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# Development and validation of a novel derivatization method for the determination of lactate in urine and saliva by liquid chromatography with UV and fluorescence detection

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ABSTRACT

We developed a novel and straightforward derivatization method for the determination of lactate by reversed phase high-performance liquid chromatography (RP-HPLC) with fluorescence and UV detection in biological matrices as urine and saliva. The derivatization of lactate was achieved employing 9-chloromethyl anthracene (9-CMA) as fluorescence reagent, which has never been previously used to obtain a lactate derivative. Lactate reacts with 9-CMA with high selectivity in a very short time, without requiring extraction procedures from the aqueous solution, and the reaction reaches 70% completion in 30 min. The ester derivative obtained can be easily determined by RP-HPLC with fluorescence detection at 410 nm ($\lambda_{ex}$=365 nm) and UV detection at 365 nm. The method was also optimized in order to allow for the simultaneous determination of lactate and creatinine for the application to urine samples. The lactate calibration curve was linear in the investigated range $2\times10^{-4}$–$3\times10^{-2}$ mM and the limit of detection, calculated as three times the standard deviation of the blank divided by the calibration curve slope, was 50 nM for both fluorescence and UV detection. Intra-day and inter-day repeatability were lower than 5% and 6%, respectively. The method proposed was successfully applied to the analysis of urine and saliva samples.


## 1. Introduction

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

The quantitation of lactate in biological fluids is an important topic in sport medicine to monitor the maximum performance level of athletes [1–3], and in clinics [4–6].

The concentration of blood lactate is usually 1–2 mmol/L and increases during intense exercise due to the switch of muscle cells to anaerobic metabolism. Once a certain level of lactate is reached (lactic acidosis), exhaustion occurs and there is a rapid decline in exercise capacity [7].

Lactic acidosis is also found in all the diseases involving inadequate intake of oxygen to tissue (hypoxia) and may thus be used as an early warning for organ failure and dysfunction: acute congestive heart failure, renal or hepatic failure, respiratory failure, etc. Hyperlactemia is typical of patients with severe sepsis or septic shock, it can be secondary to the anaerobic metabolism due to the hypoperfusion and it has a prognostic value. In all these conditions the repeated measurements of lactate at intervals of time is of fundamental importance to keep the conditions of the patients under control, also in response to a therapy [8,9].

Lactate has a chiral center and, thus, it has two enantiomers (L-lactate and D-lactate). L-Lactate is the major enantiomer measured in blood whereas D-lactate exists normally in very low concentrations under normal physiological conditions because it is produced from bacteria metabolism [10]. It has been reported that D-lactate concentration increased significantly in the serum of patients suffering from diabetes or short bowel syndrome [11–13]. Furthermore, D-lactate obstructs mitochondrial utilization of L-lactate in some tissues, such as brain and heart [14]. Therefore, L-lactate and D-lactate should be investigated separately in biological sample in several disease conditions.

The determination of lactate is usually performed in blood or plasma and based on enzymatic methods which are expensive and lack in sensitivity and specificity [15], or by separative techniques. It can be detected by gas chromatography [16,17], capillary electrophoresis [18,19], liquid chromatography with mass spectrometric detection [20,21], high-performance liquid chromatography with UV or fluorescence detection [22,23].

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In the last years the importance of monitoring of metabolites in media other than blood is increasing in parallel with the demand for non-invasive analysis. Such analytical protocols are fundamental to avoid physical and mental stress for the patients, risks of infection and at the same time do not require the presence of a medical staff. Matrices as urine and saliva are already used either to control daily parameters in hemophiliacs, neonates and elder patient, either to monitor the training of athletes [24,25].

The determination of lactic acid in complex matrices as urine and saliva is challenging. The concentration of urinary and salivary lactate is relatively low (about 0.1–0.3 mM) and for most of the reported analytical procedures, the sample has to be diluted to avoid matrix effects. Furthermore, the direct detection of lactate is unspecific because it is based on the intrinsic absorbance at 210 nm. There are only few works published on the determination of lactate in urine and saliva samples. A derivatization step is always required in order to improve the selectivity and the sensitivity of the methods (HPLC-DAD/FD)[26], to make lactate volatile for GC–MS analysis [16,17] or to increase the mass of the analyte for LC–MS/MS measurements [20,27].

In this work we describe a new, fast and simple derivatization procedure for the determination of lactate by HPLC-DAD/FD employing 9-chloromethyl anthracene (9-CMA) as fluorescent reagent, previously used for the determination of fatty and bile acids [28] and organic acids (i.e. formic, acetic, propionic, butyric, pentanoic and benzoic acids) [29]. The actual formation of the lactate derivative and the nature of by-products of the derivatization reaction were confirmed by HPLC-ESI-Q-ToF.

The optimized derivatization procedure is competitive with other derivatization methods for the determination of lactate previously reported [22,26,30–36]. The method was applied to the determination of lactate in saliva samples from healthy volunteers. Our method was also applied further optimized to achieve the simultaneous determination of lactate and creatinine in urine samples from healthy volunteers, in order to normalize the lactate concentration with respect to the physiological differences among urine samples [37,38].

