph.

2.3 CORRELATION BETWEEN LACTATE AND GLUCOSE AND DIABETE DIAGNOSIS

Lactate has been investigated both in blood and HV mainly related to glucose metabolism pathologies. In fact, disorders of carbohydrate metabolism are strongly related to D-glucose concentration and D-glucose is anaerobically glycolyzed to L-lactate (Peclet, Picotte and Jobin, 1994). As a consequence, a parallel increase of L-lactate occurs after death. The ante-mortem concentration of D-glucose is difficult to be determined due to its rapid decrease in the early post mortem period (Belsey and Flanagan, 2016); conversely, lactate increases in both HV and blood up to 60 times (Karlovsek, 2004). Glycolysis mechanism is influenced by environmental temperature (De Letter and Piette, 1998; Hess, Musshoff and Madea, 2011).

As previously discussed, the concentrations of glucose and lactate are useful to determine ante mortem diabetes and hyperglycaemia, sometimes with the parallel analysis of acetone in blood (Peclet, Picotte and Jobin, 1994) or β-hydroxybutyrate in HV (Belsey and Flanagan, 2016).

Thus, in order to better estimate glucose concentration at the time of death, it has been proposed the determination of the D-glucose and L-lactate sum in HV (Belsey and Flanagan, 2016). This value initially increases after death, then it remains constant for 1 week (Hess, Musshoff and Madea, 2011), thus glycaemic concentration can be evaluated up to 150 hours after death (Karlovsek, 2004). The upper threshold values to determine diabetic condition can be described by the following expression (Karlovsek, 2004):

Glucose (mmol/L)+ lactate (mmol/L) /2 > 23.7 (mmol/L)

However, glucose level could be altered by other diseases or different mechanisms after death, leading to misinterpretations of results (Karlovsek, 2004; Hess, Musshoff and Madea, 2011; Belsey and Flanagan, 2016) and both glucose and lactate values in HV look quite dispersed. Since lactate was found to increase after death, glucose level alone should be preferred for the quantification of glucose in blood (Zilg *et al.*, 2009), while for the determination of the cause of death, the most statistically significant parameter is the sum value (De Letter and Piette, 1998).

3. ANALYTICAL TECHNIQUES

The methods traditionally employed to determine the analytes related to PMI (sodium, potassium, chloride and urea nitrogen) could be responsible of the great variability observed among the different results of post mortem time course in electrolytes concentration (Coe, 1993). Some of these techniques are: ion-selective electrode (Coe, 1993; Thierauf, Musshoff and Madea, 2009), flame photometry, direct and indirect potentiometry, colorimetric reactions, spectrophotomety enzymatic methods, or oxygen sensor for glucose (Coe and Apple, 1985). Nowadays, new techniques are under investigation such as NMR, flow cytometry, immunohystochemistry, optical fluorescence, detection of racemization/functionalization, thermogravimetrc analysis (TGA) (Materazzi *et al.*, 2017), biosensors (Pundir, Narwal and Batra, 2016), IR and Raman spectroscopy (Kokavec *et al.*, 2016) and finally chromatographic methods (HPLC, GC-MS and CZE) (Pundir, Narwal and Batra, 2016). Insulin, thyroglobulin and calcitonin have been studied by immunohystochemical techniques (Madea, 2005). This allows PMI estimations over periods of days, in the later stages of decomposition (Hayman *et al.*, 2016).

3.1 METHODS FOR LACTATE DETERMINATION

Lactic acid can be analysed both as substrate in enzymatic assays and as organic molecule target. Thus, enzymatic methods and separation techniques are the most employed for lactate detection.

The earlier lactate detections were performed by colorimetric and voltammetric methods. In the former, this is possible by monitoring the acetaldehyde produced by lactic acid treatment in H_2SO_4. After reaction of acetaldehyde with p-hydroxyphenyl with cupric ions, a visible band can be detected at 560 nm (Pundir, Narwal and Batra, 2016). Voltammetric methods are conducted in electrochemical cells. The measured current provides information about the species involved in red-ox processes (Pundir, Narwal and Batra, 2016). Coulorimetric and voltammetric techniques are simple and cheap but suffer low specificity and sensitivity (Pundir, Narwal and Batra, 2016).

Then, enzymatic techniques became the most frequently used. They are based on a specific biochemical reaction of lactate where a product proportional to lactic concentration is measured (Pundir, Narwal and Batra, 2016). The detection can be spectrophotometric, fluorometric or colourimetric. Glucose and lactate in HV can be determined by quantitative enzymatic methods (hexokinase and lactate dehydrogenase, respectively) (Karlovsek, 2004). Enzymatic microassay represents another traditional way to measure lactate. In this method, biochemical reactions occur in a microliter plate reader allowing the optimal reaction conditions and are quantified by a colourimetric reaction (Pundir, Narwal and Batra,

2016). Enzymatic methods are more specific, sensitive and accurate but more expensive and require qualified personnel and time for sample preparation (Galli *et al.*, 2003; Pundir, Narwal and Batra, 2016).

Some innovative techniques are under examination. One is High Resolution Proton Magnetic Resonance technique (Pundir, Narwal and Batra, 2016) that should be useful for the identification of metabolites that increase after death (like lactate, histidine, amino acids and adenine nucleotide). This does not provide absolute concentration of these molecules (Pundir, Narwal and Batra, 2016). To overcome this drawback, the substances whose amount remains constant (such as creatine and N-acetyl aspartate) can be used as reference molecule (Madea, 2005; Hayman *et al.*, 2016). Brain tissue has been selected due to the protection offered by the skull, giving the concentration changes of metabolites dependent on time in a reproducible behaviour (Madea, 2005). This technique is fast, reproducible (Pundir, Narwal and Batra, 2016), non-invasive and can be performed in-situ with the same equipment of magnetic resonance imaging (Madea, 2005). Early studies accomplished on rabbit and sheep found this technique promising for PMI determination (Hayman *et al.*, 2016). The second innovation is biosensors technology. Biosensors are integrated devices with a receptor based on biological recognition element in contact with a transducer. They provide quantitative or semi-quantitative determination and are very specific. They boast advanced performances that have been recently increased by using nanomaterials. They are classified on the basis of their working principle in electrochemical, electrochemiluminescent, fluorescent, microband and reagentless. There is a huge variety of biological recognition transducers. They are simple, selective and sensitive, allowing very low detection limits (10^{-7} M and 10^{-12} M with the electrochemical and electrochemiluminescent - based, respectively). Moreover, they are rapid and completely automated. The main disadvantages of biosensors are their cost, sometimes lack of selectivity and interferences from other components or environmental conditions, or dye photo bleaching (Pundir, Narwal and Batra, 2016).

Separation methods are widely employed, in order to discriminate and quantify the analytes of interest in various and complex matrices. Organic acids and carboxylic acids, among them lactic acid, have been separated by HPLC (Wu *et al.*, 1995; Sharman, 1997), anion exchange chromatography or ion-exclusion chromatography (Galli *et al.*, 2003), and GC followed by MS analysis (Huang *et al.*, 1989; Galli *et al.*, 2003). However, HPLC needs extensive sample preparation (Galli *et al.*, 2003; Hiraoka, Ishikuro and Goto, 2010), often involving solid phase extraction or liquid-liquid extraction (Hiraoka, Ishikuro and Goto, 2010), derivatization (Hiraoka, Ishikuro and Goto, 2010) and has the limit of possible co-elution of some carboxylic acids (Galli *et al.*, 2003). GC analysis is difficult to be performed for lactate as for others carboxylic acids, due to the high polarity of these molecules; this needs molecule derivatization for this purpose (Huang *et al.*, 1989; Galli *et al.*, 2003; Zhang *et al.*, 2018). Alternatively, capillary isotachophoresis was successfully employed for carboxylic acids. This technique applies electrophoresis

between two different electrolytes for analyte separation (Huang *et al.*, 1989). It has been used to study lactate in HV and for other organic acids in other matrices (Harper, 1990). CZE has almost replaced isotachophoresis, since it demonstrated advantageous in the detection of pharmaceutical substances and impurities including carboxylic acids (Dutra *et al.*, 2006); it was also helpful to successfully separate lactate in complex biological matrices, thus it appears promising for analysis in humour vitreous for this thesis purposes. Thus, its working principles and operating modes will be described in the following chapter.

3.2 CAPILLARY ELECTROPHORESIS

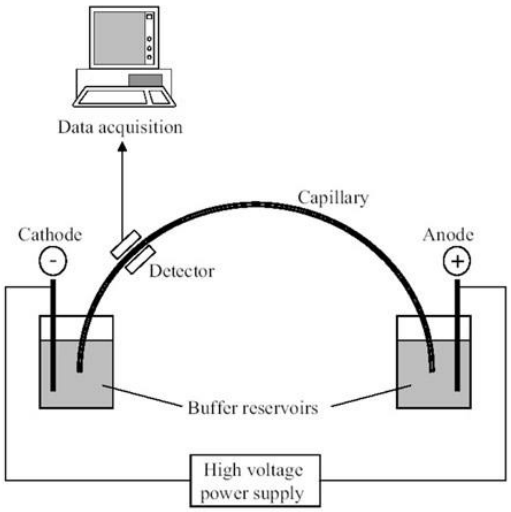


Figure 3: Schematic representation of CZE system (Source: http://www.princetechnologies.eu/products/ce-systems/ce-technologies/ce-introduction/).

