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On-line 'protein shaker': A multicommutated flow analysis system for fluorometric creatinine determination in deproteinized serum

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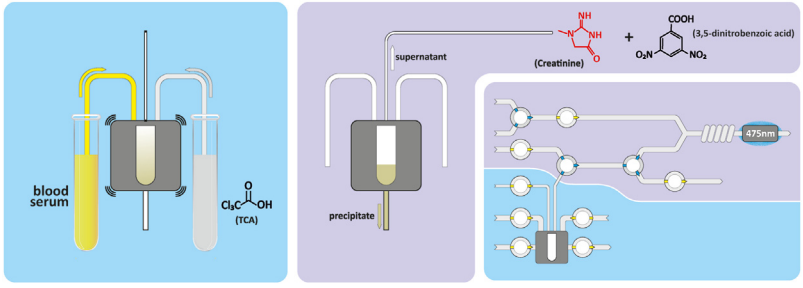
HIGHLIGHTS

- MCFA system for fluorometric creatinine determination in serum has been developed.
- Serum samples were subjected to on-line deproteinization in a flow reactor.
- Deproteinization was accomplished with TCA/NaCl precipitation.
- The flow system was validated with control sera.

GRAPHICAL ABSTRACT



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ABSTRACT

A fully mechanized multicommutated flow analysis (MCFA) system for fluorometric determination of creatinine in serum samples is introduced in this paper. The flow system was constructed with micro-solenoid pumps and valves and with a 3D-printed flow cell. Fluorometric assay relied on creatinine reaction with 3,5-dinitrobenzoic acid and hydrogen peroxide in an alkaline environment. To overcome significant interference from protein, a flow reactor for serum deproteinization was designed and implemented in the flow system. The deproteinization was carried out by precipitation with trichloroacetic acid and the addition of sodium chloride facilitated the precipitate sedimentation. The supernatant representative sample was pumped out and subjected to fluorometric creatinine assay. The obtained linear range was from 1.6 to 500 μmol L^{-1} and the precision, expressed as RSD, was below 3%. The proposed MCFA system was used to determine creatinine concentration in control serum samples. The results obtained with flow deproteinization correlated well with results obtained with conventional deproteinization ($y = (0.91 \pm 0.09)\ x + (37 \pm 28)$) with Pearson's r 0.979.

1. Introduction

The field of flow systems development for the determination of creatinine concentration in various biological samples has been blooming since the 1980s resulting in multiple papers published on this matter. It is not surprising considering the substantial diagnostic importance of creatinine levels. In fact, it is the second most frequently determined compound in clinical laboratories. Creatinine is a glomerular filtration marker and its concentration in blood serum and urine indicates kidney condition. The reference range of creatinine in serum is 45–90 μmol L^{-1} and 60–110 μmol L^{-1} for female and male, respectively. The healthy level of urinary creatinine is 4.4–18 mmol L^{-1} [1].

Four general trends can be distinguished in detection methods

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employed in these flow systems: (i) photometric detection; (ii) potentiometric detection; (iii) amperometric detection, and (iv) chemiluminometric detection. Among photometric methods, the Jaffé reaction is readily used, relying on formation of a red-orange complex of creatinine and picric acid. It was applied in flow systems in both single-point [2–5] and kinetic modes [6,7] in various techniques such as FIA [2,3,7], MCFA [4,6,8,9] or SIA [10]. Alternatively, enzymatic photometric methods are used, relying on enzymatic conversion of creatinine to ammonium ion and a subsequent determination of the reaction product [11,12].

The latter reaction can also be employed in flow systems with potentiometric detection of the liberated ammonium ion. These systems, however, suffer from interference caused by endogenous ammonia. This issue can be overcome by signal subtraction [13], predialysis [14] or chemical removal of ammonia [15].

