Lactate determination with ferric ions in biological liquids is restricted to high concentrations or samples with controlled composition

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Abstract

Lactic acid enantiomers, both L- and D-, are markers that often need to be controlled in such areas as medicine, food industry and related microbiological research. Besides the enzymatic methods for highly selective and stereospecific lactate determination, simpler alternatives with lower selectivity have been proposed. The spectrophotometric method involving ferric, i. e. iron(III), ions forming complexes with lactate has recently become popular for measurement of lactic acid in complex biological samples, although it has never been tested for selectivity under various conditions. Here we estimated the influence of some other common metabolites on readout of the method and showed that identical concentrations of some carboxylic acids, such as pyruvate and citrate, produce very similar color reactions as lactate. Although amino acids demonstrated lower interference, their combined influence in biological fluids can also have a substantial effect on this analytical reaction. This method is often used for the study of microbiological culture media, and it returned higher lactate estimates specifically at low lactate concentrations in LB growth medium due to one of its components, yeast extract. Thus, this method for lactate measurement requires some *a priori* knowledge about the amounts of interfering substances in the tested biological mixtures for its correct application.

Keywords: growth medium, $FeCl_3$, ferric ions, lactate, lactic acidosis.

Introduction

Lactic acid is a chiral hydroxycarboxylic acid, existing as two enantiomers (L(+)-lactic acid and D(–)-lactic acid) or their racemic mixture (DL-lactic acid). L-lactate is metabolically produced by the overwhelming majority of multicellular organisms and microorganisms during glucose breakdown and is largely accumulated specifically under anaerobic conditions (Rabinowitz and Enerbäck, 2020). However, some microorganisms also possess D-lactate dehydrogenases besides common L-lactate dehydrogenase and are able to both metabolically oxidize these enantiomers to pyruvate or reduce them from pyruvate (Pohanka, 2020; Biryukova, Arinbasarova, and Medentsev, 2022). This is particularly relevant for mammalian intestinal microbial communities, which can produce significant amounts of D-lactate and are the main source of D-lactate in the organism of humans and livestock (Pohanka, 2020).

Both L-lactate and D-lactate are widely used as important markers in medical diagnostics, food production and in the other fields. In particular, lactic acidosis in human or animal bloodstream can be caused by either L- or D-enantiomer in a variety of medical conditions, including carbon monoxide poisoning, anemia, severe trauma, progressive heart failure, liver diseases, short bowel syndrome, sepsis and cancer (Ewaschuk, Naylor, and Zello, 2005; Kraut and Madias, 2014; Kowlgi and Chhabra, 2015; Nath, Kubendiran, Mukherjee, and Kundu, 2023). Lactate concentrations are also widely monitored in the food industry and related microbiological research in food preservation and production of wine and fermented milk products (Pundir, Narwal, and Batra, 2016). Specifically L-lactate is used as a marker of organismal stress in seafood farming (Rassaei et al., 2014) and in environmental research on aquatic animals (Sokolova et al., 2012; Vereshchagina et al., 2021). Therefore, all these industries have an urgent need for a reliable, fast and cost-effective method of lactate measurement.

Lactic acid concentration, both combined and individual enantiomers, can be measured by different methods, which fall into two main groups: non-enzymatic and enzymatic. The enzymatic methods are based on high specificity of lactate dehydrogenase (Hohorst, 1965) or lactate oxidase (Rosenstein, Tennent-Brown, and Hughes, 2018; Vereshchagina et al., 2021) towards lactate enantiomers and use spectrophotometry or spectrofluorometry for monitoring of lactate oxidation derivatives. Non-enzymatic methods often use various colored chemical reactions with lactic acid for further colorimetric analysis of obtained derivatives (Barker and Summerson, 1941; Ewaschuk, Zello, Naylor, and Brocks, 2002). Other non-enzymatic methods of lactate detection include the voltammetric method (Schmitt, Molitor, and Wu, 2012), proton nuclear magnetic resonance (Nishijima, Nishina, and Fujiwara, 1997) and gas chromatography or high-performance liquid chromatography (Omole et al., 1999; Ewaschuk, Zello, Naylor, and Brocks, 2002; Pundir, Narwal, and Batra, 2016). The merits of non-enzymatic colorimetric methods are relative simplicity and low cost of the analysis, which is usually accompanied by low specificity. On the contrary, enzymatic methods are sufficiently specific (including stereospecificity) and sensitive to be used for accurate lactate determination in multicomponent biological fluids, but are relatively complicated and expensive (Pundir, Narwal, and Batra, 2016).