Capillary electrophoresis (CE) is a separative technique that derives from gel electrophoresis (Tagliaro *et al.*, 1998) but with the separation occurring in a narrow capillary usually made of fused silica (Hayman *et al.*, 2016). The main performances of this system are determined by the features of this thin capillary. The analytes reach the other electrode with different velocities depending on their mass and charge, allowing their separation (Hayman *et al.*, 2016). CE instrument is thus composed of an injection system, a capillary filled with a carrier buffer, a high voltage source, two electrodes and detection equipment (Tagliaro *et al.*, 1998). A schematic illustration of the equipment is in Fig. 3. The analytes move along the silica capillary carried by a buffer solution and under the application of a high current from cathode to anode. The detection mostly occurs by measuring their UV absorbance through a transparent window at the other extremity. In the electropherogram are reported the peaks corresponding to the signals of moving analytes as they are revealed by the detector. Several separation modes can be achieved with a CE instrument, based on different physical-chemical principles.

The simplest and most used of them is **capillary zone electrophoresis**, or CZE, thanks to the variety of application field. In this mode, open capillaries are employed and the buffers have low viscosity (Whatley, 2001). A high voltage is applied to the running buffer and the analytes migrate along the capillary with different velocities *v* that depends principally on their mass and charge:

$v = \mu_e E$

where E is the applied electric field and μ_e is the electrophoretic mobility. In turn, μ_e linearly depends on the ionic charge q and is inversely proportional to solution viscosity η and the ionic radius r:

$$\mu_e = \frac{q}{6\pi\eta r}$$

Moreover, a peculiar feature of CZE deriving from silica narrow capillary is the electroomotic flow. At the capillary walls the negative charged silanol groups can interact with the buffer cations (Tagliaro *et al.*, 1998) creating a double layer. When a fluid is under the effect of an electric field near a charged surface, there is the production of a net bulk movement near that surface (Whatley, 2001). The cations more poorly bound to the wall migrate toward the cathode dragging water molecules and thus creating a liquid flow named electroendoosmotic flow (EOF). It is normally directed from the anode to the cathode, but can be reversed if the buffer cations make the wall become positive. The charge on the capillary wall, which depends on the capillary material and buffer composition and is related to the charge density on the capillary surface, is the zeta potential (ζ).

The velocity of the EOF linearly depends on the dielectric constant (ε), zeta potential and electric field (E) and it is inversely proportional to medium viscosity:

$$v_{eo} = \frac{\varepsilon}{4\pi\eta}\ \zeta E$$

The most important feature of EOF is its flat profile, which is of fundamental importance for avoid band broadening and increase efficiency.

The analytes velocities are thus influenced by both their mobility and the EOF mobility, following the equation:

$v = (\mu_{eo} + \mu_e) \times V \times L^{-1}$

where L is the capillary length and V the applied voltage.

As regard the direction, v_e and v_{eo} can also have opposite directions, and analytes follow the flow with a greater contribution (vectorial sum of the two). An uncharged molecule is under the influence of EOF

and not to the electric field. Thus, the electrophoretic separation is affected by molecule charge, mass, hydrodynamic ratio, buffer pH, temperature and viscosity (Whatley, 2001).

Two are the more used injection modes, i.e. electrokinetic and hydrodynamic; the electrokinetic allows the injection of analytes depending on their mass and charge, thus allows greater selectivity (Tagliaro *et al.*, 1998). The sample volume injected is in nanoliters range.

The capillary is usually made of fused silica, that allows resistance, good thermal conductivity, excellent joule heat dissipation and in order to perform the detection in-column, it must be UV transparent (Tagliaro *et al.*, 1998). CZE peculiar performances derive from the narrow (<100 μm) (Hayman *et al.*, 2016) inner internal diameter of the capillary (Whatley, 2001). In fact, it gives a high surface to volume ratio, allows anti-convective and heat controlling features reducing thermal differences along the diameter and lateral diffusion phenomena, increasing the separation performances (Whatley, 2001). The external wall is covered by a 10-25 μm layer (Whatley, 2001) of polyimide for protective purpose, making the capillary flexible. The internal surface may indeed be uncoated or coated in order to prevent the interaction of the reactive silica with analytes, for example protein absorption (Tagliaro *et al.*, 1998; Whatley, 2001).

The carrier buffer is chosen accordingly to the physical-chemical characteristic of the sample (Whatley, 2001). The buffer pH should be high or low enough to guarantee the complete ionization of the analytes (Craston and Saeed, 1998). Moreover, pH stability is required to have constant ionization (Whatley, 2001).

The detection is made on-column through a window that is linked to the detection system and the capillary is the detection cell itself (Whatley, 2001); the window is obtained by burning the external coating (for a little extent) of the capillary near to the cathode. The most common is the absorbance detector. UV detection is sensitive to concentrations down to 10^{-6} M and masses in the pg order (Tagliaro *et al.*, 1998). Other employed systems are fluorescence, amperometric, conductivity and mass spectrometry.

To obtain reproducibility is essential to maintain constant the separation conditions (i.e. buffer pH) and the inner surface charge. The latter is obtained by regeneration processes by washing procedures before every measurement session and/or between runs (Whatley, 2001). This is useful also to prevent sample sticking.

Additives can be employed for several purposes: avoiding interactions with the capillary wall, helping analyte solubility, changing the viscosity of the medium and the migration time and so on.

CZE allows great efficiency, selectivity, resolution and precision (Tagliaro *et al.*, 1998). It is insensitive to chemical variations in pH, ionic strength, ions and analytes contamination of the matrix, which in cadavers can occur due to putrefaction processes (Tagliaro *et al.*, 1999, 2001; Madea, 2005); thus, this represents an important feature in the view of analysis of complex biological matrices. Sample preparation or derivatization are not required (Galli *et al.*, 2003). In addition, with respect to other separative techniques, it allows better separation (Craston and Saeed, 1998), simple and rapid procedures with a low consumption of samples and buffer (Craston and Saeed, 1998; Tagliaro *et al.*, 1999, 2001; Galli *et al.*, 2003), allowing to carry out the analysis also when only a little sample amount is available, as could be the case in forensic analysis. Thanks to the rapidity of molecules elution, the capillary are completely cleaned after each run, and washing procedures between runs allow reproducibility (Galli *et al.*, 2003). Thanks to its performances, it can be largely employed in a variety of fields, such as chemical, pharmaceutical and forensic; in fact in can analyse biopolymers, drug and ions (Tagliaro *et al.*, 1998). The huge numbers of factors that influence the separation enhance the probability to find the solution to an analytical problem after understanding their right combination (Whatley, 2001).

3.3.1. Other CE separation modes

Besides CZE, several modes of CE exist, each of them exploiting a different principle, in order to determine species that cannot be separated by CZE (Whatley, 2001).

Capillary Gel Electrophoresis, in which a viscous gel fills the capillary, allows the separation of molecules characterized by the same charge per unit but of different length, such as DNA (Whatley, 2001).

Capillary Isoelectric Focusing is used to separate protein species and glycoprotein isoforms by their pI. These molecules present a net charge at pH of the separation buffer corresponding to their pI. To allow their separation, the buffers contain ampholytes molecules characterized by different pKa, that under an applied voltage create a pH zone inside the capillary. The proteins migrate along the capillary until they reach the capillary region at a pH corresponding to their pI, and no charges stop moving. In this way, the analytes are separated by ampholytes on the basis of their pI (Whatley, 2001).

Capillary Isotachophoresis exploits two buffers with distinct mobility. This creates zones of analyte ions at constant concentration along the capillary. This technique is typically coupled with conductance detectors or MS, and is used to separate proteins and peptides (Whatley, 2001).

Micellar electrophoresis separates uncharged compounds. Micelles having a hydrophobic core and hydrophilic shell are added to the running buffer. The analytes can be embedded inside the micelles and migrate at micelles velocities, otherwise their migration occurs at the EOF velocity (Whatley, 2001).

Chiral electrophoresis distinguishes chiral molecules, which have the same molecular weight, on the base of their different affinity towards other chiral compounds, named chiral selectors, usually cyclodextrins. With this technique, enantiomers of pharmaceutical substances and aminoacids can be separated (Whatley, 2001).

Non aqueous chromatography resort to organic solvents with a small percentage of buffer solution, in order to exchange some properties of analytes such as pKa, solubility, solvation… This allows distinguishing substances (such as drug, dyes and preservatives) that are not separated in water (Whatley, 2001).

Capillary electrochromatography is a partitioning technique in which the inner surfaces of the capillaries are coated with particles that constitute a stationary phase. In this way, analytes that move along the capillary under the EOF are separated depending on their different affinity to the stationary phase. This mode of CE has been used for polyaromatic hydrocarbons (Whatley, 2001).

Carboxylic acids detection

Analysis of low molecular weight organic acids, such as lactic acid, is required quality for control and processes monitoring in various fields. In fact, they are often present as intermediates in metabolic reactions or industry processes or as waste products of amino acids, carbohydrates and fats degradation (Galli *et al.*, 2003). Moreover, their presence can indicate alterations in enzymatic activities in metabolism when certain diseases are present (Galli *et al.*, 2003). These compounds are often detected by means of CZE, exploiting electro-osmotic separation and indirect UV detection. Up to now, lactic acid has been separated by CZE from other toxic compounds in complex matrices such as environmental (Craston and Saeed, 1998) food, beverage and cosmetic (Sharman, 1997; Dutra *et al.*, 2006). Moreover, CZE has been used to separate L- and D- lactate in plasma (Saavedra and Barbas, 2002; Tan *et al.*, 2005), urine, CSF, amniotic fluid (Saavedra and Barbas, 2002) for diabetes or other diseases detection (Tan *et al.*, 2005). CZE have been also employed to separate olygomers either natural or synthetic (Vidil *et al.*, 1995). Commercial lactic acid solution is composed of a mixture of monomer, dimer, trimer… and so on (Vidil *et al.*, 1995). These components have been successfully separated by CZE and this technique demonstrated to be more efficient than others as size exclusion chromatography (Vidil *et al.*, 1995). For low molecular mass organic acids, the typical set up includes a cationic surfactant to reverse EOF and a chromophore able to absorb the UV radiation.

