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# From the bottom of an old jar: A fluorometric method for the determination of creatinine in human serum

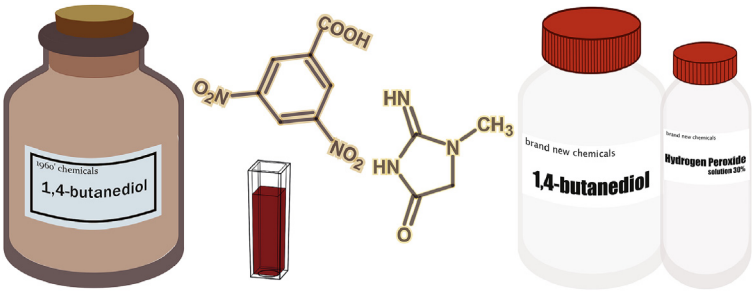
Izabela Lewińska , Michał Michalec *, Łukasz Tymecki

*University of Warsaw, Faculty of Chemistry, Pasteura 1, 02-093, Warsaw, Poland*

## HIGHLIGHTS

- An enzymeless, fluorometric method for creatinine determination was developed.
- The developed method exhibits superior selectivity to known photometric protocols.
- Method was optimized and applied for creatinine determination in real serum samples.

## GRAPHICAL ABSTRACT

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## ABSTRACT

In this paper we have investigated and optimized a non-enzymatic fluorometric creatinine assay. The method was originally described by Blass in the 90s, but we found that besides the reagents mentioned in the paper, an addition of hydrogen peroxide is required to obtain a fluorescent compound. The excitation and emission maxima of the fluorophore are 405 nm and 475 nm, respectively. The optimal conditions for creatinine quantification are as follows: 25 mmol $L^{-1}$ 3,5-dinitrobenzoic acid dissolved in 1,4-butanediol with 58 mmol $L^{-1}$ $H_2O_2$ and aqueous solution of 2 mol $L^{-1}$ NaOH mixed in 1:1 ratio. A linear calibration curve ($y = 18{,}7x + 446$ for 300 s of incubation) was obtained in the range from 2.6 to 750 μmol $L^{-1}$ of creatinine with LOD and LOQ equal to 0.7 and 2.6 μmol $L^{-1}$, respectively. The method was found to be selective towards the analyte in the presence of compounds such as urea, uric acid, bilirubin, albumin and glucose. The developed protocol was applied for creatinine determination in 13 real human serum samples. A correlation ($y = (0.94 \pm 0.03)\,x + (3.66 \pm 3.22)$ with Pearson's r 0.996) and statistical agreement (two-tailed Student's t-test at 95% confidence interval with 12 degrees of freedom) was reached between the obtained results and the reference enzymatic method.



## 1. Introduction

Creatinine is one of the most frequently determined analytes in clinical laboratories. It is synthesized in the muscles in a non-enzymatic dehydration and dephosphorylation of creatine phosphate [1] and is then transported with the blood to the kidneys, where the glomerular filtration occurs and creatinine is excreted in the urine. It is considered to be a glomerular filtration marker and its concentration in blood serum is used to diagnose kidney damage. However, the utility of this metabolite level as a diagnostic parameter is not limited to kidney diseases. It can also serve as a urine dilution factor for spot collection [2], as a parameter to

* Corresponding author.
*E-mail address:* m.michalec@uw.edu.pl (M. Michalec).

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diagnose muscular dystrophy [3], to predict patients' well-being after a cardiac surgery [4] or to monitor the efficiency of hemodialysis treatment [5]. The physiological range of creatinine concentration in the blood depends on several factors like age, sex, muscle mass or ethnicity but usually is between 45 and 90 μmol $L^{-1}$ for women and 60–110 μmol $L^{-1}$ for men [6].

The routinely employed method for the determination of creatinine (colorimetric Jaffé method) suffers from a significant limitation, namely, it lacks analytical selectivity [7]. Compounds like glucose, bilirubin, cephalosporins and many others strongly interfere this assay. To improve the performance of Jaffé protocol, a kinetic (two-point) [8] mode of measurements is usually employed as well as a mathematical compensation of the determined creatinine concentration by subtracting a constant value [9].

Another solution to this problem, reported occasionally in the literature in the previous century [10–16], might be exchanging picric acid, used in the Jaffé reaction, for 3,5-dinitrobenzoic acid. This approach is supposed to help eliminate some of the interferences present in the conventional Jaffé method. What is more, contrary to picric acid, 3,5-dinitrobenzoic acid is non-flammable. Recently we thoroughly examined the 3,5-dinitrobenzoate method and applied it for the determination of creatinine concentration in urine [17]. However, the interference arising from the presence of albumin and bilirubin in the target samples did not allow to employ the reported method to serum.