Recently a non-enzymatic colorimetric method was suggested, which, according to the authors, is applicable for measurements of lactic acid levels in complex biological mixtures such as culture liquids and fermented dairy products (Borshchevskaya, Gordeeva, Kalinina, and Sineokii, 2016), and therefore became popular. As of 4th of June 2024, the numbers of citations of this method on major platforms such as Web of Science, CrossRef, and Scopus were 120, 122 and 136, respectively. The protocol has been widely used to measure lactic acid concentration in microbiological culture media (Msuya et al., 2018; Di Sotto et al., 2018; Vijayakumar and MuhilVannan, 2021; Afzaal et al., 2019; Tak et al., 2019; Guo et al., 2019; Bijle, Ekambaram, Lo, and Yiu, 2020; Karnaouri et al., 2020; Ismailov et al., 2020; Ong et al., 2020; Erkaya et al., 2020; Chasoy, Chairez, and Durán-Páramo, 2020; Thi Minh Thu, Thi Thanh Vinh, Anh Dung, and Hoang Khue Tu, 2021; Chang et al., 2021; Lovato et al., 2021; Ngouénam et al., 2021; Taser et al., 2021; Gandhi, 2021; Khumukcham et al., 2022; Sudhakar and Dharani, 2022; Vignesh Kumar et al., 2022; Chavarria et al., 2022; Amaraweera, Senevirathna, and Singhalage, 2022; Jamnik et al., 2022; Chawla and Goyal, 2022; Nguyen et al., 2022; Uwamahoro et al., 2022; Fentie et al., 2022; Tefara, Begna Jiru, and Bairu, 2022), as well as in waste fermentation products (Tan, Abbasiliasi, Lee, and Phapugrangkul, 2020; Islam, Mumtaz, and Hossen, 2020; Karnaouri et al., 2021; López-Salas et al., 2022), silage (Arreola et al., 2019; Kaewpila et al., 2020; Besharati, Palangi, Niazifar, and Nemati, 2020; Araiza-Rosales et al., 2021; Castellón-Zelaya and González-Martínez, 2021; Sarwono et al., 2022), yogurt (Ranjbar, Bolandi, and Mohammadi Nafchi, 2021), blood plasma (Mato Mofo, Essop, and Owira, 2020), saliva (Shtumpf et al., 2021), sweat (Kuswandi, Irsyad, and Puspaningtyas, 2023) and even brain samples (Krishnaswamy, Alugoju, and Periyasamy, 2020; Mocanu et al., 2022).

This method relies on the ability of lactic acid to form complexes with ferric, i.e. iron(III), ions with change in the solution color. However, according to the previously published data, iron(III) ions are capable of forming complexes not only with lactate, but also with other carboxylic acids, including the metabolites of glycolysis, the citric acid cycle and some amino acids (Hamm, Shul, and Grant, 1951; Hazell and Johnson, 1987; Abrahamson et al., 1994; Salovaara, Sandberg, and Andlid, 2002; 2003). Since these metabolites are common in biological samples, these data question the selectivity of the lactate determination with ferric ions and applicability of this method for lactate measurement in certain cases. So, in this study we tested the influence of these potentially interfering metabolites on the colorimetric method readout and compared its selectivity with the common enzymatic alternative for lactate determination in the media that are widely used for microbial cultivation.

Materials and methods

Reagents and equipment

In this study we used ~90 % L-lactic acid solution (AppliChem, Germany), sodium D-lactate ≥ 99 % (Sigma-Aldrich, USA); pyruvic acid sodium salt ≥ 99 % (Acros organics, USA); sodium chloride USP grade ≥ 99.5 % (Helicon, Russia), fermented peptone (DIA-M, Russia); yeast extract without salt, type D 95.2 % (DIA-M,