## 2. Experimental section

### 2.1. Chemicals and materials

L-Lactic acid (L-6402, purity 98%), creatinine anhydrous (C4255, purity > 99.0%), acetate (51791), butyrate (08089) and propionate (51716) (1000 mg/L in $H_2O$), 9-chloromethyl-anthracene (196517, purity > 98%) and tetra-*n*-butylammonium bromide (426288, purity > 98%) were purchased from Sigma-Aldrich-Fluka (Milan, Italy), pyruvate sodium salt (8593589) from Merk and triethanolamine (purity > 99%) from Ashland Chemical Italiana. Stock solutions of L-lactic acid, creatinine and pyruvate were prepared in ultra-pure water obtained by a Elga Purelab-Uv system (Veolia Environnement, Paris, France). Stock solution of 9-chloromethyl anthracene, tetra-*n*-butylammonium bromide and triethanolamine were prepared in acetonitrile (ACN, LC–MS Chromasolv, 34967, purity > 99.9%, Sigma-Aldrich-Fluka). Phosphate buffer solution (PBS) at pH 2.5 was prepared from monobasic monohydrate sodium phosphate (BDH Laboratory Supplies, Poole, England) and phosphoric acid (345245, Sigma-Aldrich-Fluka).

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

### 2.2. Calibration standards and controls

A lactate standard stock solution was prepared by dissolving the lyophilized powder in Milli-Q water. Six standard solutions diluted from the stock solution were employed for the calibration curves and three of them were used as low, medium and high quality control: 0.05 mM, 0.15 mM (LQC), 0.5 mM (MQC), 1.5 mM, 5 mM (HQC) and 15 mM.

A creatinine standard stock solution was prepared by diluting the lyophilized powder in Milli-Q water. Eight standard solutions diluted from the stock solution were employed for the calibration curves and three of them were used as low, medium and high quality control: 0.05 mM, 0.15 mM, 0.5 mM, 1.5 mM (LQC), 5 mM (MQC), 15 mM (HQC), 50 mM and 150 mM.

Low, medium and high quality controls were used to evaluate the intra-day and inter-day repeatability for lactate and creatinine.

### 2.3. Biological samples collection

All the biological samples (urine and saliva) were provided by healthy volunteers.

Urine samples from healthy volunteers were collected before and after training into a 10 mL Eppendorf tube, centrifuged at 5000 rpm for 10 min (Eppendorf Centrifuge 5804R) and divided into 1 mL aliquots.

Saliva samples were collected by STARSTED Salivette® for Cortisol Testing.

All the samples were frozen at −20 °C until analysis. Lactate was proved to be stable for more than 1 year.

Urine and saliva samples were made to thaw at room temperature and centrifuged at 14,000 and 3000 rpm for 10 min respectively. The supernatant was pipetted into a 1 mL Eppendorf tube and vortexed (Velp Scientifica) for 30 s before the derivatization procedure.

### 2.4. HPLC-DAD/FD

An HPLC gradient pump (P4000, ThermoFinnigan) was coupled with a vacuum membrane degasser (SCM1000, ThermoFinnigan), an AS3000 autosampler (ThermoFinnigan), a UV6000 diode array detector and a FL3000 fluorescence detector (ThermoFinnigan).

Separations were carried out using a reversed-phase HPLC column GEMINI C18 (250 mm × 4.6 mm, 5 μm, Phenomenex), equipped with a guard cartridge (KJ0-4282, Phenomenex) with the column temperature set at 40 °C. Separations were performed with an isocratic elution in 65% ACN and 35% ultra-pure water at a constant flow rate of 0.8 mL/min. The chromatographic run was complete in 32 min, including a rinsing of the column in 100% ACN and the re-equilibrating step (Method 1). An injection volume of 20 μL was used for each sample. The detection of the lactate derivative was performed in absorbance at 365 nm and in fluorescence setting the excitation and emission wavelengths at 365 and 410 nm, respectively.

For the simultaneous determination of lactate derivative and creatinine the separations were performed with a 5 min isocratic elution in 20% ACN and 80% ultra-pure water followed by an isocratic elution with 65% ACN and 35% ultra-pure water (flow rate of 0.8 mL/min). The chromatographic run was complete in 42 min (Method 2) and the detection of creatinine was performed at 234 nm. The column temperature was 40 °C.

The direct determination of lactate in a standard solution was performed in order to estimate the derivatization yield and it was carried out using a reversed-phase HPLC column Synergi-Hydro RP-C18 (250 mm × 4.6 mm, 4 μm, Phenomenex), equipped with a guard cartridge (KJ0-4282, Phenomenex) with the column temperature set at 30 °C. An isocratic elution in 99% 0.05 M PBS (pH = 2.5) and 1% methanol was used (0.8 mL/min flow rate) as previously reported [1]. The injection volume was 20 μL and detection was performed at 210 nm.

### 2.5. HPLC-ESI-Q-ToF

All the analyses were carried out using a 1200 Infinity HPLC (Agilent Technologies, USA), coupled by a Jet Stream ESI interface (Agilent) with a Quadrupole-Time of Flight tandem mass spectrometer 6530 Infinity Q-ToF detector (Agilent Technologies).

Separations were carried out using a reversed-phase Zorbax Extend C18 column (2.1 mm × 50 mm, 1.8 μm) with a Zorbax Extend C18 guard column (2.1 mm × 12.5 mm, 5 μm).