Interestingly, according to Blass [18,19], 3,5-dinitrobenzoic acid can be also employed as a reagent for the fluorometric determination of creatinine. When 3,5-dinitrobenzoic acid is dissolved in an organic solvent (for example 1,4-butanediol), in the presence of an inorganic base at concentrations above 0.25 mol $L^{-1}$, a fluorescent compound is formed, which excited at 410 nm emits with a maximum at 470 nm. The level of detection of creatinine was reported to be below 1 μmol $L^{-1}$.

Fluorometric detection has several advantages over spectrophotometry as it tends to be more sensitive and selective. However, non-enzymatic, fluorometric methods for the determination of creatinine are rarely reported in the literature. Besides the mentioned Blass' [18,19] work, Pal et al. [20] developed a fluorescent turn-on probe based on a palladium complex with a naphtilimide derivative. It the presence of creatinine, a Pd(creatinine)$_2$Cl$_2$ species are formed and the resulting free ligand exhibits fluorescence at 530 nm. Two other approaches were presented by Ellairaja et al. [21,22]. One of them relies on a Michael addition reaction between creatinine and chalcone derivative and shows an exceptionally low limit of detection [21]. The other one bases on a turn-on response in the presence of creatinine of a non-fluorescent complex of rhodamine B with Au(III) ions [22]. The last work found in the literature employs thioglycolic acid capped Mn/ZnS quantum dots as sensitive to creatinine fluorescent probes [23].

Considering the numerous advantages of fluorometric detection and the simplicity of the method described by Blass, there should be numerous papers continuing his work and further developing this assay. However, to our best knowledge, there are none. We have attempted to recreate work done by Blass [18,19], but in the conditions described in the original article the fluorescent compound has not been obtained. Surprisingly, we found that the addition of a very common chemical, hydrogen peroxide, to the reaction media enables a fluorescent product formation. In this paper, the altered, fluorometric dinitrobenzoate method for the determination of creatinine is deeply investigated and optimized in order to apply it for selective and sensitive quantification of creatinine in serum samples.

## 2. Materials and equipment

Creatinine, 3,5-dinitrobenzoic acid (DNBA), 1,4-butanediol (BTD), bilirubin (in the form of conjugated disodium salt), picric acid and bovine serum albumin (BSA) were obtained from Sigma Aldrich (USA). Methanol, ethanol, ethylene glycol, n-propanol, 1,2-propanediol, n-butanol, hydrogen peroxide, urea, uric acid, glucose, disodium phosphate, potassium iodide and sodium hydroxide were obtained from Avantor Performance Materials (Poland). All reagents were used without further purification. Water used in all the experiments was passed through the HLP5 water purifying system (Hydrolab, Poland).

For fluorometric measurements, a spectrofluorometer FluoroMate FS-2 (Scinco, South Korea) was employed. The photomultiplier tube voltage was set to 700 V in all experiments and the excitation and emission slit were both set to 2.5 nm. Disposable, polystyrene fluorometric cuvettes were used in all experiments (Sarstedt, Germany). For photometric investigations a UV-2401 PC spectrophotometer (Shimadzu, Japan) was employed and disposable, UV and VIS transparent cuvettes (Eppendorf, Germany) were used.

Human normal (HN) and human pathological (HP) control sera in the form of lyophilisate were obtained from Cormay (Poland) and reconstituted according to the manufacturer's instructions.

Serum samples were obtained from patients of Public Central Clinical Hospital in Warsaw and were analyzed in the Central Clinical Laboratory using an enzymatic method (Creatinine plus ver. 2, Roche Cobas 6000 [24]) which was taken as a reference method.

## 3. Results and discussion

### 3.1. Optimization of the reaction conditions

In the conditions reported by Blass (50 mmol $L^{-1}$ DNBA in BTD and 1 mol $L^{-1}$ NaOH, excitation wavelength 410 nm) and using brand new chemicals, we were unable to register any analytical signal. However, we managed to obtain an aged 1,4-butanediol (ca. 60 years old) and, to our surprise, we found that when this solvent was used, measurable fluorescent signal was registered. We deducted that over such an extended period of storage 1,4-butanediol partially underwent dehydration and cyclization to form tetrahydrofuran, which is a known peroxide forming solvent [25]. This lead to a conclusion, that an addition of a small amount of hydrogen peroxide to the reaction media might enable fluorophore formation, what resulted in a 15-fold raise in the fluorescence intensity observed for 250 μmol $L^{-1}$ of creatinine. To confirm the hypothesis, that peroxide was formed in the aged BTD, a simple oxidation test of potassium iodide in acidic environment was performed. In the old BTD iodine was formed readily after KI addition. This effect was not observed in the new BTD. Starch was subsequently added to both tests to visually enhance the effect.