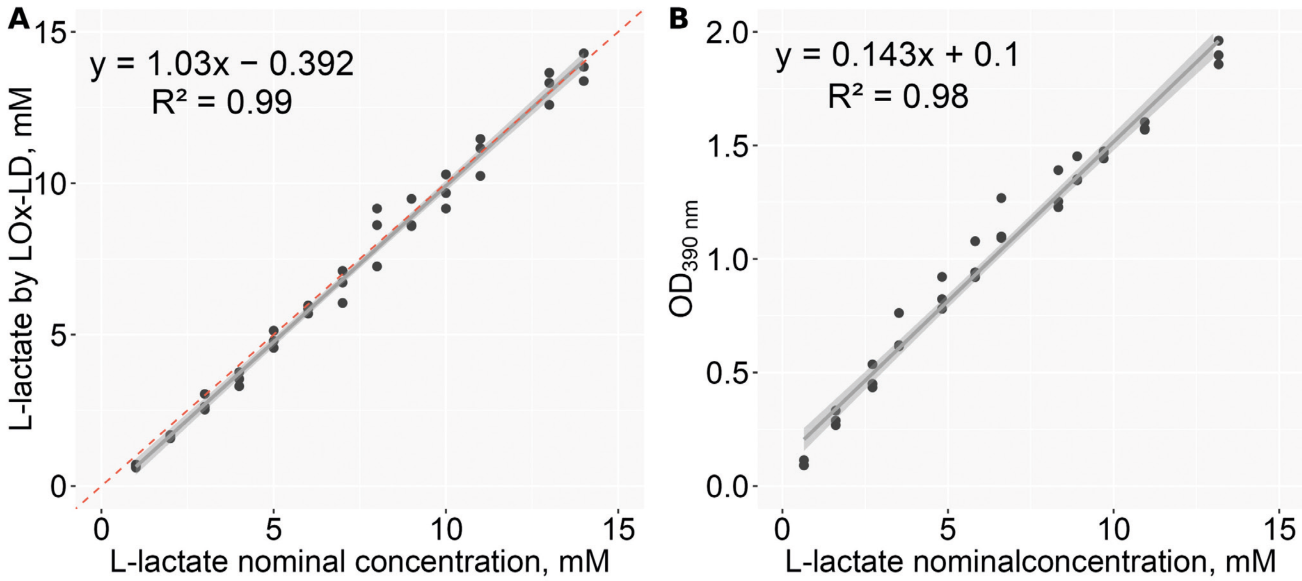

Fig. 1. Calibration of the FI-LD method. (A) Comparison of the nominal L-lactic acid concentrations and measurements by the enzymatic LOx-LD method. The orange dashed line indicates the theoretical ideal coincidence between the nominal and measured concentrations. (B) The calibration curve for the FI-LD method. The gray lines and lighter gray shades represent linear regressions and their confidence intervals; the dots are analytical replicates.

Russia); purum amino acids: threonine, valine, glutamic acid, leucine, isoleucine, alanine, methionine, glutamine, glycine, phenylalanine, arginine, lysine ("AO REACHIM", Russia); iron(III) chloride hexahydrate purified, and ddH_2O. In addition, we used a commercial kit "Lactate-Vital" (L-lactate oxidase 200 units/L; peroxidase 2000 units/L; tris-HCl 50 mM; 4-chlorophenol 6 mM; 4-aminoantipyrine 0.4 mM and bovine serum albumin 0.4%) for L-lactic acid measurement ("VITAL DEVELOPMENT CORPORATION" JSC, Russia). Spectrophotometric studies were performed using a cuvette spectrophotometer Cary 50 (Varian, USA) and a microplate reader CLARIOstar^{plus} (BMG Labtech, Germany).

Measurement of L-lactic acid concentration using the enzymatic spectrophotometric method

The enantiomer-specific lactate oxidase-based lactate determination (LOx-LD) was performed using the commercial kit according to the manufacturer's protocol with slight modifications. Spectrophotometric measurement was performed using the microplate reader. Thus, the experimental samples were added to the microplate well in a volume of 1 µl and diluted with prepared solution consisting of 200 units/L L-lactate oxidase; 2000 units/L peroxidase; 50 mM tris-HCl; 6 mM 4-chlorophenol; 0.4 mM 4-aminoantipyrine and 0.4% (weight/volume) bovine serum albumin in a volume of 100 µl. Then the mix was stirred and incubated for 5 minutes. After the incubation, absorbance measurements were conducted at the wavelength of 505 nm.