Indirect absorbance detection

Organic acids have scarce or no UV radiation absorption above 220 nm (Sharman, 1997; Dutra *et al.*, 2006), appearing hardly detectable in standard CZE detection condition. For this reason, indirect detec-

tion is usually employed for these analytes (Sharman, 1997) and, an appropriate counter ion (or co-ion), often named probe, is added as BGE, and must satisfy two requirements:

1) Firstly, it should have a strong **UV absorbance** (Wu *et al.*, 1995; Sharman, 1997; Dutra *et al.*, 2006) in order to increase sensitivity (Rolan-Assad and Gareil, 1995; Johns, Macka and Haddad, 2003). In such a system the strong probe molar extinction coefficient results greater than the analyte's, giving negative absorbance (Rolan-Assad and Gareil, 1995; Wu *et al.*, 1995).
2) Secondly, its **mobility** have to match those of the analytes of interest that do not absorb (Rolan-Assad and Gareil, 1995; Wu *et al.*, 1995; Sharman, 1997); this avoids electromigrational dispersion and allows the elution of symmetric peaks. In fact, peak shape is strongly dependent on the relative mobilities of the analyte and the probe. Discrepancies in the respective mobilities will be reflected in slightly different elution times that generate asymmetric peak: concentration of analyte will be differently distributed at the front with respect to the peak tail, with profile appearing diffused at one side and sharp in the other. Only matching mobilities will allow symmetric peaks (Johns, Macka and Haddad, 2003).

Moreover, the **concentration** of the probe should be two orders of magnitude greater than the analyte's, in order to minimize electrodispersion phenomen (Johns, Macka and Haddad, 2003).

There are several modes to buffer the BGE for indirect detection (Johns, Macka and Haddad, 2003). For example, the probe could be a buffering substance itself; otherwise, common approaches consist in the addition of a co-ion as a buffer, or alternatively a counterionic buffer; last tactic makes use of an ampholytic substance.

Chromate, benzoate, phthalate, naphatalene mono-, di- and tri-sulphonate are among the investigated BGE (Wu *et al.*, 1995). Benzoate, p-anisate, as well as benzoic acid with Tris, have been employed as chromophore and buffer for compound such as carboxylic acids (Galli *et al.*, 2003).

For weak acids, the pair of the non-ionized specie and its conjugate base existing in equilibrium has to be considered. In buffer solution, they are distributed depending on medium activity and dissociation constant. Under an applied voltage, they migrate together with an effective mobility defined as the sum of the mobility of each species weighted for the respective molar fraction (Dutra *et al.*, 2006).

When either the EOF or analytes molecular mass is low (90.08 g/mol for lactic acid), the electrophoretic mobility of the compounds may be higher than the EOF mobility so that they are not detected (Huang *et al.*, 1989). This could happen in particular when these kind of analytes, such as small organic acids, are fully ionized and their pKa is much lower than pH (Huang *et al.*, 1989). They may be separated by **reversing polarity** (Sharman, 1997), or by adding appropriate **modifiers** in the buffer, usually

cationic surfactants such as **Alkyltrimethylammonium bromide (CTAB)** (Sharman, 1997; Galli *et al.*, 2003; Tuma, Samcová and Stulík, 2011) dodecyltrimethylammonium bromide (DTAB), tetradecyltrimethylammonium bromide (TTAB)(Klampfl, 1999) and diethylenetriamine (DETA) (Wu *et al.*, 1995). As they pass through the capillary, the inner surface that present negative charge could be neutralized or even reversed depending to the concentration (Wu *et al.*, 1995). This leads to a reverse EOF so that electrophoretic and electrosmotic velocities have the same direction (Dutra *et al.*, 2006). This allows speed up the analysis duration (Huang *et al.*, 1989; Dutra *et al.*, 2006). Anyway, surfactant concentration is optimal under the CMC value (Wu *et al.*, 1995). Concentration of EOF modifier should be appropriate in order to have a stable base line (Dutra *et al.*, 2006).

Furthermore, ions have been successfully separated with this technique. In fact, despite they do not absorb the UV light, this has been accomplished by means of indirect detection, using an additive in the buffer in very low amount as UV absorber (Tagliaro *et al.*, 1999, 2001; Whatley, 2001). This allows the detection of ions such as potassium in HV (Tagliaro *et al.*, 1999). With this strategy, **lactic acid** has also been separated from other small acidic substances in complexes matrices (Craston and Saeed, 1998). As an alternative to the indirect absorbance method, conductivity detectors have also been employed for lactic acid in food (Huang *et al.*, 1989).

p-anisic acid (4-methoxybenzoic acid)

Benzoate derivatives are characterized by a molar absorption coefficient with a maximum around 254 nm. Among them, p-anisic acid, (4-methoxybenzoic acid), has a maximum absorption at about 250 nm (Fig 4). The detector wavelength set at this value would permit to minimize baseline noise and increase sensitivity (Johns, Macka and Haddad, 2003). Anisate is characterized by a high molar absorptivity (Collet and Gareil, 1995) and a mobility around $30 \times 10^5 cm^2\ V^{-1}\ s^{-1}$ (Rolan-Assad and Gareil, 1995; Craston and Saeed, 1998). This is almost equivalent to lactic acid ionic mobility, reported to be around $36.5 \times 10^{-5}\ cm^2\ V^{-1}\ s^{-1}$ (Dutra *et al.*, 2006). Benzoate employed for lactate determination (Johns, Macka and Haddad, 2003) can also be used as chromogenic specie for indirect absorption technique (Rolan-Assad and Gareil, 1995). Tris- anisate buffer was used to detect free fatty acids (Rolan-Assad and Gareil, 1995).

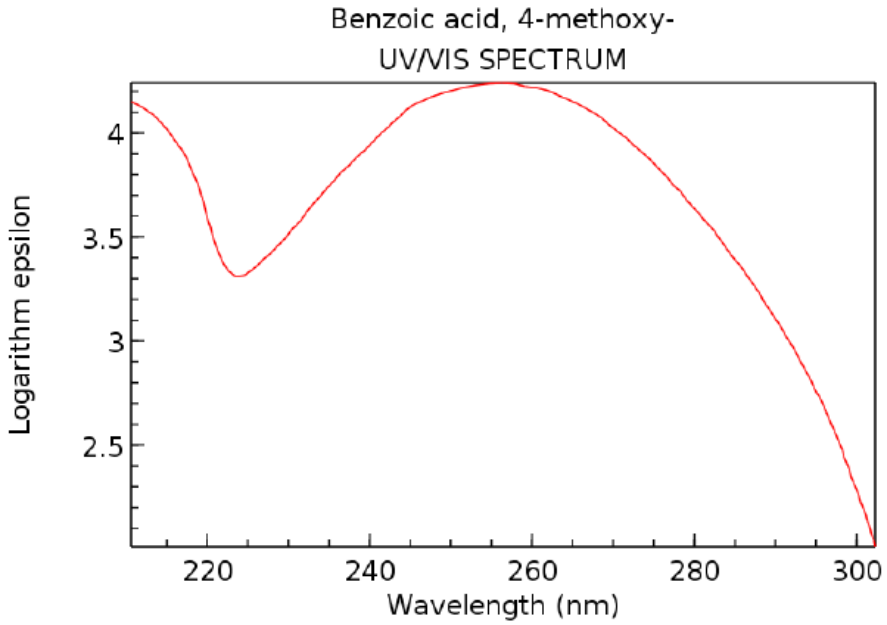


Figure 4: UV absorbance spectrum of p-anisic acid. Source: NIST Chemistry WebBook (https://webbook.nist.gov/chemistry)

In table 3 some examples of lactate detection conditions in previous works, based on indirect detection and similar to this thesis work, are reported.

Table 3: experimental methods for lactate detection by CZE in various matrices

Matrix	Buffer composition	pH	EOF modifier	Chromophore	Ref
Dermatological formulation	Tris	8	CTAB	p-anisic acid	Sharman, 1997
H2O	10 mM Phtalate salt	4.1	CTAB 0.5 mM		(Dutra, Santoro, Micke, Tavares, and Kedor-Hackmann, 2006)
Urine	20 mM MES/NaOH	6	CTAB + polybren 0.001% m/v		Tuma, Samcová, and Stulík, 2011
Beer	7.5 mM 4-aminobenzoic acid (with histidine for pH optimization)	5.75	TTAB to reverse EOF		Klampfl, 1999
Silage	2,6-Pyridinedicarboxylic acid	12	Cetyltrimethylammonium hydroxide		Hiraoka, Ishikuro, and Goto, 2010
coco oil	Tris 20 mM	8.1		p-Anisate 10 mM	(Roldan-Assad and Gareil, 1995)

4. EXPERIMENTAL

4.1 MATERIALS

N-butyric Acid, Alkyltrimethylammonium bromide, 4-methoxybenzoic acid (p-anisic acid) were purchased from Sigma Aldrich; Tris PlusOne was from Fischer Scientific; lactic acid was purchased by pharmacy (farmacia comunale Arbizzano, VR). HV samples came from autopsies conducted by specialists working at Verona Legal Medicine Department. HV samples were obtained from autopsies of cadavers died for different causes.

