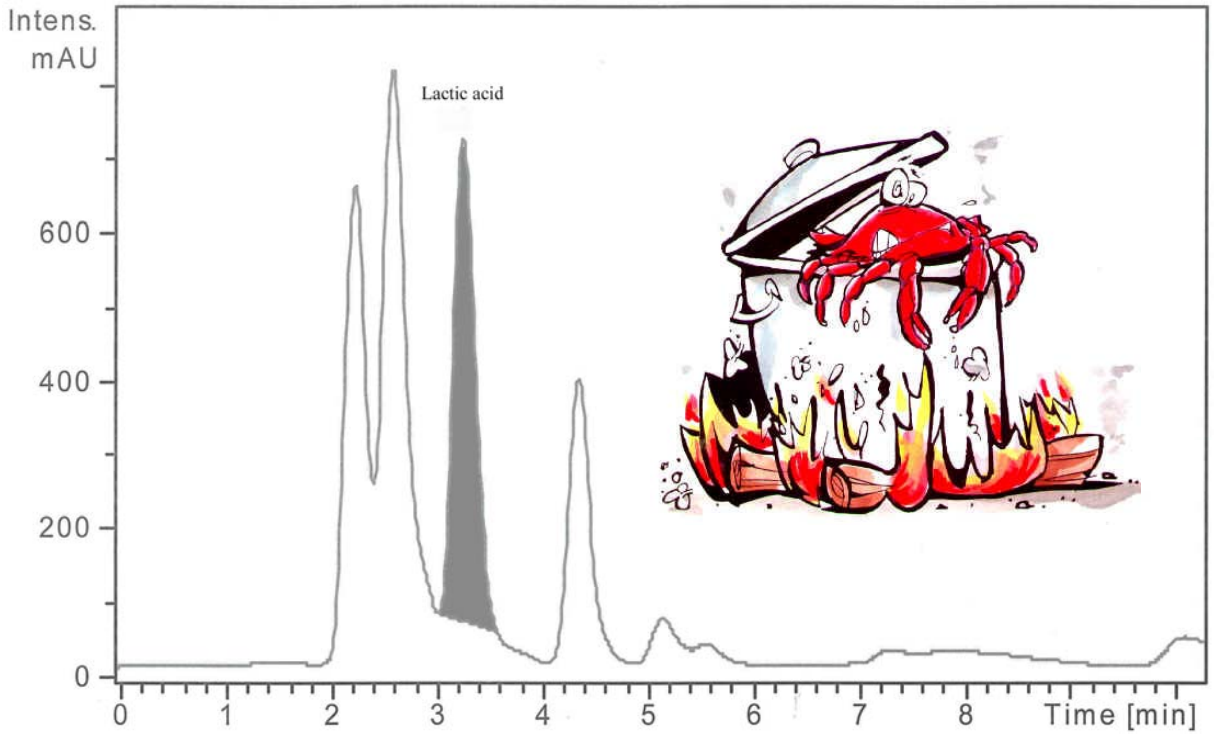
Report Å0610

A simple and rapid high performance liquid chromatography method for determination of lactic acid in blood serum from edible crab (*Cancer pagurus*).



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Ålesund, November 2006

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REPORT

Title: A simple and rapid high performance liquid chromatography method for determination of lactic acid in blood serum from edible crab (*Cancer pagurus*).	**Report nr.:** Å0610
Project financed by (name and adress): The Research Council of Norway Stensberggt. 27 Box 2700, St. Hanshaugen N-0131 OSLO	**Pages:** 15 **Reference collaborater:** 150202/510
Phone./Fax.: +47 22 03 70 00 + 47 22 03 70 01 (fax)	**Date:** 03.11.2006
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Summary:
In the present study, a simple and rapid HPLC procedure has been developed for determination of a wide range of lactic acid concentrations in blood serum from edible crab (*Cancer pagurus*). The lactic acid concentration is used as one of several stress indicators in edible crab.