Several attempts to employ creatinine amperometric detection in flow systems have been undertaken. The main advantage of such systems considers relatively short response time, assuring high sample throughput. Usually, creatinine is subjected to enzymatic conversion to ammonium ion (with creatinine deaminase/creatinine iminohydrolase, EC 3.5.4.21) or to hydrogen peroxide (with a cascade of enzymatic reactions employing creatininase EC 3.5.2.10, creatinase EC 3.5.3.3, and sarcosine oxidase EC 1.5.3.1, with a correction for endogenous creatine). While the latter reactions sequence allows for direct determination of H_2O_2 [16,17], the former requires either an electrode appropriately modified with a conducting polymer [18,19] or a further enzymatic ammonia conversion to an oxygen-consuming reaction. The decrease of dissolved oxygen is then detected with an oxygen electrode [20,21]. Also, flow systems for non-enzymatic amperometric creatinine determination using a Nafion coated copper electrode [22] or a molecularly imprinted polymer [23] have been introduced. Last but not least, flow injection systems for chemiluminometric creatinine determination were proposed employing either the enzymatic reactions cascade mentioned earlier with peroxide detected via subsequent reaction with luminol [24] or a non-enzymatic reaction of creatinine with cobalt ions and hydrogen peroxide [25].

Deproteinization is frequently a required step in blood serum analysis due to interference from protein in many clinically relevant analytical assays. Moreover, high protein concentration results in high viscosity of the solution, which is not desirable in flow analysis systems. A chemical deproteinization can be carried out by adding numerous precipitants, such as e.g., trichloroacetic acid, zinc sulfate, acetonitrile [26] or tungstic acid [27]. Physical deproteinization can be achieved by using membranes [28] which allow on-line separation of proteins during the flow of the sample through the porous barrier.

In our previous study, we have developed a novel fluorometric method of creatinine detection basing on its reaction with 3,5-dinitrobenzoic acid and hydrogen peroxide in alkaline conditions [29]. The method allows for selective determination of creatinine concentration in appropriately diluted serum samples. Here we further improve the performance of this assay by its implementation in a multicommutated flow system with on-line deproteinization. To our best knowledge, this is the first reported non-enzymatic flow analysis system for fluorometric creatinine determination, as well as the first reported chemical deproteinization in a flow system.

2. Materials and methods

2.1. Materials and reagents

Creatinine, 3,5-dinitrobenzoic acid (DNBA), trichloroacetic acid (TCA) and bovine serum albumin (BSA) were obtained from Sigma Aldrich (USA). Sodium hydroxide, ethanol, urea, methanol and sodium chloride were purchased from Avantor Performance Materials (Poland). Control human sera of normal (HN) and pathological (HP) composition were obtained from Cormay (Poland) and reconstituted according to the instructions provided. Water used in all experiments was doubly distilled.

2.2. Flow system construction

The flow system was constructed using microsolenoid pumps (20 μL stroke volume, model 120SP1220-5 TV) and three-way valves (model 100T3MP12-62-5) from Bio-Chem Fluidics (USA). The elements were connected with PTFE tubing (06417-21, ID 0.56 mm and 06417-41, ID 1.07 mm, Cole-Parmer, USA) and a 3-way Y-shaped joint (Carl Roth, Germany). The actuation was accomplished with a lab-made circuit based on Arduino Mega and ULN2803 transistors array (Texas Instruments, USA). The scheme of the electrical circuit is shown in Fig. S1.

2.3. Detection

Flow fluorometric cell was manufactured in-house according to Ref. [30]. Briefly, a cuvette insert was printed using NijnaFlex filament (NijnaTech, USA) on Dreamer 3D printer (Flashforge, China). Then pieces of Tygon tubing (ID 1.52 mm, Saint-Gobain, France) were placed in the holes in the insert and PTFE tubing was introduced to Tygon tubing. 3D printed insert was glued into disposable microcuvette - UVette® (Eppendorf, Germany, Cat. No. 0030106318) with epoxy glue. Tubing was sealed in the insert using the same glue and left to dry for 24 h. Epoxy glue was used instead of cyanoacrylate glue due to superior resistance to organic solvents. When cyanoacrylate glue was used to seal the flow-through cuvette, leakage was observed after a short usage period. The cuvette was placed in an adapter (Eppendorf, Germany, Cat. No. 4099001009) and then in the spectrofluorometer.

Fluorometric detection was accomplished with FluoroMate FS-2 (Scinco, South Korea) fluorometer. Unless stated otherwise, excitation and emission slit were both set to 5 nm. The photomultiplier tube voltage was 700 V. Fluorophore was excited with 405 nm wavelength and the emission at 475 nm was monitored.