After the discovery, that the presence of hydrogen peroxide is required to obtain a fluorescent compound, the optimization of reaction conditions was undertaken. The excitation and emission maxima were 405 nm and 475 nm, respectively. These values are similar to those described by Blass (410 nm and 475 nm, respectively), which allow to assume that the fluorophore obtained is the same as in his work [18]. The spectra of obtained in the reaction compound are shown in Fig. S1.

The reagents employed for the reaction were as follows: DNBA dissolved in BTD with an addition of aqueous $H_2O_2$ solution and aqueous solution of NaOH. For that reason, parameters, which required optimization were: the concentration of hydrogen

peroxide, the amount of aqueous $H_2O_2$ solution in the organic reagent, the DNBA concentration and the NaOH concentration. The sample to reagents volumetric ratio was fixed at 1 to 14 and the creatinine concentration throughout the optimization experiments was set at 250 μmol $L^{-1}$.

Firstly, several different concentrations of hydrogen peroxide in the reagent were tested, ranging from 0 to 290 mmol $L^{-1}$, to examine its influence on the reaction kinetics. The obtained results are shown in Fig. 1. From this data it is evident, that the presence of hydrogen peroxide is crucial for fluorescent signal development. The fluorescence intensity and therefore the sensitivity of the method is highly dependent on the employed concentration of $H_2O_2$. Higher concentrations of $H_2O_2$ lead to fast decomposition of the fluorophore, whereas when a lower concentration is used the rate of product formation is significantly slower. In that scenario, a sensitive detection would require an extended incubation time. Taking under consideration mainly sensitivity, 58 mmol $L^{-1}$ hydrogen peroxide was selected for further experiments. The volumetric percentage of aqueous solution in BTD-based reagent was fixed at 10% (v/v).

Next, the effect of the concentration of DNBA on method sensitivity was examined. The reaction kinetics registered in different concentrations of DNBA dissolved in BTD are shown in Fig. S2. 25 mmol $L^{-1}$ DNBA was chosen as the most effective. However, any DNBA concentration ranging from 10 to 25 mmol $L^{-1}$ would provide satisfactory sensitivity. The reagent itself was transparent and yellowish, but turned orange after mixing with NaOH.

Finally, several different solutions with the NaOH content ranging from 0 to 4 mol $L^{-1}$ were tested. Registered kinetics are shown in Fig. S3 A. Again, the rate of product formation, as well as the final fluorescence intensity, are highly dependent on the employed concentration of NaOH (Fig. S3 B). 2 mol $L^{-1}$ NaOH was selected for further experiments. What is worth noticing, after 60 min fluorescence intensity reaches value corresponding to the fluorescence of the blank sample suggesting either a complete decomposition of the fluorescent product or a consecutive reaction resulting in formation of non-fluorescent compounds.

To sum up, the most effective combination of reagents was: 25 mmol $L^{-1}$ DNBA dissolved in BTD with 58 mmol $L^{-1}$ $H_2O_2$ and aqueous solution of 2 mol $L^{-1}$ NaOH. The reagents are mixed in 1:1 ratio prior to the measurement. The calibration curves for 3 different reaction times are shown in Fig. 2 and analytical parameters are summarized in Table 1. The mean precision of the method expressed as relative standard deviation (n = 6) at 100 μmol $L^{-1}$ of creatinine was found to be a remarkable 1.5%.

The proposed fluorometric method exhibits both lower limit of quantification and is more precise than routinely used Jaffé kinetic as well as enzymatic photometric protocols. However, a limitation arising from using fluorometric detection is that these methods usually have a significantly narrower linear range, which is also evident in the developed assay. The analytical parameters comparison of the developed and the routinely employed methods is presented in Table 2.

### 3.2. Mechanism investigation

An attempt to partially explain the mechanism of the reaction was undertaken with the aid of spectrophotometry. The absorption spectra registered every 2 min in the course of the reaction in the range from 220 to 600 nm are shown in Fig. S4. The intense peak at 240 nm is a superposition of the absorption of the benzene ring of DNBA, hydrogen peroxide anion and creatinine. Two signals forming around 375 nm and 440 nm are visibly emerging throughout the reaction. However, for further analysis signals between 375 and 435 nm (the width of the excitation peak of the fluorophore) were discarded due to possible interference from fluorescence.