A stock solution of n-butyric acid (MW = 88.11) (hereinafter referred to as butyric acid) was prepared at a concentration of 5.7 mM by taking 26 µL from the commercial bottle and putting it in a 50 mL of water. Then it was diluted 1:100. The obtained 57 µM solution of butyric was used as internal standard. This standard solution was injected before each experimental session, in order to check the corrected functioning of the instrument and the buffer peak stability. According to this check, the stock solution was prepared every 15 days.

Lactic acid (MW = 90.08) stock solution was prepared at 30 mM concentration by dilution in water. This stock was further diluted to obtain the lactic acid standard samples in butyric acid medium 0.15 mM, by adding 10 µL to 1990 µL of butyric acid 57 µM. For some tests, successive further dilutions were performed, as later specified in the corresponding experiment descriptions.

As regard biological samples, 5 µL of each collected HV was diluted 1:150 with 745 µL butyric acid 57 µM solution. The obtained aliquot was again diluted three times (100 µL with 200 µL of butyric acid), in order to obtain a final dilution of 1:450 in the same medium.

4.2 METHODS

CZE separation was performed on a P/ACE MDQ system (Beckman Coulter Inc., Brea, CA, USA) by using a 60 cm length silica capillary with an inner diameter of 75 µm. The buffer was composed of Tris 37.15 mM, as the main buffering compound, 4-methoxybenzoic acid 3.94 mM, to improve sensitivity (Sharman, 1997) and alkyltrimethylammonium bromide 1.19 mM as electrosmotic flow modifier (Sharman, 1997). Buffer solution was freshly prepared once a week. Before each run, the capillary was washed with NaOH 0.1 M, H_2O and buffer for 1 minute each. Then, the sample was injected for 10 s at 0.5 psi. The applied voltage was 30 KV for 3.5 minutes. The detection was performed by UV-DAD detector by measuring the absorbance at 254 nm by indirect detection, since lactic acid do not absorb above 250 nm (Sharman, 1997). The capillary cartridge temperature was 25 °C.

4.3 DATA PROCESSING

Peaks areas were integrated with the instrument software 32 Karat 8.0.

Only the first peak was considered for lactate (migrating around 1.9 minutes), corresponding to the monomer. Standard lactic acid diluted in water at the final concentration of 0.15 mM was analysed by integrating only its monomer peak. The analysis concerning the internal standard butyric acid, were accomplished considering the absolute value of the corresponding peak area. On the other hand, for lactic acid standard solution diluted in butyric acid the ratio between lactate (monomer) and butyrate peak area was calculated. The same was performed for real samples, diluted 450 times in butyric acid as well, and Fig. 5 illustrates an example of HV electropherogram with integrated peaks. As shown in this figure, the instrument software elaborates the negative peaks to the adsorbing p-anisate by reversing the detection signal, generating positive peaks. Moreover, the values of the manually integrated area, time of elution and resolution are reported above each peak.

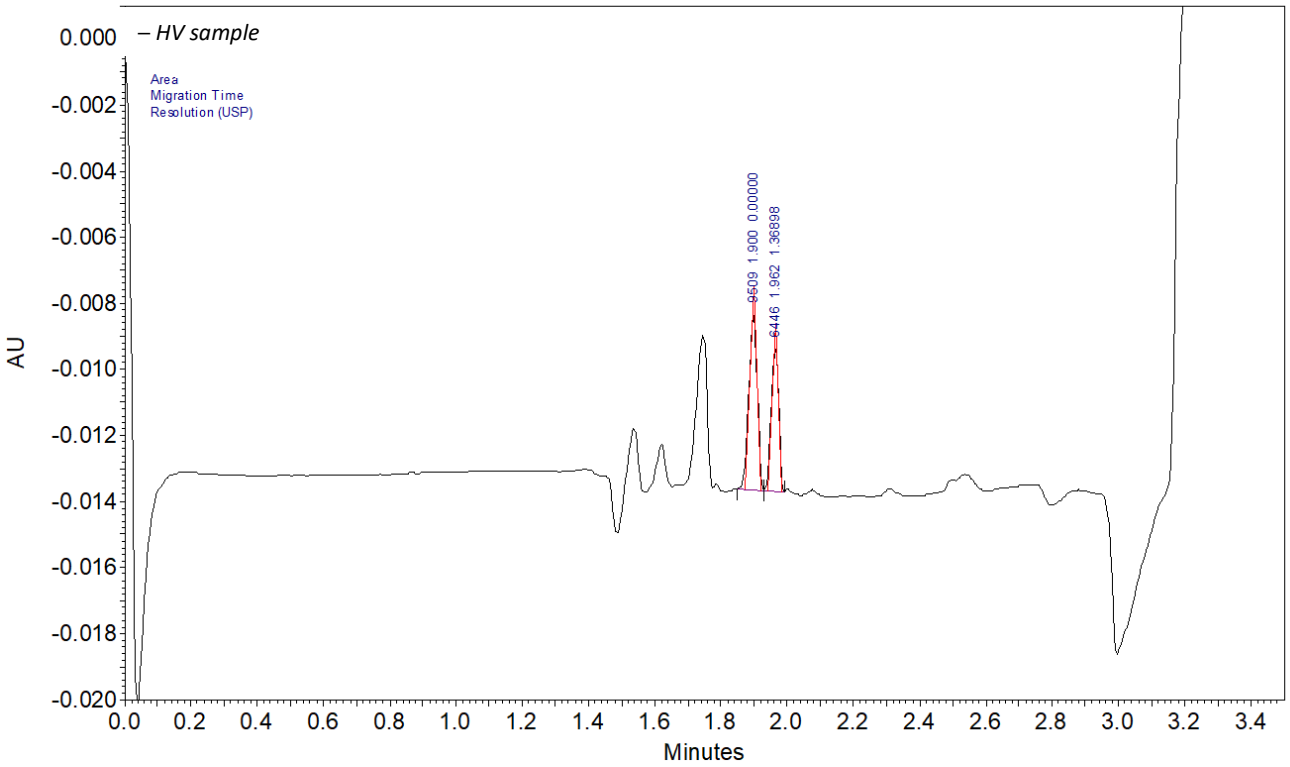


Figure 5: Electropherogram of a HV sample diluted 450 times in butyric acid 0.057 mM. The peak at 1.9 minutes corresponds to lactate monomer, while the peak at 1.962 minutes is the internal standard. Conditions: silica capillary (75 µm ID), 60 cm length. Electrolyte: Tris 37.15 mM, 4-methoxybenzoic acid 3.94 mM, alkyltrimethylammonium bromide 1.19 mM. PH 9.5. Applied voltage: 30 KV. Injection: 10 s at 0.5 psi. for 3.5 minutes. Cartridge temperature was 25 °C. Detection at 254 nm.

Calibration curve

The linearity of the method was evaluated by preparing 6 solutions containing different concentrations of lactic acid in butyric acid, in detail 4.9 μM, 9.8 μM, 24.5 μM, 49 μM, 63.7 μM and 78.4 μM. The ratio between peak area of lactate and butyrate was calculated for each concentration of lactate in order to obtain the calibration curve.

4.4 METHOD VALIDATION

Lactate standard was prepared in water and further diluted in butyric acid for the curve validation, while for stability analysis it was diluted both in butyric acid and water.

Intra run test

The same concentrations of lactic acid standard were prepared as before in order to accomplish intra run stability test. Each dilution was measured for 8 repetitions and butyrate stability was evaluated too.

Inter run test

Lactic acid at 4.9, 24.5 and 63.7 μM was prepared in butyric acid. Nine different experimental sessions were carried out in order to evaluate the inter run stability.

Temperature effect

A standard solution of lactic acid 29.3 mM was prepared, and five aliquots were stored at -20°C. Each of them was diluted at a final concentration of 0.15 mM both in water and butyric acid just before the analysis. The solutions stored at -20°C were monitored for 72 days. The area of lactate electrophoretic peak was integrated for the solution of lactate in water, while the ratio between lactate and butyrate was considered for the butyric acid medium.

4.5 HV SPECIMENS

Preliminary tests were carried out on HV that were been freezed from months and were been thawed several times for other studies; these samples are indicated as "long-time stored". Due to the diverse purposes of their previous analysis, their pre-treatment, if performed before freezing, were unknown. Long-time stored samples were diluted at various concentrations in butyric acid in order to select the best dilution for the analysis. To evaluate the best conditions for HV storage, some HV samples were stored at +4°C, while others were divided in several aliquots all stored at -20°C; each of them was individually thawed just before the CZE, at interval times of 10-15 days.

A second group of samples were indeed promptly analysed, that is less than 5 days after collection and during this period they were stored at 4°C without any freezing procedure. As pre-treatment, they were

mixed by means of vortex agitation. For stability investigation purposes, five aliquots of five of these samples were stored without any dilution at -20°C in order to evaluate the storage of HV in this condition. Aliquots were thawed nearly every 15 days, and diluted in butyric acid just before the CZE analysis. Moreover, some of the test tubes containing the original samples were kept at +4°C.