2.4. Deproteinizating flow reactor

Due to significant interference from the presence of protein in the sample, a flow reactor was designed to carry out deproteinization of serum. As deproteinizating agent 10% (w/v) trichloroacetic acid was used. As a result, formation of a coarse precipitate of denaturized protein can be observed. The implemented analytical procedure requires the following steps: (i) pumping an appropriate volume of the sample or a calibrant (serum or binary mixture of creatinine and BSA); (ii) addition of an excess of deproteinizating agent; (iii) vortexing the solutions to allow their mixing; (iv) waiting for a certain time to allow sedimentation of the formed precipitate (8 min in the optimized conditions); (v) pumping the supernatant to the injection coil; (vi) cleaning the reactor with 8 mol L^{-1} urea and ethanol in several iterations.

The reactor architecture was designed in Fusion 360 (Autodesk, USA). The drawing is appended as .stl file in Supplementary Material. The components of the flow reactor were sliced using FlashPrint 4.6.1 software and printed on FlashForge Guider II (Flashforge, China) 3D printer. The reactor itself was printed with ABS (acrylonitrile butadiene styrene) filament and the supporting parts were printed with PLA (polylactide) filament, both obtained from Orbi-Tech (Germany). The printing parameters did not significantly differ from the recommended ones for the used

filaments. In both cases printing was executed in a position allowing to limit the necessity of using supports. The detailed printing parameters can be found in Electronic Supplementary Information (ESI). Initially, the reactor was also manufactured using PLA. Unfortunately, the formed TCA-protein precipitate, instead of gravitationally sedimenting, stayed dispersed in the whole volume of the solution in the reactor, probably due to hydrophilic nature of PLA and electrostatic interactions between PLA and the precipitate. Substitution of PLA with hydrophobic ABS allowed for reproducible sedimentation of the TCA-protein precipitate.

The detailed external and internal shape of the reactor is shown in Fig. 1 and the technical sketches are shown in Figures S2 and S3. The volume of the reactor was 1.5 mL. The reactor has 6 holes for liquids transportation – 4 at the top of the reactor and 2 on the sides. The top holes are for pumping in sample, TCA reagent and ethanol and for pumping out supernatant to the injection coil. To limit sample consumption, tubing for sample introduction and pumping the supernatant had a smaller diameter than the one used in the rest of the flow system. The side holes are for pumping in urea (top) and for pumping out (bottom) waste. The tubing is inserted in the reactor in the same way as in the detector, as described in Section 2.3. Additionally, a small hole on the top without any tubing was necessary to work as a vent. The front side of the reactor was left open and secured with a gasket and polystyrene 'glass' to enable visual inspection of the processes occurring in the reactor. The shape of the gasket was analogous to the shape of the inspection window. The casing for the reactor was designed to allow a tight fit between glass and reactor and to mount the reactor on a vortex (model Micro-shaker type 326 m, Premeo, Poland). It is essential to highlight that a slight adaptation of the case will allow using the flow reactor with other brands and types of vortexing devices. The vortex was controlled via a relay (JQC-3FF-S-2-5V, Tongling, China) connected with Arduino Mega.

2.5. Manual deproteinization method

To conduct manual precipitation of protein in the sample, 10% (v/w) TCA was added to the serum sample in 1:3 (serum: reagent) volumetric ratio. Then the samples were vortexed for 20 s and, after 20 min of waiting, centrifuged for 10 min [26].

3. Results and discussion

3.1. Flow system design and operation

Fluorometric creatinine assay relies on its reaction with DNBA and hydrogen peroxide in a strongly alkaline environment. In these conditions a fluorophore is formed, which emits at 475 nm when excited with 405 nm. Fluorescence intensity depends strongly on the solvent used. The best results can be obtained in methanol and 1,4-butanediol. However, omitting any organic solvents and using water instead is also possible and gives satisfactory results [29].