During the lactic acid analysis, the analytic column, Ace 5 C18, was operating at 20ºC. The mobile phase, 10 mM KH_2PO_4:acetonitrile (99:1), pH 2.5, was delivered isocratic, at a flow rate of 0.2 ml/min, in a 10 min run. Detection and identification were performed using a photodiode array detector at 220 nm. Detection limits were found in the range of 0.02 mM (s/n=8) per 10 µl sample injected, while linearity was measured up to 118 mM. Statistical evaluation of the method was examined performing intra-day (n=10) (VC% = 0.3-3.3%) and inter-day (n=5) (VC% = 0.0-1.3%) calibration and was found to give high accuracy and precision results.

Blood serum samples previously analysed by Vitros 950 chemistry station, were analysed by the developed HPLC method (r=0.94)

Keywords:
HPLC, lactic acid, haemolymph.

Distribution:
Closed

PREFACE

This report is a part of the ongoing project “Increased value from marine resources using new technology” which has been a 5 year strategic research project at Møreforsking. The project is supported by the Research Council of Norway.

Wenche Emblem Larssen at Møre Research is gratefully acknowledged for skilful laboratory work. Inge Fossen at Møre Research is gratefully acknowledged for critical review of the manuscript and good help with statistics.

Ålesund, November 2006

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Jannicke Fugledal Remme

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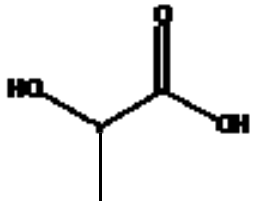
1 INTRODUCTION

Lactic acid (α-hydroxypropanoic acid) is a chemical compound that plays a role in several biochemical processes. It is well known that it accumulates in muscles during anaerobic exercise due to lack of oxygen (inadequate oxygen supply). Lactic acid accumulation during emersion and associated physiological and haemocyte-related effects, have been identified as a major correlate of mortality for some crustaceans [1], including edible crab.

When relating the physiological state of an animal to the environmental conditions, it is an advantage to have a quick and reliable method for the physiological measurements. A number of different analytical methods are used for determination of lactic acid [2,3,4]. A frequently used enzymatic method for lactic acid determination (Vitros 950 chemical station) is based on its reaction with β-NAD^+ in the presence of lactate dehydrogenase to produce pyruvate and NADH [5]. NADH concentration is then monitored colometrically (540 nm) by UV detection. This method is, however, developed for human lactic acid levels, which are low compared to the concentrations that may occur in crustaceans. A reliable method, with wide linearity, was therefore desirable.



lactic acid

For commercial exploited aquatic crustacean, oxygen consumption can become limited during holding and transport, due to emersion [6] or as a result of exposure to reduced oxygen levels in the holding water [7,8]. A switch to anaerobic pathways of energy production then occur, causing changes to the blood biochemical composition and acid-base balance [9,10,11]. In particular, an increased utilization of carbohydrate and lactic acid production may be observed followed by the onset of blood acidosis.

The lactic acid concentration in edible crab (*Cancer pagurus*), in normoxic seawater, is about 1.5 mM [12]. Lactic acid concentrations at 11.1 mM [13] and above 20 mM [14] have been reported after dry transport to market and after 6.5 h emersion, on deck, respectively. However, the temperatures during emersion were not given in these references. The increase in lactic acid concentration seems to depend both on time exposed to air, the air temperature and humidity [15,16,17,18].

Lactic acid concentration in the range of 50 to 60 mM have been observed for crabs exposed to air, over night, at temperatures between 14-17°C [19], a practice that occur in the Norwegian crab fishery.

The individual variation in the lactic acid concentration is large in the crab haemolymph, and therefore, a large number of samples have to be analysed. An easy and quick method is therefore required. An easy and quick HPLC method, which enables lactic acid measurement over a wide range of concentrations, has therefore been developed.

2 MATERIALS AND METHODS

2.1 Instrumentation and chemicals

An Elite LaChrom (Hitachi, USA) liquid chromatograph consisting of a system controller, binary pump (L-2130), autoinjector (L-2200), column oven (L-2300) and photodiode array detector (DAD) (L2456) was used. All solvents used were of HPLC grade. Acetonitrile, KH_2PO_4 and lactic acid were purchased from Sigma (USA).