The rate of the formation of the product absorbing at 440 nm is directly proportional to creatinine concentration, suggesting that this compound might be the final product of the reaction between

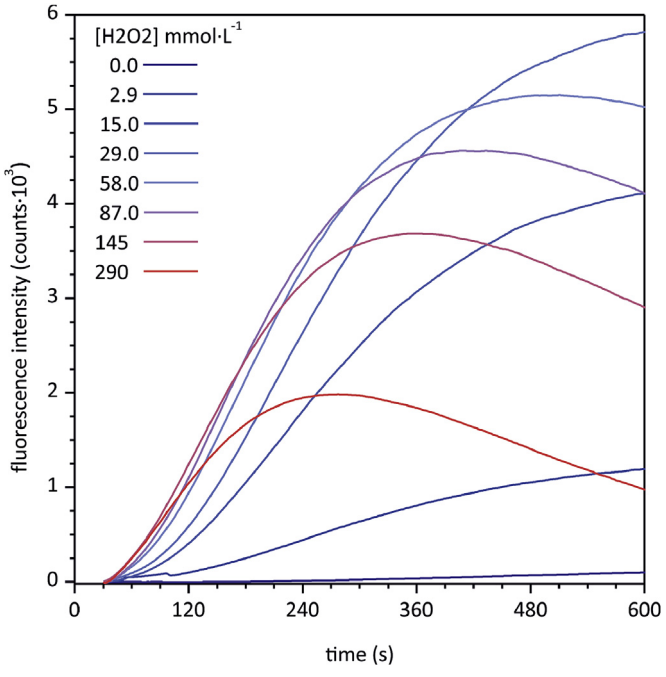


**Fig. 1.** The dependence of fluorescence intensity on the employed concentration of hydrogen peroxide. The kinetics were registered at 475 nm with excitation wavelength 405 nm.

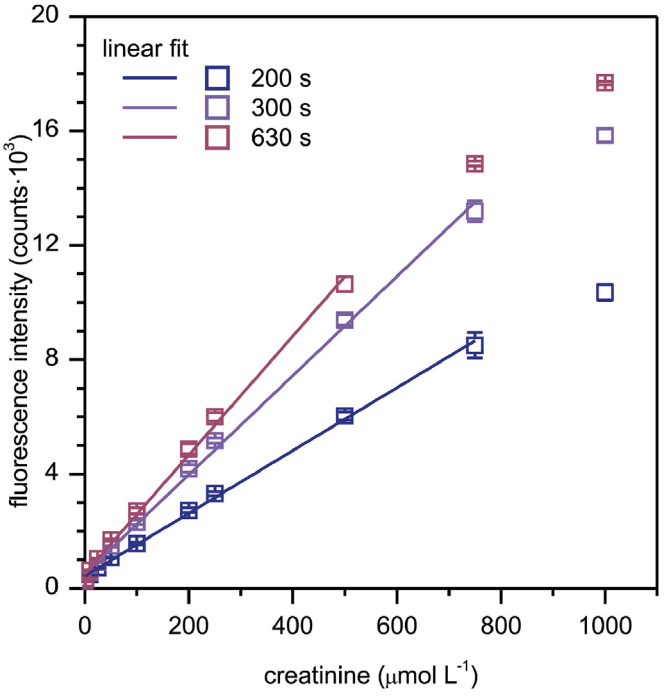


**Fig. 2.** Calibration dependencies registered at selected times for consecutive creatinine standards (0, 1, 10, 25, 50, 100, 200, 250, 500, 750 and 1000 μmol $L^{-1}$) at 475 nm, n=3.

**Table 1**
Analytical parameters of the developed creatinine assay. LOD and LOQ values were calculated as the mean signal of 10 blank measurements plus 3 or 10 times standard deviation of these measurements, respectively.

| Reaction time [s] | Sensitivity [counts/μmol·L$^{-1}$] | $R^2$ | LOD [μmol·L$^{-1}$] | LOQ[μmol·L$^{-1}$] | Linear range [μmol·L$^{-1}$] |
|---|---|---|---|---|---|
| 200 | 11.8 | 0.994 | 2.3 | 6.8 | 6.8–750 |
| 300 | 18.7 | 0.989 | 0.9 | 2.6 | 2.6–750 |
| 630 | 23.0 | 0.981 | 0.3 | 0.9 | 0.9–500 |

**Table 2**
Comparison of some analytical parameters of the developed method, the DNBA-photometric method and two routinely used protocols (kinetic Jaffé and enzymatic). M is mol·L$^{-1}$.