The MCFA system to conduct the above-mentioned reaction was constructed with 8 microsolenoid pumps and 3 microsolenoid valves as shown in Fig. 2, presenting also the corresponding operation program. The flow system can be divided into two parts – part A for conducting the fluorometric determination of creatinine and part B for protein precipitation with TCA. Part A of flow manifold consists of P1 and V1 for mixing and pumping reagents – DNBA with peroxide and aqueous NaOH solution. These reagents cannot be premixed because of a reaction occurring between them, resulting in a 38% decrease in signal after 3 h of storing premixed reagents (as shown in Fig. S4). For that reason, the reagents have to be mixed on-line in 1:1 ratio (step C in Fig. 2, bottom). The sample, which is the supernatant formed above the protein precipitate in the reactor, is pumped to the injection loop between V2 and V3 by P2 (step D in Fig. 2, bottom), with V3 in normally closed (i.e. V3 is energized) position and V2 in normally open position. Then the sample is injected into the flowing stream of reagents, owing to alternating actuation of P1 and P3 (step E in Fig. 2, bottom) with V3 in normally open position and V2 in normally closed position. The flow is stopped when the sample zone reaches the detector for 450 s and the reaction kinetics is registered. After this time passes, the flow cell is washed with a fresh portion of reagents (step P in Fig. 2, bottom).

Part B of the MCFA system consists of 5 pumps marked as P4, P5, P6, P7 and P8. The thinner tubing shown in Fig. 2 has an internal diameter of 0.56 mm and was used to limit sample consumption. The procedure of protein precipitation begins with the actuation of P4 and P5 in 1:3 ratio. Then the precipitated solution is vortexed twice in 20 s time interval and left to sediment for 8 min (steps A and B in Fig. 2, bottom). Such conditions were determined

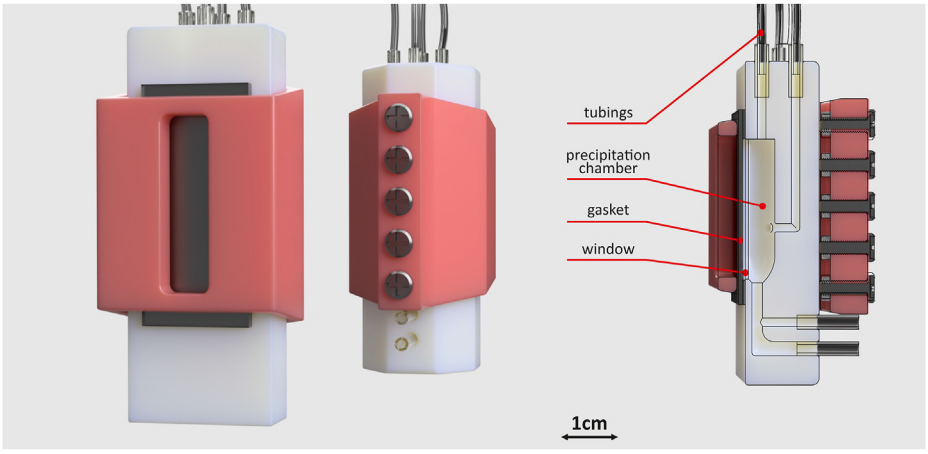

Fig. 1. Visualization and cross-section of the deproteinizing reactor. The white part is printed from ABS, whereas the red part is printed from PLA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