The injection volume was 1 μL and the *needle wash* injection type was used. The column temperature was 40 °C and the flow rate was 0.3 mL/min. Separations were performed with an isocratic elution with 20% ACN (LC–MS grade, Sigma Aldrich, US) and 80% LC–MS grade water (Sigma Aldrich, US) for 0.8 min followed by a 0.2 min linear gradient up to 65% ACN for 2.4 min. The chromatographic run was complete in 10 min, including the rinsing of the column in 100% ACN and the re-equilibrating step (Method 3).

The ESI operating conditions were as follows: drying gas ($N_2$, purity > 98%): 350 °C and 10 L/min; nebulizer gas 35 psig; sheath gas ($N_2$, purity > 98%): 375 °C and 11 L/min. Nitrogen (purity 99.999%) was used as collision gas for MS/MS analysis. The capillary and the nozzle voltages were set at 4 and 1 kV, respectively. The fragmentor potential was set at 100 V and the Skimmer 1 and the Octopole RF were set at 65 and 750 V, respectively. Two time acquisition segments were used: from 0 to 1 min for creatinine and from 5.5 to 7 min for the lactate derivative.

High resolution MS spectra were acquired in positive mode in the range 100–1700 *m/z*, while tandem MS/MS spectra were acquired in the range 85–600 *m/z*. The data were collected with a MS scan rate of 1.46 spectra/s. The mass axis was calibrated using the Agilent tuning mix HP0321 (Agilent Technologies) in ACN. Mass spectrometer control and data acquisition were performed with MassHunter® Workstation Software (B.04.00).

### 2.6. Samples preparation and derivatization procedure

The final optimized derivatization procedure is summarized below.

Biological samples were centrifuged at 14,000 rpm for 10 min. 10 μL of sample were added to the reaction vial with 20 μL of 5% triethanolamine stock solution in ACN (TEA), 90 μL of 90 mM tetra-*n*-butylammonium bromide stock solution in ACN (TBAB), 370 μL of 10 mM 9-CMA stock solution in ACN and 10 μL of ACN (final volume 500 μL).

The reaction solution was incubated at 70 °C for 30 min in a thermostatic water-bath in the dark to facilitate the derivatization reaction. The reaction solution was cooled at room temperature, diluted 1:10 in the mobile phase, filtered through a PTFE syringe filter (0.45 μm, GRACE) and injected.

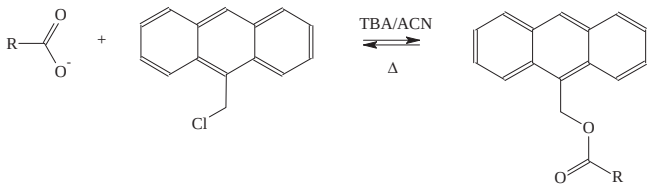
## 3. Results and discussion

### 3.1. Optimization of the derivatization procedure.

#### 3.1.1. Reactivity of lactate with 9-chloromethyl anthracene.

Carboxylic acids react with 9-chloromethyl anthracene (9-CMA) to form fluorescent esters according to Reaction 1.

The reaction is a nucleophilic substitution that proceeds in aprotic solvents (as ACN or cyclohexane) to avoid the protonation of carboxylates and due to the low solubility of 9-CMA in protic solvents. The reaction is accelerated in the presence of tetra-*n*-butylammonium bromide or hydroxide because the formation of an ion pair with the analytes makes them soluble in the organic solvent. The obtained esters have the same spectroscopic




**Reaction 1.** Reaction of carboxylic acids with 9-CMA.

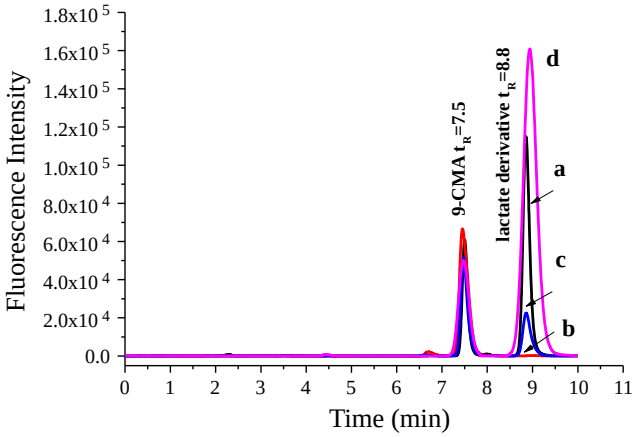


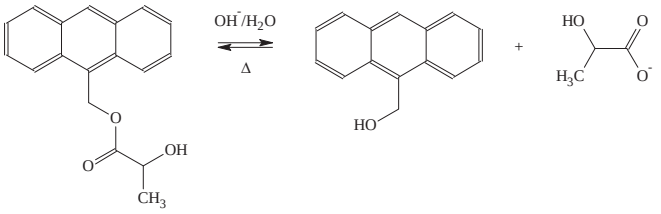

**Fig. 1.** Fluorescence chromatograms at 410 nm ($\lambda_{ex}$=365 nm) of 1 mM lactate aqueous standard solution after derivatization (a, black line), the corresponding blank solution obtained by applying the derivatization procedure to 10 μL of ultrapure water (b, red line), the lactate derivative obtained from the ethyl acetate extract (c, blue line), lactate standard solution prepared in ACN (d, pink line). Chromatographic conditions: see Section 2.4 (Method 1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

properties of 9-CMA and they can be detected easier and with higher sensitivity than the corresponding carboxylic acids with spectrophotometric detectors [28].