Measurement of lactic acid using the non- enzymatic spectrophotometric method with iron(III) ions

The lactic acid (D- and L-enantiomers) concentration was measured using the ferric ions-based lactate determination (FI-LD) method (Borshchevskaya, Gordeeva, Kalinina, and Sineokii, 2016). For this experiment, we added 100 µl of iron (III) chloride hexahydrate solution (22.2 mM) and 100 µl of the experimental sample to a plastic 96-well plate (impermeable to ultraviolet). The plate was then incubated for 5 minutes at 25 °C, following the proposed protocol (Borshchevskaya, Gordeeva, Kalinina, and Sineokii, 2016). After incubation, the absorbance at 390 nm was measured using the microplate reader CLARIOstar^{plus}.

To construct the calibration curve, 100 µl of 22.2 mM aqueous iron(III) chloride hexahydrate solution and 100 µl of 1 to 14 mM aqueous L-lactic acid solution in 1 mM increments were added to the microplate well. The blank sample contained 100 µl of iron(III) chloride hexahydrate solution and 100 µl of deionized water (Fig. 1B). In addition, the applied nominal concentrations of lactic acid solutions were verified using the LOx-LD approach, which confirmed the high accuracy of the prepared concentrations (Fig. 1A).

Experiments to evaluate the selectivity of the FI-LD method

Spectral analysis of 22.2 mM aqueous iron(III) chloride hexahydrate solution and 0.2–20 mM pyruvic and D-lactic acids was performed. Blank measurement was

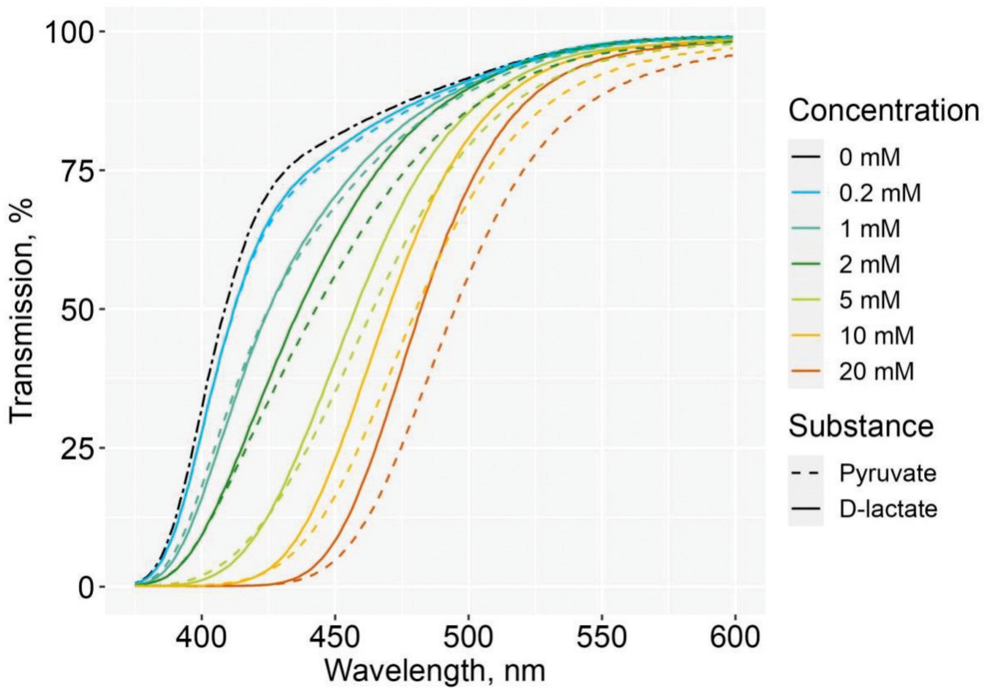

Fig. 2. Comparison of spectra of aqueous $FeCl_3$ solution mixed with D-lactate and pyruvate (from 0.2 to 20 mM). The concentration of 0 mM (black dash-dotted line) indicates the transmission of pure $FeCl_3$ solution.

achieved as follows: 500 μl of iron(III) chloride hexahydrate solution and 500 μl of deionized water were added to a quartz cuvette and the baseline was recorded. Experimental measurements were performed by mixing 500 μl of iron(III) chloride hexahydrate solution and 500 μl of an experimental sample that contained either pyruvic acid or lactic acid of known concentration. Spectral analysis was conducted using a quartz cuvette with a Cary 50 cuvette spectrophotometer within the wavelength range of 700 to 190 nm.