2.2 Chromatographic conditions

For the determination of lactic acid, an ACE 5 C18 (150 mm x 2.1 mm) column (ACT, Scotland) at 20 ºC was used. The mobile phase was 10 mM KH_2PO_4: Acetonitrile (99:1), pH 2.5, at a flow rate of 0.2 ml/min. Samples and standards (10 µl) were injected using an autoinjector. Lactic acid was detected with a DAD at 220 nm. The lactic acid standards were diluted in dH_2O. A stock solution of 118 mM was made, and standards (n=30) were prepared from the stock solution. All solutions were stored at room temperature. The chromatographic conditions for lactic acid were chosen in terms of peak shape, chromatographic analysis time, selectivity and resolution.

2.3 Method validation

The method described has been validated with respect to accuracy, within-day and between-day precision, linearity and sensitivity. Linearity was studied by a series of standards, covering the entire work range; each solution was injected three times. Regression analysis revealed calibration equations with the respective correlation coefficient. The detection limits are considered to be the quantities that are producing a signal of peak height eight times the size of the background noise.

2.4 Sample preparation

Edible crabs were caught in traps at Lepsøy, Norway, stacked in boxes on deck and transported to the laboratory facility at Møre Research Ålesund, on the northwest coast of Norway. After 48 hours revitalisation in seawater at ambient temperature, groups of crabs were exposed to air at different temperatures. Samples of haemolymph were collected consecutively every 6^{th} hour from the base of the 3^{rd} or 4^{th} walking leg as described by Danford [8].

The blood was immediately transferred to centrifuge tubes with glucose inhibitors and mixed before centrifugation at 6000 rpm (Eppendorf, Centrifuge 5810 R) for 5 min. Supernatants (serum) were collected and frozen. Serum samples for HPLC analysis were prepared according to a method by Ichihara et al [20]. Hundred microliters of thawed serum was mixed with 900 µl ethanol. The solution was vortex-mixed vigorously and centrifuged at 10 000 rpm for 10 min. After 900 µl of the supernatant was evaporated to dryness in a vacuum-centrifuge (Eppendorf, Concentrator 5301), 90 µl dH_2O was added to the residue and vortexed. The lactic acid concentration (10 µl sample) was determined in triplicates. To study the stability of lactic acid in serum, samples were frozen and thawed (3x) before analysis.

2.5 Vitros 950 chemistry system versus HPLC

Based on the lactic acid concentrations previously obtained by Vitros 950 chemistry system, 19 haemolymph samples, in the concentration range from 3 mM to 74 mM were also selected for analysis by HPLC.

2.6 Statistical analysis

The mean value and standard deviation for each component analysed were calculated and reported. All samples were analysed in triplicates. Coefficient of variation (CV%) is calculated as (standard deviation x 100)/mean. The calibration curve was obtained by linear regression analysis of the peak area versus the lactic acid concentration. Data from the freeze-thaw cycles were subjected to t-tests (Microsoft Excel) to determine differences in concentration between cycles, whereas the lactic acid concentrations from the Vitros 950 chemical station and HPLC were subjected to Wilcoxon Signed Ranks Test, Systat, to determine differences in concentration between the two methods.

3 RESULTS AND DISCUSSION

The described method achieves separation of lactic acid from blood serum in 10 minutes, where lactic acid has a retention time of 3.3 min (figure 1). The lactic acid peaks in blood serum were identified by comparison of retention times. The lactic acid peak was also confirmed by mass spectrometry (MS).