The electrophoretic peaks corresponding to lactate and butyrate of each HV were integrated. Afterwards, the concentration of lactate was calculated from the lactate to butyrate area ratio, by using the the calibration curve. Finally, the achieved values were related to the PMI of the corresponding HV samples.

5. RESULTS

In general, the best conditions for acidic analytes by means of CZE are obtained by using highly hydrophobic surfactants (Craston and Saeed, 1998) and an UV-absorber additive selected in order to have appropriate charge and mobility and allow indirect detection (Craston and Saeed, 1998). The main reference work to set up the experimental conditions for lactate determination in HV has been the Sharman procedure for dermatological preparations (Sharman, 1997). In that work the capillary was coated with CTAB (Cetyl triethil ammonium bromide), a surfactant bearing a positive charge, in order to obtain a reversed EOF (Craston and Saeed, 1998; Galli *et al.*, 2003). Tris-anisate was used as described by (Rolan-Assad and Gareil, 1995). Tris, in molar excess, allowed buffering capacity and a good baseline stability. Peak symmetry and sensitivity were checked thanks to p-anisic acid: in fact, its characteristic mobility and molar absorption coefficient matches the requirements for lactate detection (Rolan-Assad and Gareil, 1995). Moreover, the combination of Tris with p-anisic acid allowed the desired pH and a total absorption in the linearity range of Lambert-Beer law (Rolan-Assad and Gareil, 1995).

5.1 STANDARD SOLUTIONS

Fig.6 reports the electropherogram of the internal standard, butyric acid. A unique peak eluted at 2.0 minutes.

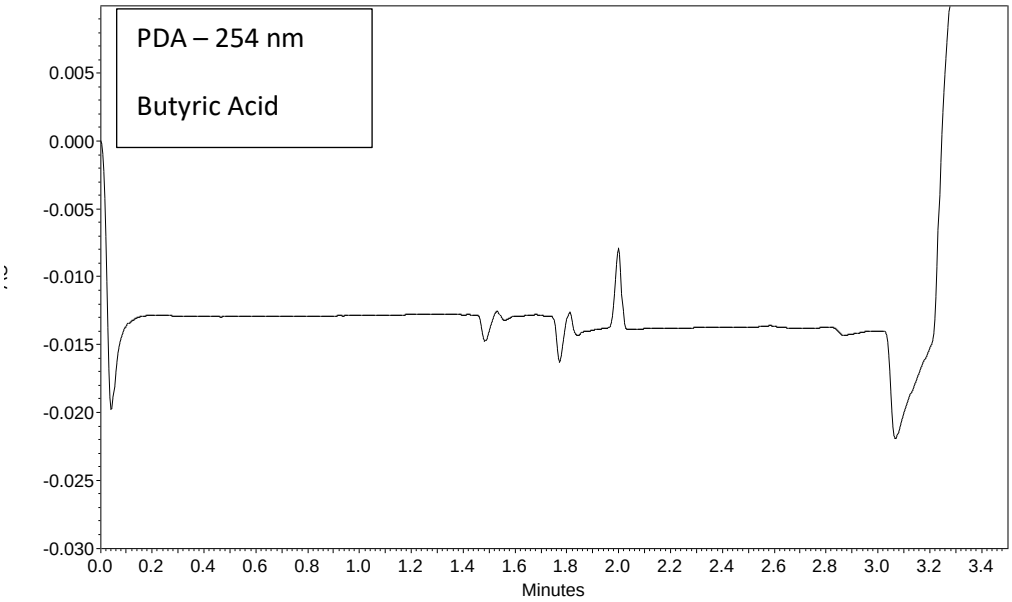


Figure 6: electropherogram of butyric acid internal standard, 57 µM. For experimental conditions, see caption of Fig. 5.

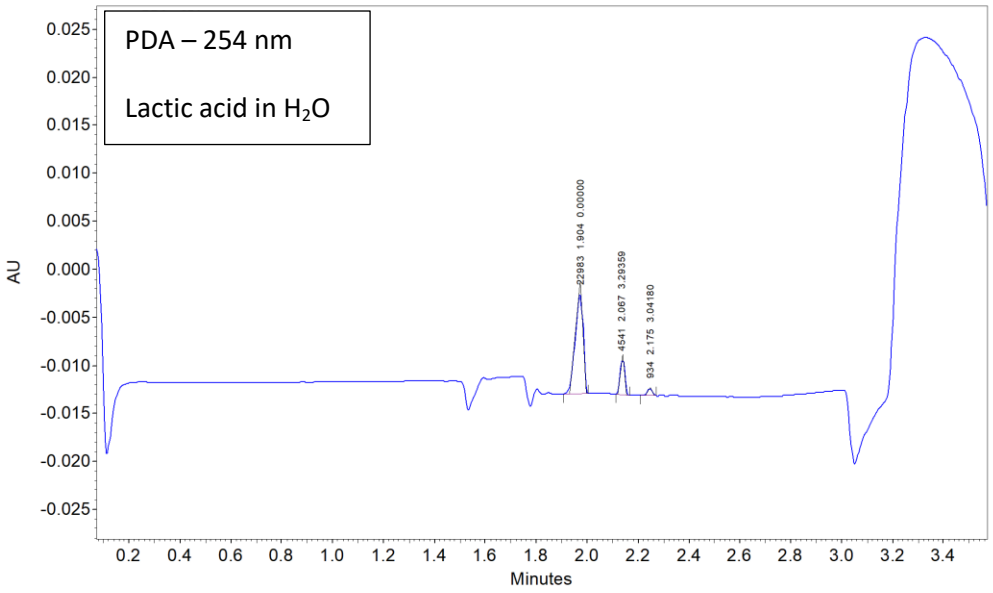


Figure 7: electropherogram of lactate 0.15 mM diluted in water. For experimental conditions, see caption of Fig. 5.

In Fig. 7 the electropherogram corresponding to 0.15 mM of lactic acid standard diluted in water is depicted. The peaks corresponding to the monomer, dimer and trimer are clearly visible, at the elution time around 1.90 minutes, 2.07 and 2.17 minutes, respectively. It should be noted that the elution order is related with the species weight, with the lighter monomer eluting first and the heavier trimer last. Also the respective intensities decrease with the increased polymerization degree, since the main specie is the monomer while condensation reaction is necessary in order to obtain the oligomers.

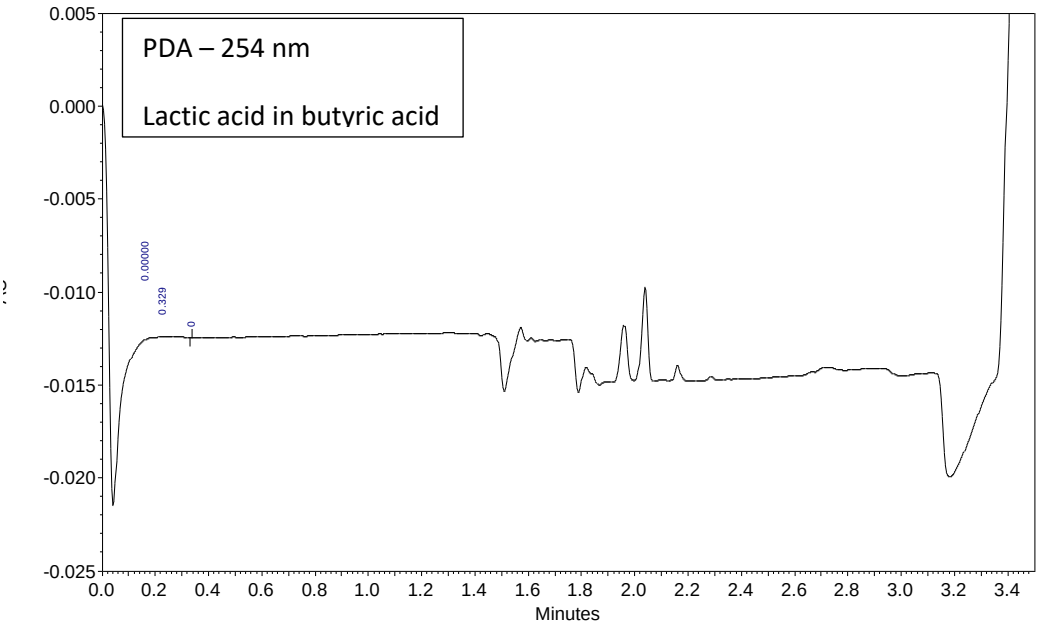


Figure 8: electropherogram of lactate diluted in 57 µM butyric acid. For experimental conditions, see caption of Fig. 5.

The electropherogram in Fig. 8 represents a sample of standard lactate 50 µM diluted in 57 µM butyric acid. By comparing with the previously results, it can be interpreted that the first peak, eluting at around 1.92 ± 0.03 minutes corresponds to lactate monomer, while the second one eluting at 2.0 ± 0.03 minutes corresponds to the butyrate peak. These two peaks are well resolved, and the resolution factor is 1.7. Lactate dimer and trimer were also distinguishable.

The current was always stable between 7 and 8 µA (reversed) during the electrophoretic run, for both standard solutions and HV samples.

Temperature effect

The freezed aliquots showed a decrease around less than the 30% of the initial value after 72 days.

Calibration curve

The correlation between the peak area ratios of each lactic acid dilution and internal standard with respect to the expected lactic concentration is reported in Fig. 9. Table 4 summarizes the main features of the obtained relationship that is linear and described by equation 1 and an R^2 value of 0.9981.