The effect of lysogeny broth (LB) as well as its components sodium chloride (2.5 g/L; in case of LB 5 g/L was used), yeast extract (2.5 g/L; in case of LB 5 g/L was used), fermented peptone (5 g/L; in case of LB 10 g/L was used) on the measured L-lactic acid concentration was evaluated. Before analysis, all solutions were autoclaved. Then, the LB medium or its components were mixed with lactic acid of known concentration (from 0 to 14 mM). The resulting mixes were used to measure the lactic acid concentration using the FI-LD and LOx-LD methods described above.

Data analysis

Data analysis was conducted in the R v4.2.2 programming environment (R Core Team, 2022). For data visualization, we utilized several packages including "ggplot2" (Wickham, 2016), "xlsx" (Dragulescu and Arendt, 2020), "tidyr" (Wickham, Vaughan, and Girlich, 2023), "ggpubr" (Kassambara, 2023), "ggmisc" (Aphalo, 2023), "jpeg" (Urbanek, 2022), "patchwork" (Pedersen, 2023), "grid" (R Core Team, 2023), and "cowplot" (Wilke, 2020). To perform linear regression, we utilized the built-in lm function. The code utilized for generating the graphs can be accessed on GitHub (https://github.com/MutinAndrei/Plots-for-the-article-Fe).

Results and discussion

Interaction of other metabolites with ferric ions

Lactate is a direct derivative of pyruvate in the metabolic process of glucose breakdown, so the two substances are usually present together in fluids of biological origin. Therefore, as pyruvic acid is structurally and metabolically very close to lactic acid, it makes distinguishing them very important during any lactate measurements. Thus, we started from testing the potential interference of pyruvate with ferric ions-based lactate determination.

Indeed, the transmission spectra of aqueous solutions of iron(III) ions mixed with the same amount of either lactic acid or pyruvic acid were found to be very similar (Fig. 2). It is worth noting that these spectra are not identical, which makes the method potentially suitable for simultaneous measurements of lactate and pyruvate in complex mixtures on two conditions: (i) *a priori* known absence of other interfering metabolites and (ii) multi-wavelength analysis of the obtained spectra. However, robust distinguishing lactate and pyruvate with FI-LD using only one wavelength is not possible.

Table 1. Composition of human blood and cow milk according to (Psychogios et al., 2011; Foroutan et al., 2019; Klein et al., 2012; O'Callaghan et al., 2018; Amer et al., 2013; Schlimme, Martin, and Meisel, 2000). Top 30 compounds are shown and ordered by their maximal concentrations

Blood		Milk	
Compound	Concentration, μM	Compound	Concentration, μM
Urea	4000–9000	Lactose	98357–153216
D-glucose	4700–6100	Citrate	3692–7435
L-lactate	740–2400	D-galactose	86–1960
L-glutamine	502–670	Glycerophosphocholine	291–1217
Glycerol	27–431.6	Urea	119–1174
L-alanine	259–427.2	Orotate	208–1002
Citrate	30–400	O-phosphocholine	0–941
Glycine	178–325.4	L-glutamate	111–740
L-valine	190–276	Creatine	312–543
Pyruvate	22–258	Acetone	9–497
L-proline	198.3–239.0	Choline	152–479
L-lysine	178.6–183.0	D-glucose	246–478
L-threonine	107–173	N-acetylglucosamine	127–371
Acetone	35–170	Hippurate	79–267
L-serine	137.0–159.8	Glucose-1-phosphate	0–216
L-glutamic acid	21.0–150.0	L-lactate	0–167
L-leucine	98–148	Ethanolamine	56–166
L-tyrosine	54.5–147	Cis-aconitate	21–157
L-tryptophan	48.7–54.5	2-oxoglutarate	37–156
Betaine	20–144	Malate	55–151
L-arginine	82.2–140.9	Creatinine	36–125
L-histidine	72–131.2	3-hydroxybutyrate	12–121
Formate	32.8–121.7	Betaine	33–115
L-Cystine	62.9–109	Acetate (C2:0)	13–113
Creatinine	74.1–86.6	L-carnitine	57–103
Acetoacetate	0.0–86.0	Creatine-1-phosphate	0–102
Isopropyl alcohol	83.3	L-alanine	18–78
L-asparagine	41–82.4	L-acetylcarnitine	37–65
2-Hydroxybutyric acid	8–80	Dimethyl sulfone	10–59
L-carnitine	26–79	Butyrate (C4:0)	20–57