| | DNBA-fluorometric | DNBA-photometric [17] | Kinetic Jaffé [9] | Enzymatic [24] |
|---|---|---|---|---|
| LOQ [μmol·L-1] | 2.6 for 300 s | 40 | 15 | 5 |
| Linear range [μmol·L-1] | 2.6–750 | 40–4000 | 15–2200 | 5–2700 |
| Precision [%] | 1.5 (at 100 μM) | 0.6 (at 100 μM) | 2.1 (at 105 μM) | 2.3 (at 83 μM) |

creatinine and DNBA in the presence of alkali and hydrogen peroxide, while the formed fluorophore is only an intermediate. The kinetics of the absorbing at 440 nm product formation are also dependent on the concentration of $H_2O_2$, but as can be seen in Fig. S5 these dependencies are not linear. For every peroxide concentration the most intense growth of the absorbance is observed during the first 8 min of the reaction, which is coherent with the increase of fluorescence intensity. After this time the dependencies are roughly linear, but the monotonicity of the changes depends on the peroxide concentration. It is constant for $H_2O_2$ concentration lower than 58 mmol L$^{-1}$, linearly increasing for 58 mmol L$^{-1}$ $H_2O_2$ and decreasing if the peroxide concentration is higher.

Interestingly, when hydrogen peroxide is utilized at higher concentration than the optimal 58 mmol L$^{-1}$, a side reaction occurs, resulting in a product absorbing at 510 nm. The kinetics of this reaction for different hydrogen peroxide concentration are shown in Fig. S6 A. The rate of this product formation depends linearly on hydrogen peroxide concentration, but is independent of the concentration of creatinine. The offset observed for low $H_2O_2$ concentrations (Fig. S6 B) is likely due to formation of an absorbing adduct of creatinine and DNBA with absorbance maximum at 500 nm [17]. The product absorbing at 510 nm is an outcome of a side reaction occurring between DNBA and hydrogen peroxide ion and its formation explains the dependencies shown in Fig. 1. The intensity of the fluorescence decreases, when the concentration of $H_2O_2$ exceeds 58 mmol L$^{-1}$, because reagents necessary for fluorophore formation are utilized in a side reaction. Presumably, this product might be a so-called Meisenheimer complex formed from dinitrobenzoic acid, hydrogen peroxide anion and an alkoxide [26]. According to Strauss [27] similar compounds exhibit an absorption maxima in the 500–600 nm region of the spectrum.

### 3.3. Selectivity investigation

Fluorometric detection is expected to be more selective than photometry. However, due to the fact that serum is a sample with an exceptionally rich matrix, nonspecific fluorescence quenching can occur. For that reason, a detailed investigation of the influence of matrix components on obtained results was conducted. Glucose, uric acid, bilirubin, albumin, phosphate ions and urea were selected as the most common interferents of Jaffé reaction and therefore their effect on the developed method was examined in both physiological and pathological concentrations. Binary mixtures of 250 μmol L$^{-1}$ creatinine and a potential interferent were prepared and analyzed according to the developed protocol. The results are presented in Fig. 3 for 3 different times of reaction (200, 300 and 630 s) as a percentage difference between fluorescence intensity measured for 250 μmol L$^{-1}$ creatinine standard and the standard enriched with the interferent.

The results are very promising. In case of the vast majority of the tested interferents measured error does not exceed ±7% of the registered signal, which represents 16 μmol L$^{-1}$ of creatinine at this level. However, the relative error reaching −25% obtained for a sample with 40 g/L of albumin could pose some questions about the selectivity of the method. For that reason, a more detailed investigation of the influence of protein on registered reaction kinetics was undertaken and the results are presented in Fig. S7 A. It is clear, that the presence of protein does not significantly affect the reaction kinetics from the beginning of the reaction. The fluorescence intensity is independent of the concentration of albumin until 200 s of reaction time. Moreover, this trend is observed for every tested

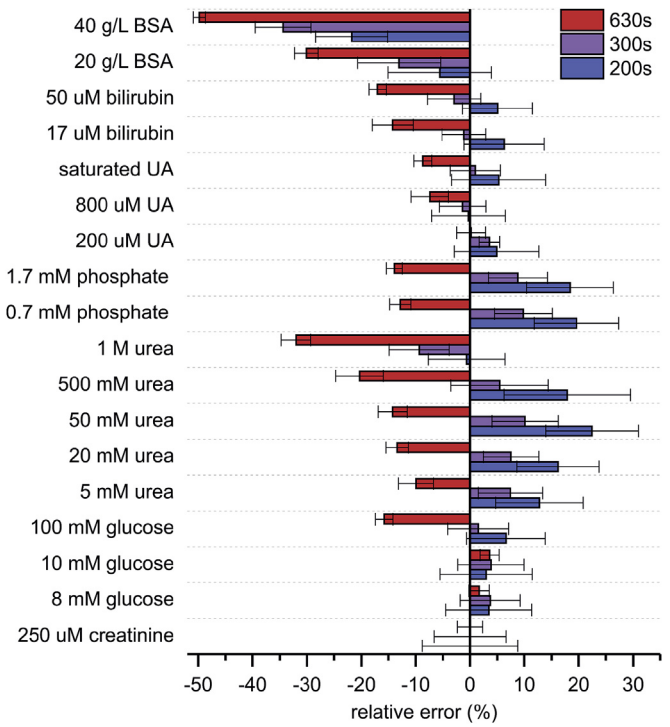


**Fig. 3.** Interference study plot. The value on the abscissa is the percentage difference between the 250 μmol L$^{-1}$ solution of creatinine and the same solution enriched with the interferent specified in ordinate in the registered fluorescence intensity, n=3. M is mol·L$^{-1}$, u is μ, BSA is bovine serum albumin, UA Is uric acid.