The derivatization procedure optimized by Xie at al. [29], which requires the extraction of analyte in ethyl-acetate, was applied to a 2 mM lactate aqueous standard solution acidified with 2 M HCl. The same derivatization procedure without the extraction step (10 μL of sample + 20 μL of 5% TEA + 420 μL of 20 mM TBAB + 50 μL of 10 mM 9-CMA – final volume 500 μL) was adapted to a 1 mM aqueous standard solutions of lactate to check if the lactate reacts with 9-CMA also in presence of trace of water. Fig. 1 shows the fluorescence chromatograms at 410 nm of a 1 mM standard solution of lactate derivatized in the presence of traces of water (a), the corresponding blank solution obtained by applying the derivatization procedure to 10 μL of ultrapure water (b), and the lactate derivative obtained from the ethyl acetate extract (c).

The fluorescence chromatogram of the extract derivatized (Fig. 1a) shows two peaks at 7.5 and 8.7 min retention time, assigned to the 9-CMA excess and to the anthrylmethyl ester of lactate, respectively. The same peaks were observed in the fluorescence chromatogram of the derivatized aqueous lactate standard solution (Fig. 1b). This result demonstrates that lactate reacts with 9-CMA also in the presence of traces of water. However, another peak at 7.7 min is present in the chromatogram of the derivatized aqueous lactate standard solution that likely corresponds to a by-product of the reaction. The fluorescence chromatogram of the blank (Fig. 1c) evidences a small interfering peak eluting at the same retention time of the lactate derivative. This peak does not affect the determination of lactate because its signal has been minimized in the optimization step (see below).

In order to study the origin of the by-product peak we applied the same derivatization procedure to a lactate standard solution




**Reaction 2.** Hydrolysis of the lactate derivative in the basic reaction medium.

prepared in ACN (Fig. 1d). The fluorescence chromatogram showed only the peaks of the 9-CMA excess and of the lactate derivative, and the area of the lactate derivative peak was double with respect to that of the derivative obtained from derivatization in an aqueous solution. These results supported the hypothesis that the peak at 7.7 min corresponds to a by-product of the decomposition reaction of the lactate derivative. The lactate derivative is indeed an ester which may hydrolyze, during the heating step, to 9-hydroxymethyl anthtracene (9-OHMA) and lactate (Reaction 2) in the basic reaction medium. This hypothesis was confirmed by the analysis with HPLC-ESI-Q-ToF (see below).

The derivatization yields of aqueous lactate standard solution and of lactate in ACN were investigated by determining the not derivatized lactate in the reaction medium using the direct method of determination of lactate [1]. We found that the yield ranged between 70% and 88% when lactate was dissolved in water and between 85% and 97% when lactic acid was dissolved in ACN.

All these results confirmed that the lactate from an aqueous solution can be derivatized and the extraction step can be bypassed.

### 3.1.2. *Effect of the reaction time*

We studied the effect of reaction time on the derivatization reaction of 0.5 mM lactate standard solution with 9-CMA at 75 °C [29]. The reaction reached a *plateau* after about 2.5 h (see Fig. S1 of the Supplementary Material). The same products were obtained also at room temperature, but the *plateau* was reached after about 20 h.

### 3.1.3. *Application of the central composite design for the optimization of temperature and reaction time*

We performed a central composite design with two factors (temperature and time) at five levels for a total number of 13 experiments. All the experiments were performed on a 0.5 mM lactate standard solution and on a blank solution, since the blank chromatogram evidenced the presence of a small peak eluting at the retention time of the lactate derivative. The concentrations of the reagents were kept constant: 1 mM 9-CMA, 16.2 mM TBAB and 10.8 mM TEA in ACN [29].

Figs. 2A and B shows the response surfaces obtained by plotting the areas of the lactate derivative and of the unknown peak detected in the blank integrated on fluorescence chromatograms ($\lambda_{ex}$=365 nm, $\lambda_{em}$=410 nm), as a function of the reaction time and temperature. The response surfaces were obtained with Origin software by converting the raw data into a 100 × 100 matrix using a *Random (Renka Cline)* gridding method. The response surfaces were calculated for both the lactate derivative (A) and the unknown peak detected in the blank (B).