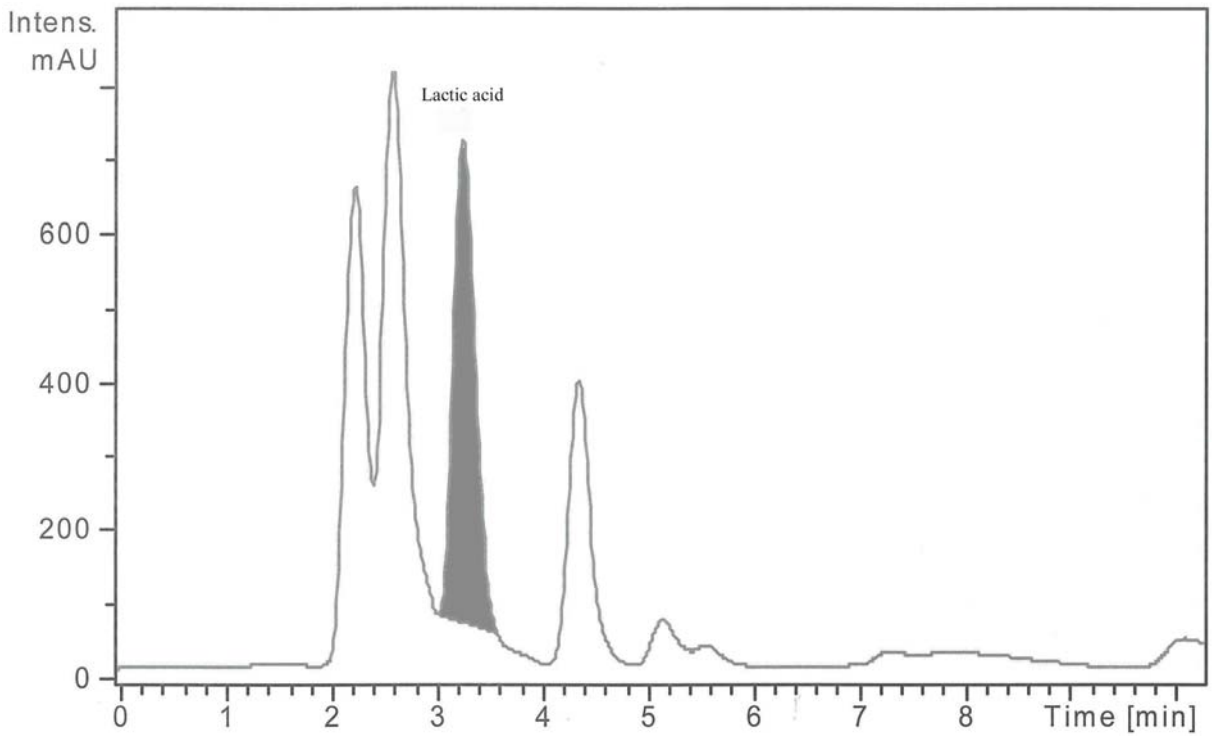


Figure 1: Chromatogram of lactic acid (retention time 3.3 min) in blood serum from edible crab.

The calibration curve was linear up to the highest concentration measured, 118 mM (y=0.01x, n=30, r^2 = 0.999). The lowest concentration analysed, 0.02 mM, had a signal to noise ratio of 8.

The precision of the method based on within-day repeatability was assessed by replicate injections of three standard solutions covering different concentration levels, low, medium and high. The results (Table 1) show a high repeatability for all concentrations. The reproducibility (day-to-day variation) of the method was established using the same concentration level as above. Determination of each concentration was conducted during a period of 5 days. During this period the concentration was determined to 108.1 ± 1.21 mM, 11.4 ± 0.11 mM and 1.2 ± 0.02 mM. The coefficient of variation was calculated to 1.1 %, 1.0 % and 1.6 %, respectively. However, without affecting the concentration, there were visible changes in the chromatograms at the lowest concentration (Figure 2), where it seems that the peak at 2 min increases during storage. These changes were not visible for higher concentrations of lactic acid.

Table 1: The concentration of three lactic acid standards, at high, medium and low concentration, analysed within-day (n=10) and between-day (n=5).

Day	*Standard 1*	*CV (%)*[1]	*Standard 2*	*CV (%)*[1]	*Standard 3*	*CV (%)*[1]
1	106.5±3.48	3.3	11.2±0.08	0.8	1.2±0.02	2.0
2	107.4±0.37	0.3	11.4±0.12	1.0	1.2±0.02	1.6
3	109.2±0.80	0.7	11.4±0.12	1.1	1.2±0.02	1.7
4	109.9±0.62	0.6	11.4±0.12	1.1	1.2±0.01	1.5
5	107.4±0.80	0.7	11.2±0.11	1.0	1.2±0.01	1.3

[1] CV(%) = Standard deviation*100/mean

Analysis of lactic acid during three freeze-thaw cycles of blood serum resulted in no significant differences in analyte concentration. Hallstrøm et al [21] estimated that samples with lactic acid can be stored for at least a week at -20ºC. However, our results showed that the concentration of lactic acid was stabile for at least one month. Time in the autosampler did not impact the accuracy and precision of the analysis (data not shown).