creatinine concentration in the physiological range (Fig. S7 B). If the serum sample was diluted, the reaction time could be elongated to 300 s, which would be beneficial because the sensitivity of the developed method increases with the reaction time. The reagents to sample volumetric ratio was found to be crucial to diminish the influence of protein. When this ratio was fixed at 1:1, the magnitude of fluorescence quenching was devastating (90% of signal for 20 g/L of BSA). The ratio of 14:1 was found to both allow for a sensitive determination of creatinine and to significantly decrease the quenching influence of the protein.

A comparison of selectivity of Jaffé (single-point and kinetic) protocol, DNBA-photometric method and DNBA-fluorometric method is presented in Table 3. The superiority of the designed fluorometric assay over photometric methods is evident.

### 3.4. Alternative solvents

Due to the fact that BTD could be a substitute for some illegal drugs, purchasing this reagent is strictly controlled in the EU. For this reason, the utility of several other organic solvents to promote fluorescent product formation was examined. As shown in Fig. 4 all of tested alcohols and diols allowed for registration of a measurable fluorescent signal. Among them, methanol or 1,2-propanediol could substitute BTD, because they would provide similar sensitivity of the assay. In case of propanol, during the 10 min of measurement, the aqueous phase and the propanol phase separated and resulted in a non-monotonic kinetics due to light scattering. The influence of the solvent on the fluorescence intensity can be of a dual nature – a change of a solvent can affect the kinetics and the yield of the product formation as well as the fluorescence quantum yield of the fluorophore [28]. We suspect that the change of the solvent does not alter the mechanism of the reaction, because the excitation and emission maxima of the fluorophore remain the same in every out of tested solvents.

Surprisingly, the presence of an organic solvent is not required for the fluorescent compound formation. Fluorescence can be registered even when DNBA is dissolved in an aqueous solution of NaOH, similarly to the recently developed photometric method [17]. However, in this case, the method's sensitivity, which is enhanced by organic solvent, is not sufficient for an accurate determination of creatinine in serum samples. Respective calibration curves as well as the analytical parameters can be found in the Supplementary Material (Fig. S8 and Table S1). In the authors' opinion, the fluorometric method in an aqueous environment could be an attractive tool for the determination of creatinine concentration in urine or dialysate samples.

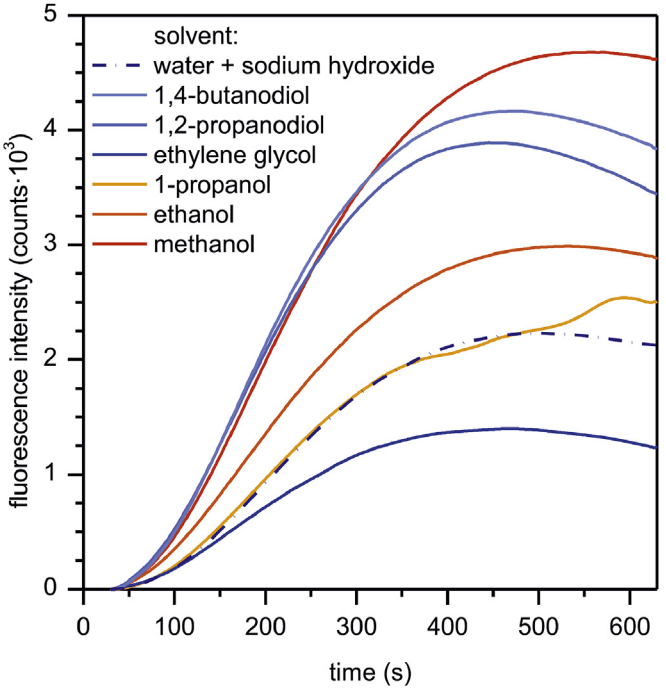


**Fig. 4.** The dependence of fluorescence intensity and reaction kinetics on employed solvent for DNBA dissolution. The concentration of DNBA was 25 mmol $L^{-1}$, the concentration of NaOH was 2 mol $L^{-1}$ and the concentration of $H_2O_2$ was 58 mmol $L^{-1}$.