The signal of the lactate derivative increases as the time and temperature increases. However, for temperatures higher than 80 °C, several new peaks appear in the chromatogram indicating the massive formation of by-products/degradation products. We selected 70 °C and 30 min reaction time as the best derivatization conditions, which provide the maximum signal of the analyte,

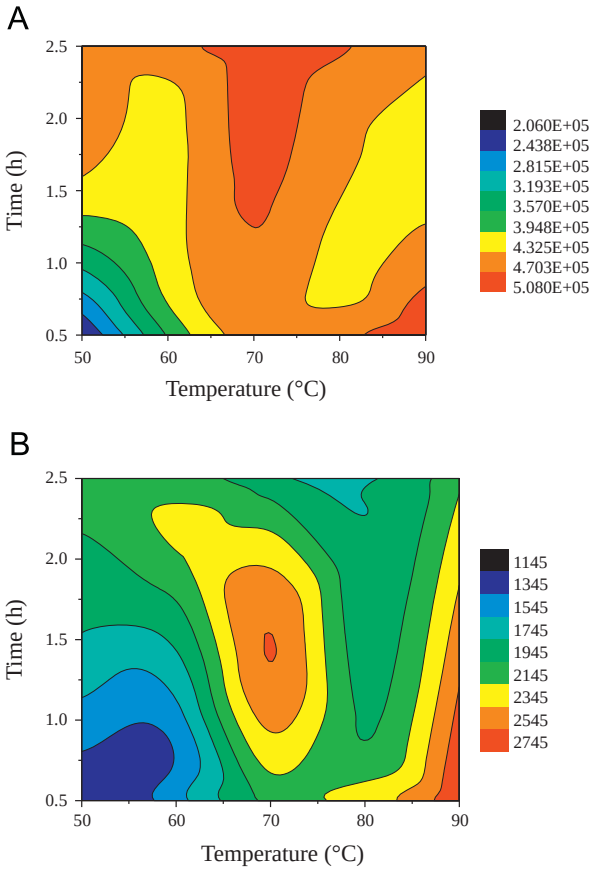



**Fig. 2.** Response surfaces obtained from the experimental design (factors: temperature and reaction time) of a 0.5 mM lactate standard solution (A) and of blank solution (B). The used concentrations were: 1 mM 9-CMA, 16.2 mM TBAB, 10.8 mM TEA. Chromatographic conditions: see Section 2.4 (Method 1).

while minimizing both the intensity of the unknown peak detected in the blank, and the formation of by products.

### 3.1.4. *Optimization of the amount of 9-CMA*

We studied the effect of 9-CMA excess on the derivatization yield.

A 0.5 mM lactate standard solution was derivatized for 30 min at 70 °C with 9-CMA by varying the 9-CMA/lactate ratio in the range 1–10. The signal reached a maximum for a 9-CMA/lactate ratio higher than 5 (see Figure S2 of the Supplementary Material).

However, we chose to operate at the maximum concentration of 9-CMA compatible with its solubility in ACN (saturated stock solution) in order to get the maximum dynamic linear range in the lactate determination, which corresponded to 7.4 mM 9-CMA in the reaction medium.

## 3.2. *Optimization of fluorescence and absorbance detection*

The influence of the excitation wavelength on the fluorescence detection at 410 nm of the lactate derivative was investigated. As the UV spectrum of 9-CMA has two characteristic absorptions maxima at 256 and 365 nm (see Fig. S3 and S4 of the Supplementary Material), both these wavelengths can be used as excitation frequencies. The excitation at 256 nm leads to a higher sensitivity, but it is a unspecific wavelength. Thus, the excitation at 365 nm was selected in order to guarantee the best selectivity.

The determination of lactate derivative can be achieved also in UV at 365 nm.

Table 1 summarizes the optimal operating conditions for the derivatization and chromatographic determination of lactate.

### 3.3. Optimization of the chromatographic conditions for the simultaneous determination of lactate and creatinine

In the case of urine samples, the monitoring of lactate for clinical test requires a normalization of the lactate level with respect to the amount of urinary creatinine in order to compare the results from different samples. Clinical determination of urinary creatinine is generally performed by the colorimetric determination of creatinine *(Jaffé method)* [39].

Several authors proposed the simultaneous determination of creatinine and other analytes in urine samples by HPLC-DAD [40–43]. Creatinine has, indeed, a characteristic absorption at 234 nm and elutes in a RP column at high percentages of water in the mobile phase [41,43].

**Table 1**
Summary of the optimal derivatization conditions of lactate.

| Derivatization conditions | |
|---|---|
| Reaction temperature | 70 °C |
| Reaction time | 0.5 h |
| [9-CMA] | 7.4 mM |
| [TBAB] | 16.2 mM |
| [TEA] | 10.8 mM |

**Table 2**
Analytical figures of merit for the determination of lactate derivative and creatinine.

| Parameter | Lactate | | Creatinine |
|---|---|---|---|
| | 410 nm | 365 nm | 234 nm |
| *Linearity* | | | |
| Range (mM) | $2\times10^{-4}$–$3\times10^{-2}$ | $2\times10^{-4}$–$3\times10^{-2}$ | $3\times10^{-4}$–$3\times10^{-1}$ |
| Slope[a] | $(3.3\pm0.1)\times10^{7}$ | $(4.4\pm0.3)\times10^{7}$ | $(4.1\pm0.3)\times10^{7}$ |
| $R^2$ | 1.00 | 0.998 | 0.999 |
| *Intra-day repeatability*[b] | | | |
| LQC (0.0003 mM), % | 5 | 5 | 7 |
| MQC (0.0010 mM), % | 2 | 1 | 2 |
| HQC (0.0100 mM), % | 3 | 1 | 1 |
| *Inter-day repeatability*[b] | | | |
| LQC (0.0003 mM), % | 5 | 6 | 8 |
| MQC (0.0010 mM), % | 6 | 4 | 7 |
| HQC (0.0100 mM), % | 5 | 3 | 6 |
| *LOD (nM)*[c] | 50 | 60 | 90 |
| *LOQ (nM)*[d] | 170 | 200 | 300 |

[a] 95% confidence level ($n=7$).
[b] Expressed as RSD%, $n=3$ (intra-day), $n=9$ (inter-day).
[c] Calculated as 3 times the standard deviation of the blank divided by the calibration curve slope.
[d] Calculated as 10 times the standard deviation of the blank divided by the calibration curve slope.