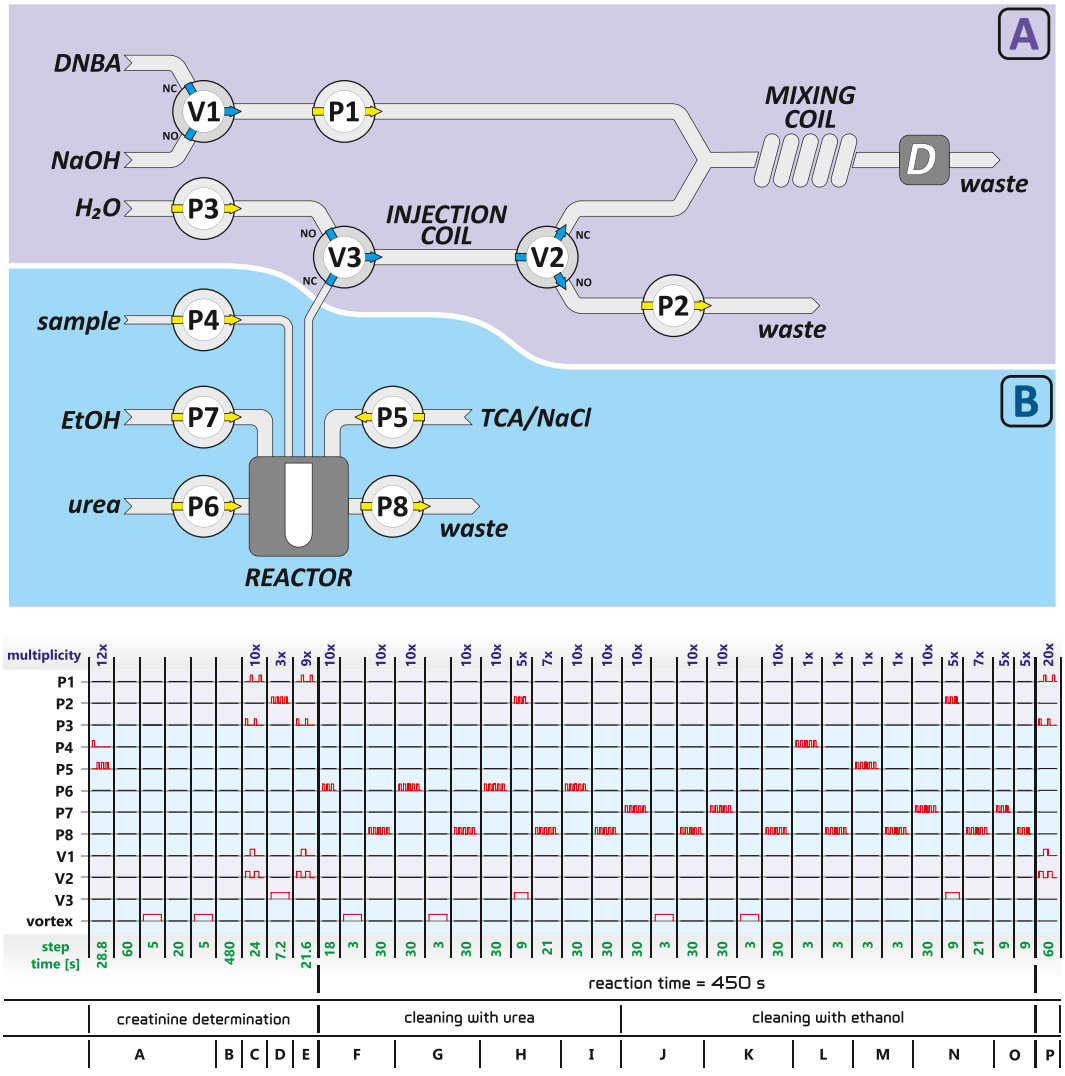

Fig. 2. Top: Scheme of MCFA system for the fluorometric determination of creatinine with on-line sample deproteinization. Bottom: Schematic representation of the Arduino program for MCFA system operation. Each step is explained in detail in the text. Abbreviations used in the figure: P – microsolenoid pump, V – microsolenoid valve, NO – normally open, NC – normally closed.

experimentally and these findings are further discussed in Section 3.3. When the reaction of the analyte with the reagents occurs in the detector, the reactor is subjected to a washing procedure. According to the literature, TCA-protein precipitate is soluble in ethanol [31] as well as 8 mol L^{-1} urea [32]. Ethanol allows for the dissolution of TCA-albumin precipitate, whereas all TCA-protein precipitates are soluble in urea. Surprisingly, we found that wetting the reactor with ethanol allowed for sedimentation of the precipitate but when this step was omitted, the precipitate was dispersed in the whole volume of the reactor. For that reason, both urea and ethanol were used to wash the reactor. The washing procedure goes as follows: the reactor is filled with the washing medium using P6 or P7, vortexed for 3 s and then the content of the reactor is pumped out using P8 (steps F,G and J,K in Fig. 2, bottom). Moreover, cleaning the injection loop with urea solution and ethanol was implemented in steps H and N, respectively. To get rid of residual precipitate, which might be present in the end of tubing after vortexing, a certain portion of sample and TCA reagent is pumped in steps L and M and then pumped out to waste with P8. In steps I and O final cleaning with urea and ethanol is executed.