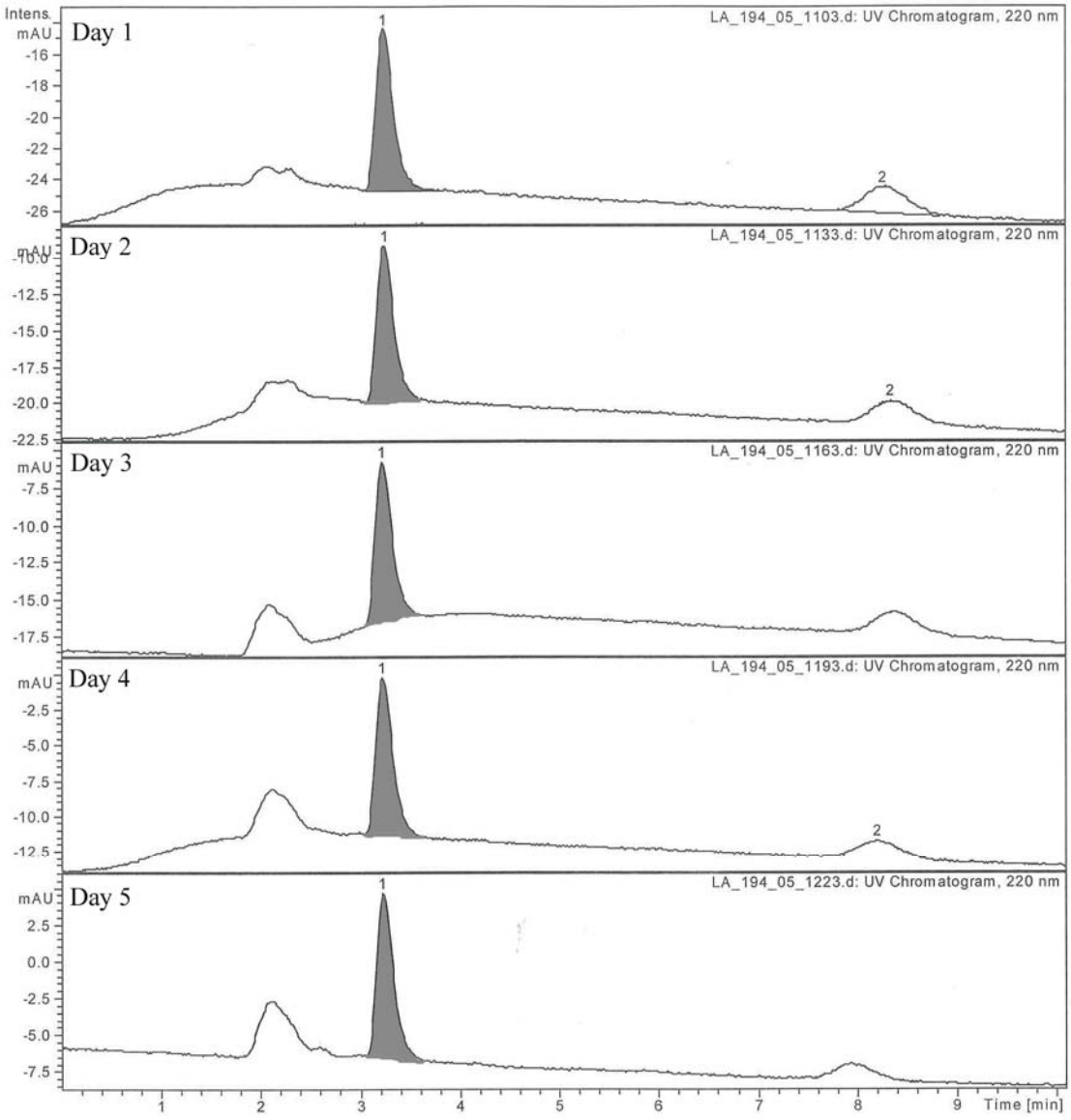


Figure 2: Chromatograms of a lactic acid (1.2 mM) standard over a period of 5 days.

3.1 Vitros 950 chemistry system versus HPLC

Several edible crab blood serum samples were analysed by both HPLC and Vitros 950 chemistry station, to compare the concentrations obtained with the two different methods. No significant differences were found. The results are shown in figure 3, and show a high correlation factor (r=0.94) for the lactic acid concentrations. Samples with high lactic acid concentration have to be diluted when analysed by Vitros 950 chemical station, and this may contribute to errors.