A 0.02 mM creatinine standard solution was added to the reaction vial in our optimized conditions. An isocratic elution in 20% ACN followed by the isocratic elution in 65% ACN (see Method 2) allowed us to separate creatinine and to determine in one chromatographic run both creatinine eluting at 3.3 min (UV detection at 234 nm) and the lactate derivative eluting at 16.0 min (fluorescence detection: $\lambda_{ex}$ 365 nm, $\lambda_{em}$ 410 nm or UV detection at 365 nm).

### 3.4. Analytical figures of merit

#### 3.4.1. Linearity, repeatability and detection limits

Calibration was performed on the lactate derivative and creatinine. Standard solutions covering the concentration ranges $2\times10^{-4}$–$3\times10^{-2}$ mM (injected concentrations) for lactate derivative and $3\times10^{-4}$–$3\times10^{-1}$ mM (injected concentrations) for creatinine were employed as calibration samples. Calibration was carried out monthly and a new stock solution of 9-CMA was prepared each time. Calibration curves for the lactate derivative and creatinine were obtained by linear regression analysis of the peak area plotted against the nominal analyte concentration (Table 2). The calibration curves were reproducible over 5 months (investigated time) with a confidence level of 95%.

The quality control standard solutions were analyzed in triplicate every day for three days in order to evaluate the intra-day and inter-day repeatability.

#### 3.4.2. Selectivity and stability

The selectivity of the method was assessed by derivatizing several organic acids that can be found in the biological matrices analyzed, i.e. propionic, acetic, butyric and pyruvic acids. We observed the formation of the pyruvate derivative only. However, it does not represent an interference for the determination of lactate because it elutes at 5.6 min. The lactate derivative was found to be stable for 24 h at 4 °C.

### 3.5. Characterization of the derivatization products by HPLC-ESI-Q-ToF

The optimized method was transferred to an HPLC-ESI-Q-ToF in order to characterize the derivatization products. The mass spectrometric conditions were optimized to find the best ionization conditions. The fragmentation of the anthracene derivatives leads to an unspecific fragment ($m/z$ 191.086),[44] which corresponds to the anthrylmethyl cation. Thus, the fragmentor (declustering potential) and capillary voltages were chosen in order to produce the highest abundance of the parent ions and a reduced fragmentation, resulting in a higher intensity of the analytes diagnostic masses (pseudomolecular ions). In particular, the fragmentor potential was set at 70 V or 100 V in order to yield the highest abundance of different pseudomolecular ions. Moreover, the mobile phase was modified by adding 1% of formic acid in order to increase the protonation of the analytes in the ESI source. The interpretation of the mass spectra was based on the determination of the exact mass (mass accuracy $<2$ ppm) and, thus, of the raw

**Table 3**
Peak attribution on the basis of the mass spectra obtained in the optimized conditions (see Method 3).

| $t_R$ (min) | Compound | MW | $m/z$ |
|---|---|---|---|
| 0.5 | Creatinine | 113.118 | 136.057 $[M+Na]^+$, 114.066 $[M+H]^+$ |
| 4.4 | 9-TEA-MA | 339.435 | 362.172 $[M+Na]^+$, 340.191 $[M+H]^+$, 191.086 [M-TEA]$^+$ |
| 5.5 | 9-OHMA | 208.260 | 247.058 $[M+K]^+$, 231.078 $[M+Na]^+$, 208.088 $[M]^{\bullet+}$, 191.086 $[M-H_2O]^+$ |
| 5.9 | Lactate derivative | 280.323 | 583.208 $[2M+Na]^+$, 319.074 $[M+K]^+$, 303.084 $[M+Na]^+$, 280.109 $[M]^{\bullet+}$, 191.086 $[M-C_3H_6O_3]^+$ |