Equation 1:

$$y = 0.0117x + 0.033$$

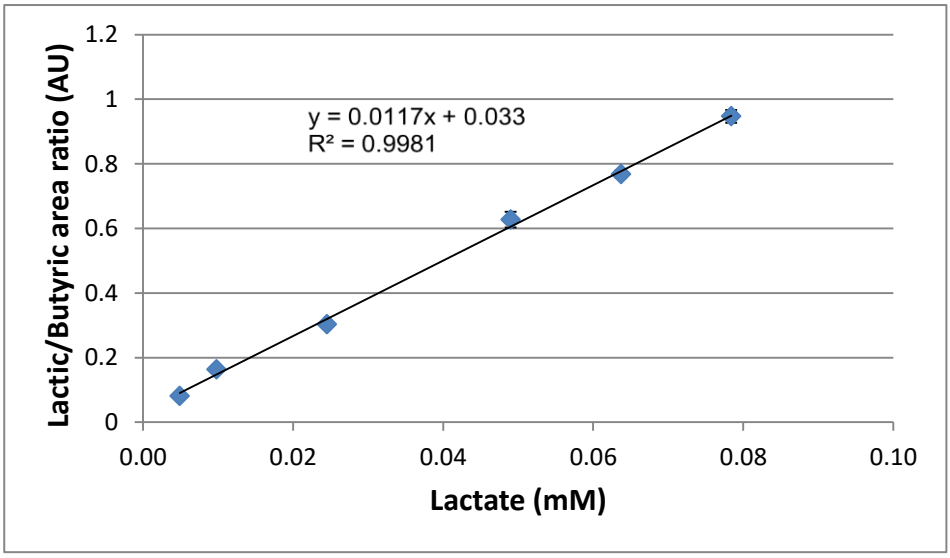


Figure 9: interpolation of lactate monomer peak area to butyrate peak area ratio versus the expected lactate levels of standard solutions diluted with internal standard solution. Lactate original concentrations in CZE vials are 4.9 µM, 9.8 µM, 24.5 µM, 49 µM, 63.7 µM and 78.4 µM.

Table 4

Working range (mM/L)	2.20 – 35.28 mM(*)
Calibration (linearity test)	
Regression analysis (slope)	0.0117
intercept	0.033
Correlation (r^2)	0.9981

(*)concentration in CZE vial

5.2 METHOD VALIDATION

Intra run tests

Only the first eluted peak, corresponding to the monomer of lactate, was considered. Intra run repetitions demonstrated reproducibility, described by standard deviation (StDEV) values and coefficient of variation percentage (CV%) for all the lactate solutions and butyric acid. The average resolution value of these two peaks was 1.4 for all the examined dilutions.

From the calibration equation, the concentration of each lactic acid dilution was calculated in order to evaluate the reliability of the method. The percentage of variation between the calculated and the expected values presented the higher values for the most diluted samples (4.9 and 9.8 µM) and was about 16% underestimated for the standard at lower concentration and overestimated for about 13% for the higher concentrated one. The other six samples varied less than 6%. The results of intra run measures are reported in table 5.

Table 5: results of intra and inter run tests on lactate standard diluted in butyric acid.

Intrarun precision	**Mean (mM × 10^{-3})**	**StDEV**	**Coeff. of variation (%)**
4.9 µM (n= 8)	4.09	0.77	18.82
9.8 µM (n= 8)	11.12	0.87	7.80
24.5 µM (n= 8)	23.08	0.43	1.87
49 µM (n= 8)	50.78	2.04	4.03
63.7 µM (n= 8)	62.83	1.03	1.63
78.4 µM (n= 8)	78.12	1.70	2.18
Inter run precision			
4.9 µM (n = 9)	6.91	2.57	37.19
24.5 µM (n = 9)	30.23	4.98	16.48
63.7 µM (n = 9)	89.69	30.67	34.20

Butyrate mean area value resulted reproducible with a CV% < 4.14 for each of these lactic acid dilutions.

Inter run tests

The inter run samples were affected by greater errors and variability, as demonstrated by the CV% and the percentage variation from the expected values. The results of inter run measures are reported in table 5.

5.3 HV SAMPLES

5.3.1 Long-time stored samples

Biological samples presented almost only the monomer peak of lactate, probably due to the complex composition of the HV matrix that hinders minor signals. Preliminary tests suggested a best dilution of samples to 1:450 in butyric acid. As disclosed in the previous paragraph, the CZE current was always stable at 8 μA also during the analysis of biological matrices. Some of these samples were monitored during time. Probably, some of them were degraded, as demonstrated by their yellow appearance or by the presence of a particulate. When this occurs, lactate peaks were no detectable, maybe due to either its absence or matrix interferences.

Stability tests of aliquots stored at both +4°C and -20°C showed a great variability of lactate concentration trend among the analized HV, with some scattered points and without a clear trend. Also the relation of the calculated lactate values with PMI was very dispersed, as shown in Fig. 10.

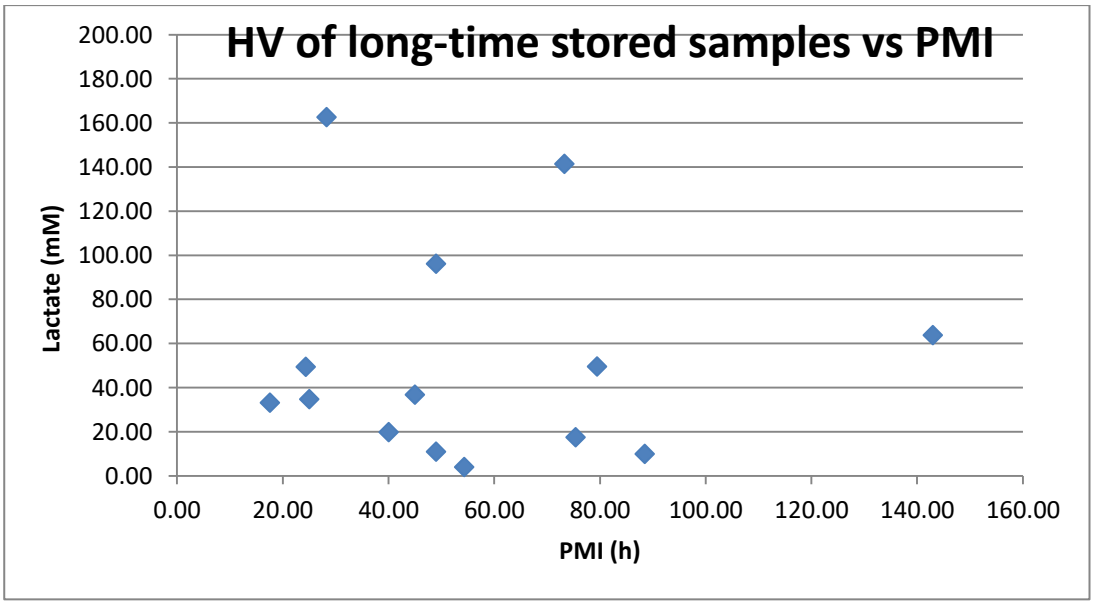


Figure 10: correlation between the lactate values and PMI of long-time stored samples. Lactate values were calculated from equation 1.

5.3.2 Promptly analysed HV samples

Ten HV samples were analysed closely after collection and before the freezing procedure: after sampling, each aliquot was stored at +4°C for a short period (generally 24 hours, with the exception of two HV, stored for four and six days, respectively) in order to avoid the beginning of degradation phenomena. Samples refrigerated for more than one week were discarded; in fact, some of them appeared degraded, presenting agglomerations. Samples were diluted 450 times in butyric acid solution as previously described. An example of an electropherogram of one of these samples is reported in Fig. 11.

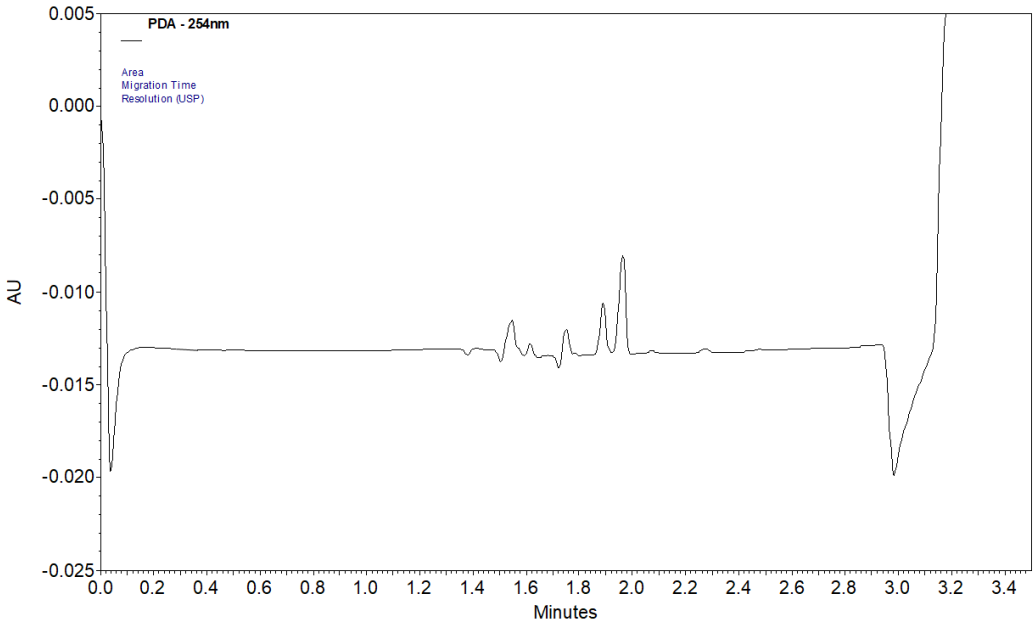


Figure 11: electropherogram of a HV promptly analysed after sampling from cadaver. Sample treatment and CZE method are described in the experimental section.