Next, we compared the optical density (OD) of the $FeCl_3$ solution mixed with other metabolically relevant carboxylic and amino acids specifically at 390 nm as suggested by (Borshchevskaya, Gordeeva, Kalinina, and Sineokii, 2016). We chose 12 amino acids taking into account their relatively high concentrations in human blood (Table 1; Psychogios et al., 2011) and representing distinct groups, including aliphatic (Gly, Ala, Val, Leu, Ile), aromatic (Phe), uncharged (Thr, Gln), negatively charged (Glu), positively charged (Lys, Arg), and sulfur-containing (Met) categories. Besides 5 mM pyruvate, citrate solution also had a high OD that was comparable with the OD of lactate solution at the same concentration of 5 mM (Fig. 3). Water solutions of suc-

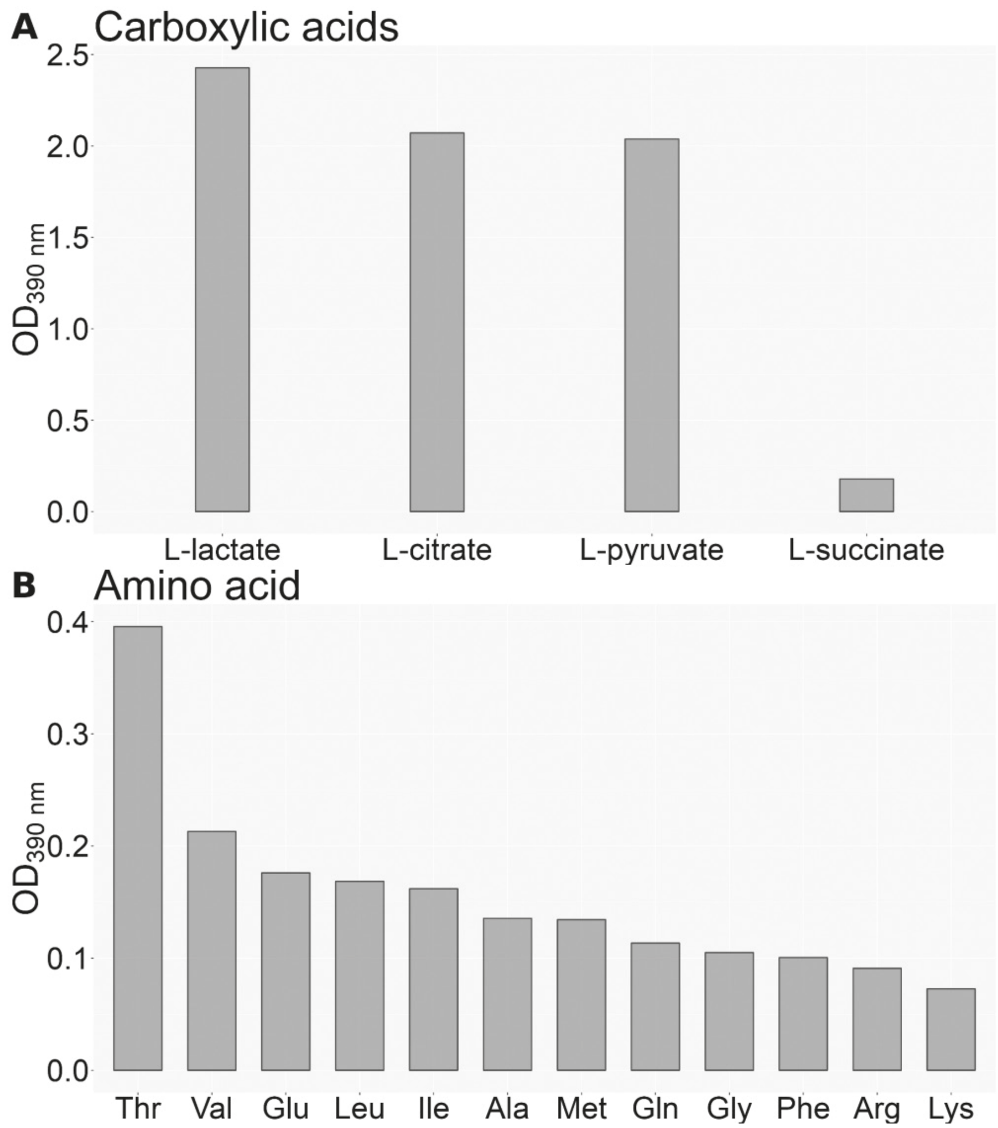

Fig. 3. Optical density (OD) of water solutions of common metabolites in complex with iron(III) ions measured at 390 nm. (A) Optical density with 5 mM carboxylic acids. (B) Optical density with 1 mM amino acids (from left to right: threonine, valine, glutamic acid, leucine, isoleucine, alanine, methionine, glutamine, glycine, phenylalanine, arginine, and lysine).