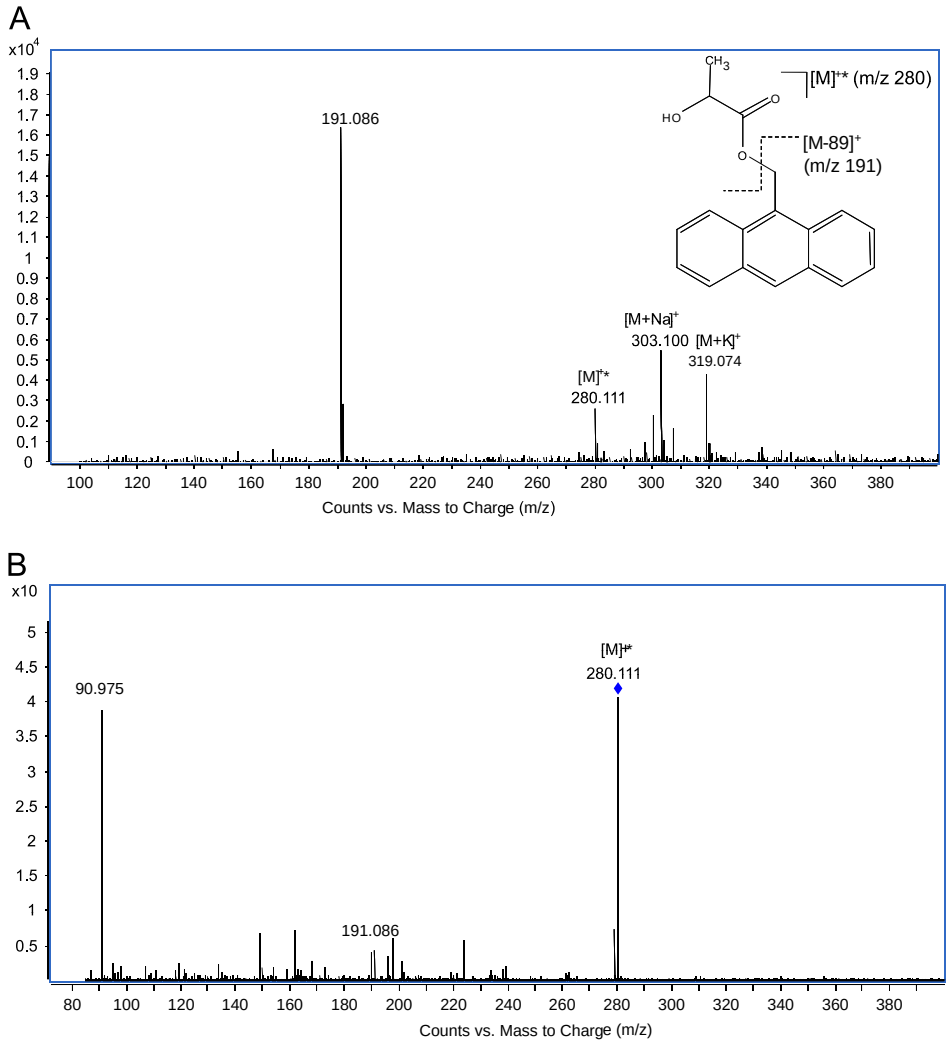



**Fig. 3.** (A) Mass spectrum of the lactate derivative with the fragmentor potential set at 100 V. (B) Target MS/MS spectrum of *m/z* 280.1 of the lactate derivative mass spectrum with the fragmentor potential set at 70 V and collision energy at 20 V.

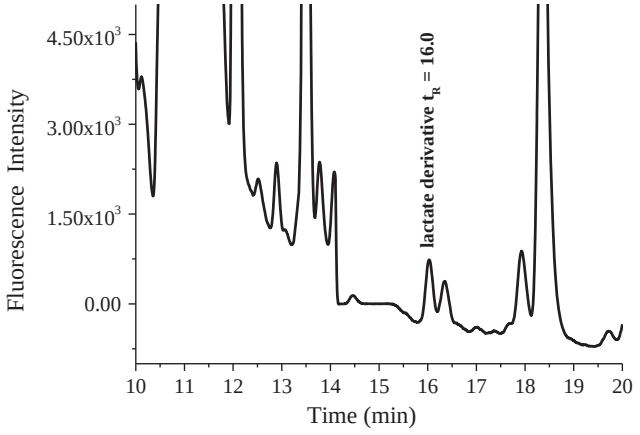



**Fig. 4.** Inset between 10 and 20 min of the fluorescence chromatogram at 410 nm ($\lambda_{ex}$=365 nm) of a urine sample diluted 50 times in ACN derivatized with 9-CMA. Chromatographic conditions: see Section 2.4 Method 2.

formula of the analytes and on the interpretation of the fragmentation patterns.

Table 3 summarizes the peaks attribution on the basis of the mass spectra obtained in the optimized conditions (see Method 3).

**Table 4**
Fitting parameters of the analytical addition method curves obtained on a pooled urine and saliva sample for fluorescence detection at 410 nm ($\lambda_{ex}$=365 nm) and UV detection at 365 nm.

| Parameter | Urine | | Saliva | |
|---|---|---|---|---|
| | FD 410 nm | UV 365 nm | FD 410 nm | UV 365 nm |
| Slope[a] | $(2.3 \pm 0.1) \times 10^7$ | $(3.2 \pm 0.1) \times 10^7$ | $(3.3 \pm 0.1) \times 10^7$ | $(4.5 \pm 0.2) \times 10^7$ |
| $R^2$ | 0.999 | 0.999 | 0.999 | 0.999 |

[a] 95% confidence interval ($n$=5).

The mass spectra of the identified compounds are reported in Fig. S5 and S6 of the Supplementary Material.

The results showed the formation of an adduct between TEA and 9-CMA (9-TEA-MA) and the formation of the 9-OHMA, indicating that the lactate derivative is partially hydrolyzed in the reaction medium.