The obtained values of lactate concentrations ranged from 9.36 mM to 55.47 mM. PMI of these samples ranged from 4.56 to 119 hours. These samples were related with PMI by following a linear trend, as showed in Fig. 12.

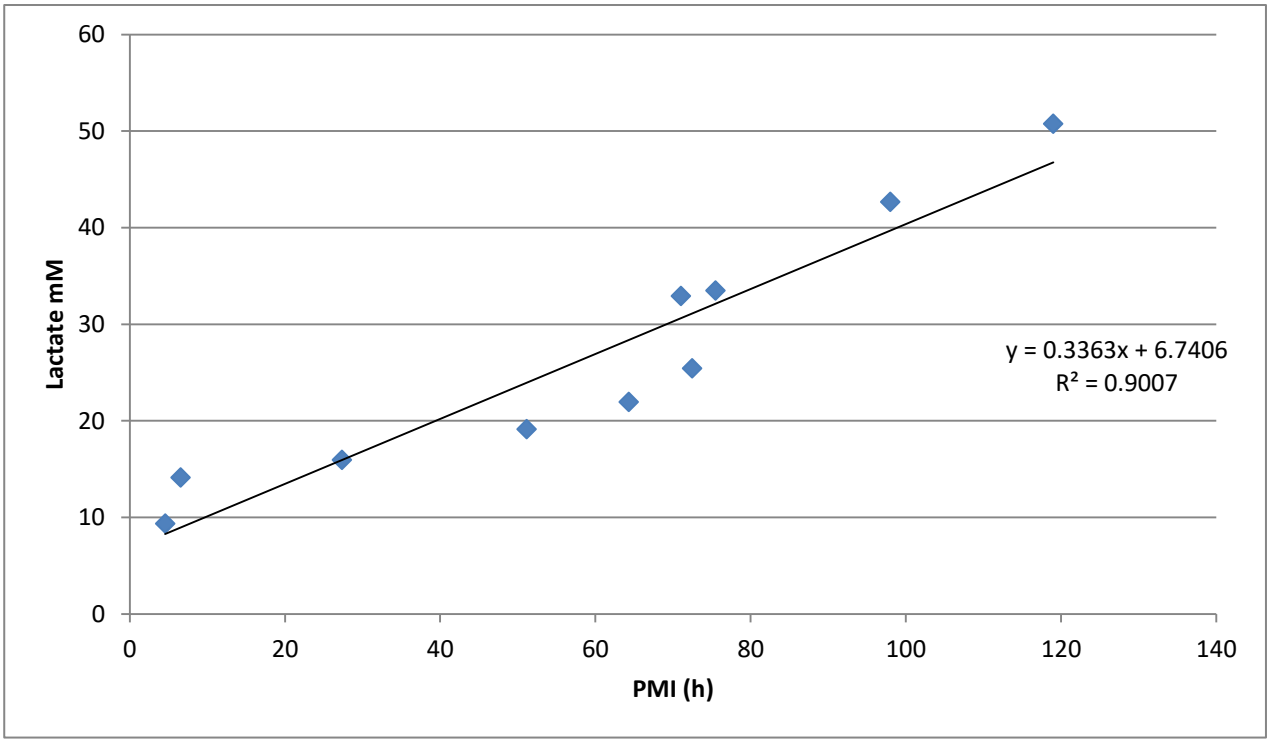


Figure 12: correlation between lactate amounts of HV analysed soon after collection and their corresponding PMI.

Butyrate stability was also evaluated in these samples, by calculating the peak area from inter run measurements of different HV specimens, all containing the same internal standard amount. The obtained values showed a CV% of 8.1.

Stability tests of HV stored at different temperature were not homogeneous for the various samples and in some cases lactate values were scattered. Thus, the obtained values of HV lactate demonstrated lack of reliability also taking into account the experimental variability. This occurred for both freezed and refrigerated samples, stored at -20°C and +4°C respectively. However, it must be noted that most of the samples appeared viscous and yellow thus probably degraded.

6. DISCUSSION

This work demonstrated that CZE is a technique suitable for lactate determination in HV. Since lactate has no absorption in UV range, p-anisic acid was added in buffer solution in order to perform the detection through indirect absorbance mode. Moreover, the capillary polarity was reversed by adding CTAB, taking into account the charge and the low molecular weight of lactate molecule. Thanks to this set up, lactate was clearly detectable in both standard solutions and in human HV samples and lactate monomer was well resolved from butyrate. The reproducibility of migration times was demonstrated for both lactic monomer and butyrate, and as a consequence, for the resolution of these two compounds. This allowed the precise identification of both molecules also in the biological matrix of interest. The traces baselines were steady, unless for damaged or very aged HV samples, demonstrating that this technique was not affected from impurities that may be present in biological fluids. The current too was constant (8 μA) during each electrophoretic run and did not significantly change depending on the individual samples. Hence the chosen method conditions demonstrated to be appropriate for the purpose to detect lactate in HV. Moreover, the procedure was fast, since each run lasted less than 10 minutes (including washing procedure, sample injection and voltage application for the actual electrophoretic run). Another point worth of note is the very low amount required to prepare the samples: in fact, only 5 μL of the collected HV were enough in order to obtain the required sample volume for the analysis. This allowed performing the analysis for all the samples, despite their often limited volume available, which makes impossible the analysis with other techniques.

The calibration curve obtained from lactic monomer to butyrate peaks area ratio versus lactate concentration demonstrated a linear relation, with a statistical significant coefficient of regression (R^2 = 0.9981). The stability tests of frozen aliquots of lactate in butyric acid medium were subjected to a slight decrease during 72 days. In future studies, -80°C could be investigated as storage temperature. Albeit the cause of this behaviour should be more deeply investigated, the above described facts suggest that storage at -20°C doesn't assure appropriate sample preservation. Intra run tests were characterized by CV% less than 7.8%. These values demonstrated the reproducibility of the method and the stability of the tests materials during measurement sessions (1-2 hours). The only exception was the more diluted standard (4.9 μM), that showed a corresponding CV% of about 18%. This is in agreement with the decrease of precision with peak size typical of CZE (Sharman, 1997). Another explanation could be the high viscosity of lactate standard that increases the experimental error during sampling. However, the lactate values calculated from the calibration equation showed variations of 16% and 13% with respect to the expected concentrations of the two more diluted samples, and less than 6% for the others.

The pH of the various solutions and buffer were checked. While the background electrolyte was basic (pH = 9.5), the internal standard used as medium for samples dilution was characterized by a pH of about 4.8. Thus, commercial lactic acid and HV biological samples diluted in butyric acid showed an acidic pH: lactic acid stock was 2.75, and values in the range between 5 and 7 for biological samples were measured. Indeed, HV appeared neutral soon after collection. Thus, lactic acid is present in the lactate form in the biological fluid and in the analysis conditions.

HV samples were analysed following the same method used for standard solutions. Since it was reported that the two eyes do not differ in potassium concentration (Tagliaro *et al.*, 2001), as well as sodium and chloride (Gagajewski *et al.*, 2004), it seems presumable that also lactate is present at the same concentration between the two eyes. This was also demonstrated in our laboratory (data not reported here). For this reason, the value of a single eye was investigated in this thesis. HV samples had stable baseline, and both lactate and internal standard were detectable at their typical migration time with good resolution. This demonstrated that the chosen methods for sample management and for electrophoretic separation were suitable. Moreover, the concentrations of lactate obtained from long-time stored HV extended from about 3.9 mM to 162 mM; the mean value was around 50 mM; most of the values were around 30-40 mM. This is in agreement with the range suggested by the literature: from hypothetical blank values less than 10 mM (Belsey and Flanagan, 2016) or about 14.7 mM (Peclet, Picotte and Jobin, 1994), up to 20-30 mM in the first hours after death (Harper, 1990; Mihailovic *et al.*, 2011; Mitchell *et al.*, 2013). Harper (Harper, 1990) reported preliminary studies in which different mean values were found depending on the cause of death. However, he found that such a correlation was not so easy to postulate, due to the high number of influencing factors on lactate concentration. In the present thesis work, samples presenting higher values (up to 162 mM) were found. One reason should be an antemortem disease or cause of death, both unknown. In addition, these samples were collected from cadavers died in a time extent of a year, so that each of them was subjected to dissimilar aging effect, depending on their storage time, on the condition and duration of storing before freezing and a different number of freezing/thawing cycles. All these presumed differences are probably responsible of the dispersion of lactate concentration with respect to PMI and of the discrepant trends obtained from stability tests. Finally, with aging, some of them visibly degraded, as became yellow and denser. Thus, it should be deduced that the lactate present in the HV, particularly if subjected to freezing and thawing, is not stable after many months.