3.2. Conditions optimization

The experiments aiming at optimization of flow system performance were conducted using only flow system manifold marked as A in Fig. 2. Two creatinine standards – 100 and 500 μmol L^{-1} were used to optimize physical and chemical conditions of the flow systems. In order to prove that such an approach can be implemented, full calibration curves were registered for various standard injection volumes. As shown in Figure S5, the height of the peak for these two standards corresponds to the sensitivity of the calibration curves.

The parameters selected for optimization were: injection volume, mixing coil length, DNBA concentration, H_2O_2 concentration and NaOH concentration. The optimization of the physical parameters (i.e., injection volume and mixing coil length) was performed using both methanol and water as solvents. While the physical parameters of the MCFA system can vary depending on the solvent, the chemical conditions should remain the same for both solvents, therefore water was used to optimize the chemical parameters of the assay. Using water has obvious advantages over methanol – it is non-toxic, cheaper and does not promote bubbles formation when

mixed with aqueous solutions. However, basing on our previous results [29], the analytical parameters of the assay performed in a fully aqueous solution might be insufficient for reliable creatinine determination in serum.

Firstly the effect of the injection volume was established. This parameter was changed by swapping the length of the tubing between V2 and V3. As expected, the higher the injection volume is, the more sensitive the response is for both solvents because it enables creating a wider sample zone and limits dispersion. On the other hand, injection volume exceeding 100 μL was not tested because the dead volume of the flow cell is 100 μL, so it was expected that further increase of sample volume would not affect the results significantly. 15 cm mixing coil was enough to allow mixing the reagents and the sample for both solvents. Using longer mixing coils enhanced sample zone dispersion and led to lower fluorescence intensities, whereas shorter coils did not allow for reproducible mixing of reagents and sample.

The optimization of chemical parameters began with investigating the influence of DNBA concentration on the peak heights. It was found that 35 mmol L^{-1} DNBA allowed for obtaining the highest fluorescence intensities. Similarly to our previous findings [29], 58 mmol L^{-1} hydrogen peroxide proved to be the most effective and allowed for obtaining the highest fluorescence intensities. Last but not least, the concentration of sodium hydroxide was optimized. This parameter had the most profound influence on the signals with the highest concentration resulting in the highest fluorescence. The optimization was stopped at 5 mol L^{-1} NaOH due to a possibility of damaging microsolenoid pumps (check valve to be precise) with more concentrated NaOH. However, no further improvement is expected with the increase of NaOH concentration. Our previous results suggest that an optimal concentration of NaOH in the reaction medium is around 1 mol L^{-1} [29]. In the case of the developed flow system, the NaOH concentration reaching the detector is 4-fold diluted in reference to the initial concentration therefore 5 mol L^{-1} initial concentration would result in 1.25 mol L^{-1} NaOH in the detector, which means that probably the optimal conditions are achieved.

To conclude, the optimal conditions for flow system operation are: 100 μL injection volume, 15 cm mixing coil, 35 mmol L^{-1} DNBA, 58 mmol L^{-1} H_2O_2 and 5 mol L^{-1} NaOH. The results of the optimization experiments are summarized in Fig. 3.

In the optimal conditions calibration curves were registered in the range from 0 to 500 μmol L^{-1} of creatinine to establish the difference of sensitivities when methanol and water are used as solvents for the reaction. The curves are depicted in Fig. S6 and as can be seen the slopes of the two regressions do not differ considerably to justify using much more toxic solvent. However, from the Authors' experience with this fluorometric assay, in a non-aqueous environment the magnitude of fluorescence quenching resulting from the presence of protein is smaller than in an aqueous environment [29]. This phenomenon was confirmed experimentally – it was found that for methanol-based reagent the peaks registered in the presence of 10, 20 and 50 g L^{-1} BSA were 11%, 27% and 51%, respectively, lower than the peaks registered in the absence of protein for 100 μmol L^{-1} creatinine. On the other hand, peaks registered in the same conditions for aqueous reagent were 33%, 50% and 69% lower than peaks for 100 μmol L^{-1} creatinine without any protein. For that reason, we decided to use methanol as a solvent for the fluorometric assay.