The lactate derivative was characterized by target MS/MS experiments on the main masses of its mass spectrum (Fig. 3A). In the MS/MS spectrum of the *m/z* 280.1 (Fig. 3B) the presence of the *m/z* 91 (corresponding to the protonated lactic acid) confirmed that the ion at *m/z* 280.109 contains a structure whose mass corresponds to that of lactate. This result confirms our attribution

**Table 5**
Comparison of LODs and dynamic linear ranges of our method and other reported in the literature.

| FL reagent | Matrix | LOD (pmol) | Dynamic linear range (mM) | Detector | Ref. |
|---|---|---|---|---|---|
| (+)-FLEC | – | – | – | UV | [25] |
| DBD-PZ | Rat serum | 0.300[a] | 0.01–20 | FL | [26] |
| NBD-PZ | Rat urine | 0.050[a] | – | FL | [21] |
| | Human serum | 25[a] | 1–10 | FL | [27] |
| NBD-PZ-Val | Human plasma | 750[b] | 0.5–4 | FL | [17] |
| α-BAP | Human plasma | 36[a] | 0.18–6 | UV | [28] |
| 2-DBAP | Body tissues | 0.034[a] | – | UV | [29] |
| PNBDI | Table olives | 1.5[a] | 0.001–0.011 | UV | [31] |
| | Fruit juices | 0.68[a] | 0.00014–0.0054 | UV | [30] |
| 9-CMA | Human urine and saliva | 1[b] | 0.0002–0.03 | FL, UV | this work |

[a] Calculated as 3 times the signal to noise ratio (S/$N$=3).
[b] Calculated as 3 times the standard deviation of the blank divided by the calibration curve slope.

of the $m/z$ 280.109 to the $[M]^{\bullet+}$ of the lactate derivative. It has to be stressed that for $m/z$ lower than 100, the resolution of the mass spectrometer is lower than for higher masses.

### 3.6. Application to urine samples

The optimized method was applied to urine samples.

Fig. 4 shows a representative fluorescence chromatogram of a urine sample diluted 50 times in ACN obtained in the optimized conditions.

The analytical addition method was applied to urine samples to study the matrix effect; the effect of the 9-CMA excess in a biological sample was also evaluated. Calibration curves were performed at three levels of urine dilution (1:10, 1:25 and 1:50) and for each dilution level, three different concentrations of 9-CMA were employed. We observed a significant matrix effect on the 1:10 and 1:25 dilution levels (for details see Figure S7 of the Supplementary Material). Hence we chose to dilute the urine sample 50 times with the maximum 9-CMA concentration (7.4 mM). The analytical addition method slopes in the optimized conditions in FD and UV detection were about 70% of the external calibration curve slope (see Table 2).

No matrix effect was observed in the determination of creatinine. The average slope in the analytical addition method was not significantly different (95% confidence level) from the slope of the external calibration curve. Thus, the concentration of creatinine was calculated by using the external calibration curve.

Table 4 summarizes the fitting parameters of the analytical addition method curves obtained on a pooled urine sample. Intra-day repeatability was 1–4% for fluorescence detection and 1–5% for UV detection. Intra-day recoveries for fluorescence and UV detection were 92–105% and 98–106%, respectively.

### 3.7. Application to saliva samples

The optimized method was successfully applied to saliva samples.

A pooled saliva sample was diluted 50 times (as urine samples) and the analytical addition method was carried out in order to study the possible matrix effect. The analytical addition method slope was not significantly different from the external calibration slope at a 95% confidence level (see Table 2) indicating that no matrix effect occurs.

Table 4 summarizes the fitting parameters of the analytical addition method curves obtained on a pooled urine sample.

Intra-day repeatability was 1–2% for fluorescence detection and 1–3% for UV detection. Intra-day recoveries for fluorescence and UV detection were 101–108% and 104–109%, respectively.

### 3.8. Comparison with other methods

In summary, the proposed method has the advantage of determining the lactate using a fast (30 min) derivatization reaction in ACN, avoiding the extraction in organic solvents and long sample handling procedures. Table 5 reports the comparison of LODs and dynamic linear ranges of our method and other methods reported in the literature.

Many of the methods reported in Table 5 discriminate between L-lactate and D-lactate, which is fundamental in the analysis of pathological samples. In this work this discrimination was not necessary because in healthy conditions the amount of D-Lactate is neglectable. However, as 9-CMA reacts with both L- and D-lactate, the method proposed can be potentially applied to the separation of the two enantiomers once a chiral HPLC column is used.

## 4. Conclusions

In summary, we developed a novel and straightforward derivatization method for the determination of lactate by HPLC-DAD/FD. The derivatization of lactate was mandatory to achieve the selectivity and sensibility required for the application of the method to complex matrices. The novelty of this work is the derivatization of lactate with 9-CMA from an aqueous solution. The derivatization reaction is performed in ACN, it occurs also in the presence of few percent of water, thus avoiding the extraction of the analyte in organic solvents and long sample handling procedures. The derivatization procedure was optimized and the method was evaluated for its linearity, precision and selectivity. The method was successfully applied and validated for the analysis of urine and saliva samples. This is also the first method that allows the simultaneous determination of lactate and creatinine.

The optimized method is fast, simple and competitive with the other reported RP-HPLC methods. It can be potentially useful to monitor the lactate levels in athletes during training and in patients with lactic acidosis.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.07.015.

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