cinate (5 mM) and 12 tested amino acids (1 mM; chosen to be comparable to their combined minimal concentrations in human blood of ~1.8 mM, Table 1) were shown to have a substantially lower absorbance at 390 nm in comparison to lactate (Fig. 3), but their combined influence also may cause a significant error in the readout of FI-LD. Taking into account other biological examples, cow milk generally contains a much higher amount of citrate than lactate (Table 1; Foroutan et al., 2019). In case of human blood, lactate concentration is relatively high but variable, and combined effects of citrate and amino acids within their physiological concentrations (Table 1; Psychogios et al., 2011) may indeed shift the output of FI-LD in some cases. Thus, since all the tested substances are common in multicellular organisms, precise lactate measurement with ferric ions is clearly not feasible in biological tissues without some *a priori* information about their chemical composition.

Interaction of culture media with ferric ions

Microbiological media for culturing various bacteria and yeast can have very diverse composition, and some of them may contain substances complexing with ferric ions in negligible amounts. Since FI-LD is very popular for lactate measurements specifically in liquid growth media (Bijle, Ekambaram, Lo, and Yiu, 2020; Khumukcham et al., 2022; Lovato et al., 2021), we further compared the readouts of FI-LD and control LOx-LD methods for LB (lysogeny broth), which is a widely used medium for the cultivation of microorganisms and has been chosen as an example of a standard microbiological medium.