**Table 3**
Comparison of the selectivity of DNBA-fluorometric, DNBA-photometric and Jaffé (single-point and kinetic) methods. Values in the table represent the percentage difference between creatinine standard and the same standard enriched with the interferent specified in the first column in the registered signal. M is mol·$L^{-1}$.

| | Single-point Jaffé | Kinetic Jaffé | DNBA photometric (120 s) | DNBA fluorometric (200 s) |
|---|---|---|---|---|
| 8 mM glucose | - 4.9% | - 4.3% | - 4.3% | +5.9% |
| 10 mM glucose | - 12.6% | - 6.3% | - 4.9% | +7.9% |
| 200 μM uric acid | - 8.5% | - 10.0% | - 3,5% | +6.6% |
| 800 μM uric acid | - 5.0% | - 9.2% | 0,0% | +4.9% |
| 17 μM bilirubin | +51.3% | - 6.3% | +17.8% | - 6.2% |
| 50 μM bilirubin | +144,6% | - 4.2% | +33.1% | - 5.5% |
| 20 g/L albumin | - 17.1% | - 24.6% | +15.8% | +3,6% |
| 40 g/L albumin | - 30.5% | - 52.6% | +33.0% | - 8.5% |
| 0.7 mM phosphate ions | - 15.9% | - 19.0% | - 0.9% | - 4.1% |
| 1.7 mM phosphate ions | - 26.1% | - 23.3% | +1.1% | - 8.6% |
| 5 mM urea | - 8.6% | - 3.6% | - 6.1% | - 7.8% |
| 20 mM urea | - 9.0% | - 3.6% | - 2.7% | - 0.6% |

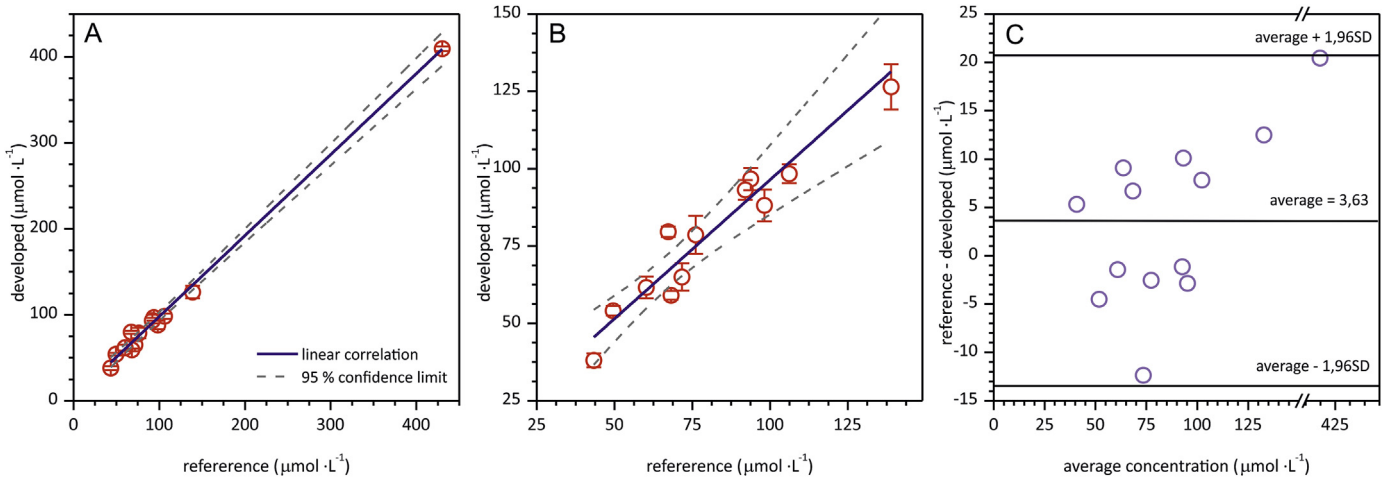


**Fig. 5.** Correlation of all (A) and selected samples (B) and Bland-Altman plot (C) of the developed fluorometric method and the reference enzymatic method.

### 3.5. Serum sample analysis

For the final verification that the developed protocol is adequate for the determination of creatinine concentration in real serum samples, two control sera as well as their mixtures in 1:1 and 1:3 ratio were analyzed. Each sample was diluted 2-fold, 5-fold and 10-fold. Moreover, one sample was analyzed without dilution. Obtained results are presented in Table S2.