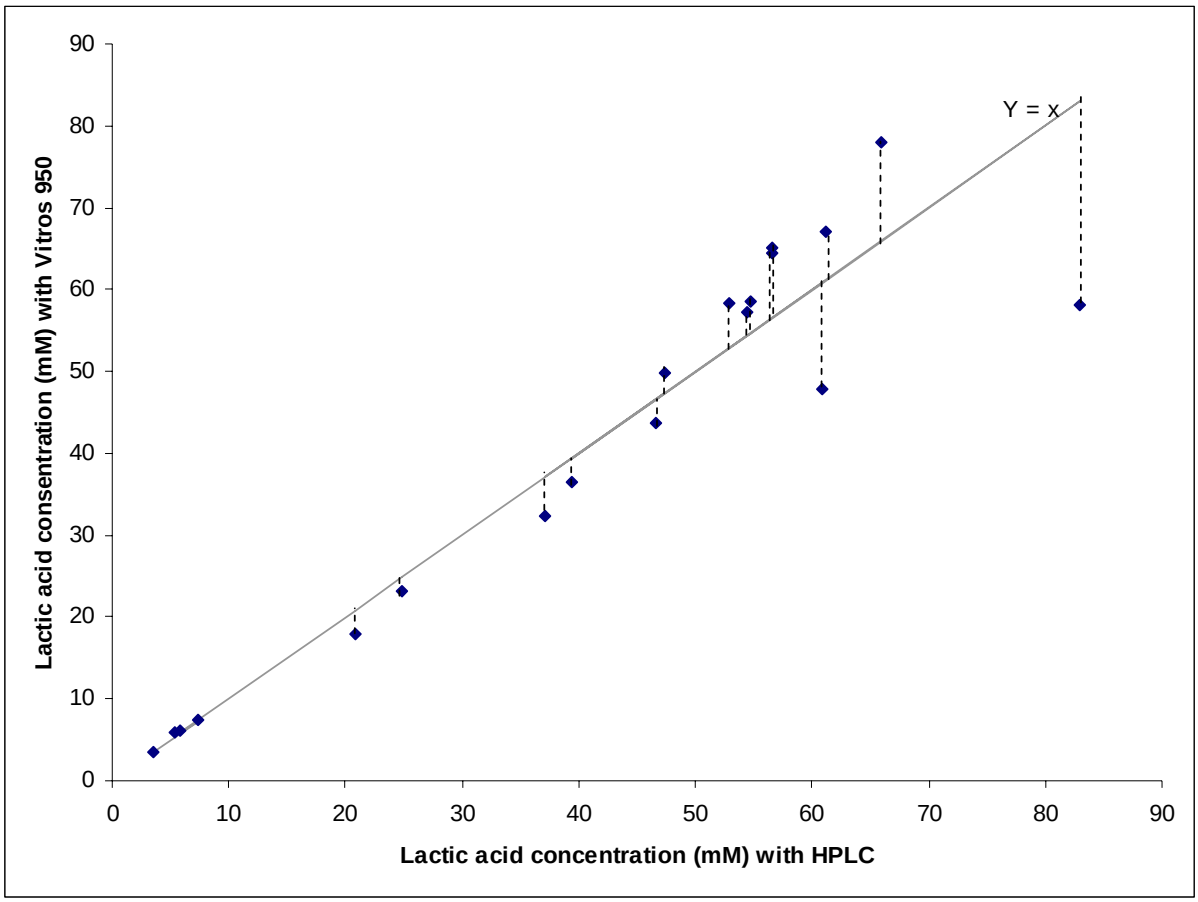


Figure 3: *The correlation of lactic acid concentration (mM) analysed by HPLC and Vitros 950 (y=0,99x, n=19, r^2=0,89). The broken lines show the difference between the obtained lactic acid concentrations by HPLC and Vitros 950 analysis.*

4 CONCLUSION

In the present study, a method for determination of lactic acid in serum is presented. Detection limits, good sensitivity and linearity and short analysis time, combined with the simplicity of the procedure, should make this method a useful tool for the determination of lactic acid in blood serum.

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