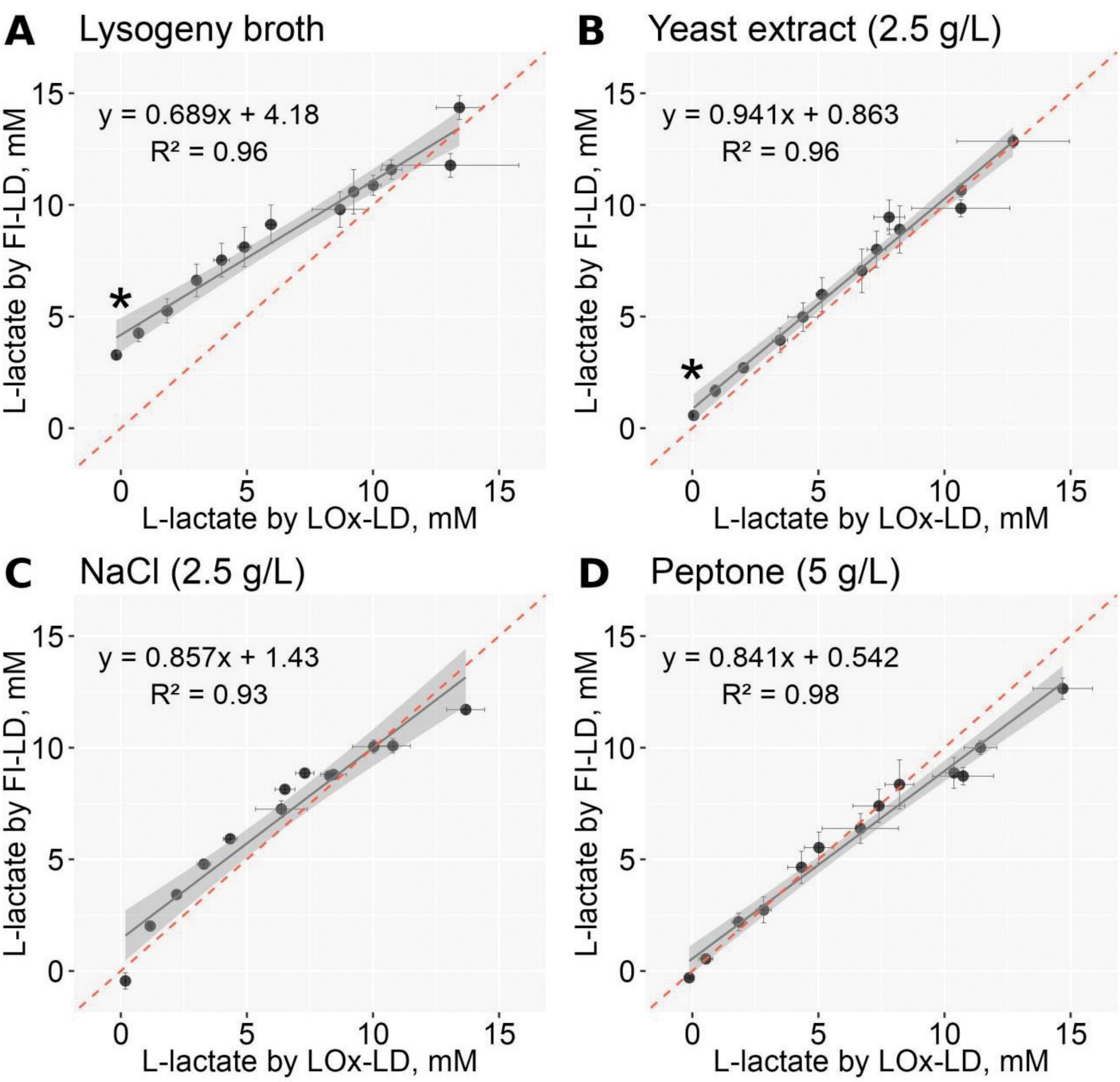

Fig. 4. The readouts of the FI-LD and LOx-LD methods for LB culture medium and its components with various added lactate concentrations (0-15 mM). The orange dotted lines indicate the theoretical ideal coincidence between the tested FI-LD and the standard LOx-LD. The gray line and light gray shades represent linear regression and its confidence intervals. The dots represent the average between the analytical replicates of the L-lactic acid concentration, and the whiskers indicate the standard deviation. Asterisk indicates statistically significant difference of the intercept in built linear regression from zero.

As expected from the previous results, a substantial discrepancy between the readouts of FI-LD and LOx-LD for LB was revealed specifically at low lactate concentrations (Fig. 4). This observation is supported by the statistically significant difference of the intercept from zero suggested by the linear regression (Fig. 4A); i.e., FI-LD indicated about 3-4 mM lactate concentration in the solution where the control enzymatic LOx-LD method showed absence of the metabolite.

To identify the component that contributes most to the increase in optical density of the LB after interaction with ferric ions at 390 nm, we separately tested three components of the culture medium: sodium chloride, peptone and yeast extract (Fig. 4B–4D). Statistically significant difference of the intercept from zero was revealed only for yeast extract, despite its tested concentration being twice lower than in LB. Therefore, yeast extract is indeed the main source of complexes of organic compounds with ferric ions as it is rich in various carboxylic and amino acids in different concentrations. It is worth noting that composition of yeast extract is variable and may depend on the batch and manufacturer. Importantly, yeast extract is often used (sometimes in combination with separately added carboxylic acids) in the media for culturing lactate-producing microorganisms (Vignesh Kumar et al., 2022; Guo et al., 2019; Tak et al., 2019), which highlights the necessity to carefully check the applicability of FI-LD for certain tasks of microbiological research. In general, measurements of lactate with concentrations lower than ~10 mM in complex liquids or in combination with high amounts of structurally similar carboxylic acids should be avoided.

Conclusions

Our experimental results corroborate the literature data on the ability of iron(III) to form complexes with a variety of organic compounds, the presence of which are expected in biologically derived media. The data obtained strongly suggest that lactate measurement using ferric ions is not a selective method and, as a consequence, should be applied with caution to complex mixtures with unknown composition. Still, in certain applications the method can indeed be very useful such as lactate determination in solutions with low amounts of interfering substances or with high lactate concentration. This can especially be the case for synthetic microbiological media with controlled formulation. Another theoretical application found in our study is potential simultaneous determination of lactate and pyruvate (or other metabolites with distinct absorption spectra) in solution with multi-wavelength spectrophotometry. However, since iron(III) ions are not selective to lactate, each application of the method strictly requires *a priori* information about the ratio between the expected lactate concentration and amounts of interfering substances in the tested samples.

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