When comparing determined creatinine concentration with creatinine concentration range declared by the serum manufacturer, several points should be mentioned. First of all, sample dilution before the measurement is necessary to obtain reliable results. Secondly, the fold of the dilution should be determined by the desired reaction time. For a 2-fold dilution fluorescence intensity measurement after 200 s of reaction is preferred, for a 5-fold dilution – 300 s of incubation is required and for a 10-fold dilution – 630 s. As we suspect, the reason that the results are dependent on both dilution as well as incubation time is because of numerous potential interferents present in the serum samples, which we did not account for (in Fig. 3 and Table 3). The concentration of these compounds would be lowered with the increase of the dilution but also the kinetics of this reaction should be taken under consideration. This outcome is coherent with the results obtained for binary mixtures of creatinine and albumin described previously (Fig. S7 A). The influence of the protein is diminished by sample dilution, but elongated incubation time is beneficial because of increased sensitivity. For samples with larger protein concentration (i.e. less diluted), the shortening of incubation time is necessary.

Thirteen real human serum samples were previously analyzed in the Central Clinical Laboratory, Medical University of Warsaw using the most reliable, but also the most expensive enzymatic method [24] All samples were 5-fold diluted and analyzed according to developed protocol with 300 s of incubation time. Each sample was analyzed in triplicate.

The obtained results were statistically evaluated. The correlation between creatinine concentration determined using the developed method and the enzymatic protocol is $y = (0.94 \pm 0.03)x + (3.66 \pm 3.22)$ with Pearson's r 0.996. Two-tail paired Student's t-test at the 95% confidence interval with 12 degrees of freedom was employed to determine the accuracy of the described assay. The calculated t-value was 1.499, while the tabulated $t_{crit}$ for this test is 2.179. This indicates that concentrations determined using the developed, single-point fluorometric protocol are not statistically different from those determined with a highly selective enzymatic assay. The correlation, as well as the Bland-Altman plot, are shown in Fig. 5.

### 3.6. Recommended protocol

To summarize this work, a detailed protocol for routine applications is presented. The developed method can be executed both with a fluorometer or with a microplate reader. The main reagent consists of 25 mmol $L^{-1}$ DNBA with 58 mmol $L^{-1}$ hydrogen peroxide in 90% BTD and 10% water solution and is stable up to 100 h stored in a fridge. The prepared reagent should be mixed prior to measurement directly in fluorometric cuvettes on in a well with 2 mol $L^{-1}$ aqueous sodium hydroxide in 1:1 volumetric ratio. 1.4 mL or 70 µL of each reagents are proposed to be used in a typical fluorometric cuvette or a microplate well, respectively. The recommended standard solution or sample volume is 200 µL for a fluorometer and 10 µL when using a microplate reader. Serum samples should be diluted 5-fold prior to mixing with reagents and the appropriate incubation time is then 300 s. The recommended dilution of urine sample should be between 25 and 100-fold to fit within the linear range of the method.

## 4. Conclusions

Despite an over century-old history of Jaffé protocol existence and the determination of creatinine in biological samples, finding a more reliable creatinine assay is still a considerable issue. Blass in his paper [18] reported a very simple and selective fluorometric method of creatinine determination. However, we found that in the conditions he described, a fluorescent signal can only be obtained when an aged solvent is used. We concluded, that an addition of hydrogen peroxide to the reaction media enables fluorophore formation. In this paper, we have developed, optimized and applied for the determination of creatinine in serum samples a non-enzymatic, rapid and selective fluorometric assay. The mechanism of the reaction between DNBA and creatinine in the presence of alkali and hydrogen peroxide still remains unclear and isolation of the products of the reaction is necessary. In the Authors' opinion, this fact does not limit the analytical applications of the developed method. However, discovering the underlying mechanism could result in an improvement of the analytical parameters of the assay, especially its selectivity.

The selectivity of the developed method is superior to the selectivity of both single-point and kinetic Jaffé protocol as well as the photometric DNBA assay, especially regarding bilirubin and

albumin. The results obtained in real samples do not statistically differ from the reference, enzymatic method. The interferences arising from the presence of albumin were reduced by increasing reagents to sample ratio and can be further diminished in a flow system working in a strict time regime [29].

What is important to highlight is that a commercially available enzymatic kit for 100 samples costs 460 EUR [30], while reagents needed for creatinine determination in 100 samples according to the developed method cost ca. 7 EUR. This significant reduction of the cost of the assay as well as circumventing other drawbacks associated with enzymatic methods is the main advantage of the developed protocol.

## CRediT authorship contribution statement

**Izabela Lewińska:** Methodology, Validation, Formal analysis, Investigation, Writing - original draft. **Michał Michalec:** Data curation, Visualization, Writing - review & editing, Project administration, Funding acquisition. **Łukasz Tymecki:** Conceptualization, Resources, Writing - review & editing, Supervision.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.08.017.

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