The promptly analysed samples, refrigerated at +4°C for short time, revealed lactate concentrations from 9.36 mM to 55.47 mM, with a mean value corresponding to 29.2 mM. As observed earlier, this value matches the literature reports. PMI range was between 4.56 hours and 119 hours. In these conditions, the correlation between lactate and PMI is linear with r-squared value R^2= 0.901. Despite the

small number of available samples for this study, those with a PMI minor than 24 hours contained less than 20 mM lactate, accordingly to Mihailovic results (Mihailovic *et al.*, 2011). These results confirm the values reported in the literature of lactate concentrations in HV during the early PMI.

The obtained data also prove lactate increase in HV after death. The comparison of these experimental results with those acquired from the long-time stored samples, reinforces the hypothesis about the importance to perform the analysis before freezing HV and as soon as possible after collection. Moreover, up to now lactate has been mainly measured by means of enzymatic methods in blood (Coe, 1993; Valenza *et al.*, 2005; Pundir, Narwal and Batra, 2016), while only rarely in HV (Zilg *et al.*, 2009; Mihailovic *et al.*, 2011; Mitchell *et al.*, 2013) or isotachophoresis (Harper, 1990).

Despite CZE proved to be suitable for the purpose of this investigation, some aspects of the method still need optimization. A wide variability of samples amount was found by monitoring storage time and temperature effects. However, as previously discussed, also standard lactate and butyrate tests showed variable values after freezing and thawing. This suggests that deep investigations of these aspects have to be performed in future studies in order to identify and avoid the involved experimental variations. On the other hand, the sampled HV volume available for this kind of analysis was not enough for the preparation of replicates. For this reason, only one diluted sample was analysed and no reproducibility assays have been accomplished on biological test materials. In the view of a further optimization of the experimental procedure, two main aspects should be faced. First of all, matrix effect on lactate. In fact, HV composition is thoroughly known, as well as the possibility of some substances to penetrate from other tissues. Nevertheless, the biochemical processes occurring after death involving lactate have not been investigated yet. The possibility that some substances present in HV may start lactate production (like enzymes) or degradation reactions after death has not been investigated. These kinds of substances could penetrate in HV from other tissues, mainly from blood circulation, but this can occur only in cadaver; thus, if HV is collected before the beginning of diffusion, no further reaction will take place in vitro. The second aspect that needs further evaluation is the possible butyric acid influences as solution medium. In fact, it's known that lactic acid is prone to polymerize in oligomers, and the dilution in internal standard medium causes samples pH decrease. It should be assessed if these changes favour polymerization of commercial standard lactic acid, but especially of lactate contained in biological matrix. On the other hand, the absence of eventual reactions among butyrate and lactate should be verified. Butyric acid was chosen as internal standard because it demonstrated appropriate use for lactate quantification by means of CZE in a dermatological preparation (Sharman, 1997). Thus, it appeared likely that it doesn't react with lactate. Moreover, it is present in biological matrix only in trace amounts, without affecting internal standard value. Furthermore, samples containing high levels of lactate (be-

longing to the long-time stored samples) were never accompanied by particularly enhanced butyrate signal with respect to the internal standard alone measured before each session run for comparison.

This work presents preliminary data for an evaluation of lactate determination in HV and its correlation with PMI. Up to now lactate has been measured only in different matrices or by means of different analytical approaches, giving discrepant results: Coe (Coe, 1993) found no significant relationship, while Mihalovic found linearity (Mihailovic *et al.*, 2011). Lactate and the sum value were found to be correlated to PMI with a logarithmic trend: they increased immediately after death and reached a plateau after 2 days (De Letter and Piette, 1998) and showed a normal (Gauss) distribution. Indeed, glucose was not correlated with the time since death and was characterized by a Poisson distribution (De Letter and Piette, 1998). Sample treatment, storage conditions and analytical technique changed among these works. For this reason, this thesis aims to find and validate the best method for estimation of lactate in vitreous in relation with PMI. At first, CZE procedure represented a suitable tool for eliminating the differences between analysis methods and sample treatment (Tagliaro *et al.*, 1999). This technique also allows the separation of more than one component and consequently multivariate analysis that improves PMI estimation precision by 5 times (Madea, 2005). Secondly, up to now lactate has been mainly determined by means of common enzymatic methods for human blood/serum analysis. This is an important point, since the scarcity of validated methods is one of the main drawbacks in HV analysis. Conversely, here CZE has been specifically employed to perform calibration study and method validation and satisfied the requirements related to this scope. On the contrary, other techniques have been mostly used to analyse this parameter in different matrices and without specific validation procedures. One exception is the research performed by Harper (Harper, 1990) that analysed lactate in HV by means of isotachophoresis. In that study, HV samples were stored at 4°C until the analysis, without any treatment procedure neither preservatives additions. This suggests that our samples management was correct. It's worthy to note that some of the samples studied by Harper were analysed also long time after death, up to 27 days. All the causes of death of the considered specimens were known and no sample was discarded. A calibration curve was obtained by injection of different standard lactate concentrations and no internal standard was employed. Lactate amounts in biological samples were obtained by comparing the length of the zone of each sample isotachopherogram with this calibration curve. The present thesis exploited CZE with an internal standard in order to provide better accuracy. Nevertheless, some degree of variability was observed and for this reason further investigations are suggested. Yet, the levels of lactate obtained with CZE are perfectly in line with the range found by Harper: mean value of 29.2 mM versus 27.4 mM. New in this thesis with respect to Harper study, is the survey about the correlation between vitreous lactate and the time of death. In fact, in Harper work all the PMI values were known and reported but their correlation with lactate concentration was not inves-

tigated. The aim of Harper study was to assess the usefulness of isotachophoresis to determine lactate concentration in HV, and to relate the obtained values to the cause of death. Thus, the paper reported the information concerning the patients (cause of death, sex and age), the time between death and cadaver examination and the period among time examination and isotachophoresis analysis (Harper, 1990). No correlation of lactate with PMI emerged by examining Harper data. This seems reasonable due to the nature of the analysed samples. In fact, the selected HV derived from patients died from various causes, chosen with the scope to embrace as many as possible various forensic cases. Thus, most of these samples contained elevated concentrations of lactate, also due to the influencing factors they were subjected to. A second reference work that indeed considered such relationship is the study performed by De Letter *et al.* (De Letter and Piette, 1998). Here, several changes in the procedure steps are present with respect to both Harper and this thesis work. In fact, HV collected from different forensic cases were treated with perchloric acid in order to avoid glycolysis processes then frozen before being analysed. Tests were performed by enzymatic reactions without validating the method. Finally, while in this thesis the maximum PMI found was 119 hours, the PMI accounted in De Letter work extended up to 11 days. Both lactate and the sum of lactate and glucose were investigated in relation to the time since death. For both these parameters, a logarithmic curve was found (De Letter and Piette, 1998); at first, this should be related to the different PMI range investigated. Secondly, but not less important, the obtained concentrations levels were very dispersed (De Letter and Piette, 1998). Origins of such variability can be supposed: the freezing and thawing cycle before the analysis; the not validated method; the evaluation of all the samples. Finally, a more recent work performed by Mihalovic et al. (Mihailovic *et al.*, 2011) explicitly investigated the vitreous lactate changes versus PMI but in cadaver. Here, small aliquots of HV were repeatedly collected from the corpse at different time intervals in order to assess the changes occurring in the corpse. The PMI levels investigated increased with increasing the time of sampling from the same corpse. The investigated range was 24 hours. Samples were only centrifuged as pre-treatment, freezed at -20°C and diluted in water for the analysis. In this case, the enzymatic method for lactate analysis was validated. The obtained lactate concentrations linearly increased with the time since death. However, the goal of this research was to study the trend in the same corpse at different time interval of this parameter. These reference works are limited but representative of the multiplicity of the procedures employed, the multiple factors involved and, as a consequence, of the obtained results. This clearly emphasizes the importance of setting up a unique procedure, from sampling to treatment and storage, as well as the method of analysis. This thesis is the first step towards this goal as regards lactate in HV.

7. CONCLUSIONS

CZE revealed to be an appropriate analytical technique, since it allowed specific detection of lactate and reproducibility and resolution with respect to the internal standard investigated. The method used allowed fast analysis time and required only a low sample amount. Up to now, this technique has been employed only for the investigation of potassium in HV and for lactic acid in other matrices. Moreover, in this work the novelty consists in the preliminary attempt of a method validation for lactate analysis of HV by means of CZE. The variability resulting from some tests suggested the need for deeper investigations about some experimental procedure details and samples management. The experimental results demonstrated lactate levels in accordance with the reported literature data. Moreover, lactate in HV increased linearly with PMI. A deep inspection of the other few investigations performed on lactate vitreous content in HV clearly demonstrated the significant number of influencing factors: ante mortem patient conditions and cause of death, sample pre-treatment, storage temperature and time, analytical technique. Variations of experimental procedures or samples features were reflected in discrepant results between this work and literature data. This highlights the importance of a unique method, validated in every detail. As an example, sample omission of HV affected by a pathology correlated with lactate depends on the intent to obtain a criterion valid for every cadaver or only from ante-mortem healthy patients. In this thesis, according to this assumption, degraded samples were discharged, while the cause of death was not considered. Different trends have been found in literature depending on the interval between death and sampling: the linearity obtained evaluating the early PMI could be present only as a first stage, and it should be interesting to evaluate its evolution at longer time intervals. This task could be probably difficult to be achieved since it would entail dealing with cadavers in a more advanced degradation state. To conclude, this work can be a valid initial base to create a precise tool to establish the time since death by quantifying lactate in HV.

8. REFERENCES

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