3.3. Reactor operation optimization and validation

To further diminish the protein interference, a flow-through reactor for conducting protein precipitation was designed and implemented in the flow system (part B in Fig. 2, top). Initial tests and optimizing experiments were conducted using binary mixture of creatinine and BSA. Protein was precipitated in the reactor with 10% (w/v) TCA in 1:1 ratio. This TCA concentration was selected as the most commonly used in TCA protein precipitation protocol (e.g. Ref. [26]). We found that TCA does not influence the reaction kinetics therefore it can be used as a precipitating agent in this reaction.

Next, the influence of vortexing was established. The peaks were registered without prior vortexing of the precipitated sample, with single vortexing for 5 s and with double vortexing for 5 s in 20 s time intervals. When single vortexing was implemented in the procedure, the precipitate did not sediment properly and attempts to pump out the supernatant resulted in clotting the tubing. Double vortexing allowed for reproducible sedimentation of the precipitate in 8 min. When comparing the results obtained in experiments with double vortexing versus no vortexing at all, it was found that the peaks were 60% higher when the precipitate was vortexed twice. For that reason, double vortexing was implemented in the experimental procedure. However, the sedimentation of precipitate formed in artificial mixtures of creatinine and BSA was somewhat different than sedimentation of precipitate of control serum. In the

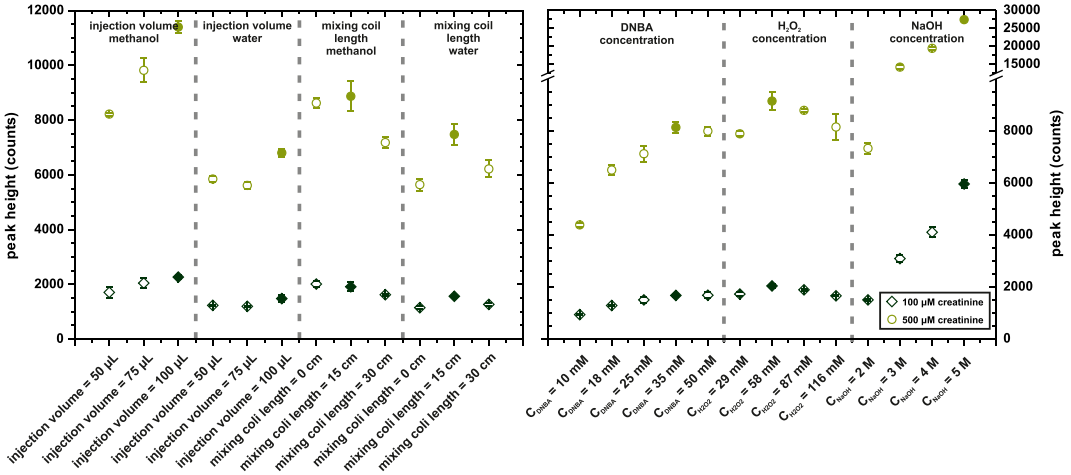

Fig. 3. Optimization of physical and chemical conditions of the MCFA system. Initial conditions: 100 μL injection volume, 30 cm mixing coil length, 25 mmol L^{-1} DNBA, 58 mmol L^{-1} and 2 mol L^{-1} NaOH. Full points represent the selected, optimal parameters. M is mol·L^{-1}.

described conditions the precipitate formed in control sera did not sediment in a reasonable time. In this case term "reasonable time" refers to a time shorter than the length of manual deproteinization procedure (i.e. 30 min).

According to the literature [33,34], the addition of ionic species to acetone significantly improves the recovery of precipitated proteins as well as speeds up the precipitation process. This is due to the ion pairs formation between added salt and the precipitated protein, which shields electrostatic forces. These forces would otherwise enhance protein solubility in an organic solvent. We hypothesized that perhaps adding a simple salt to TCA reagent would maximize precipitation and perhaps sedimentation of the formed precipitate. The mechanism of TCA-induced precipitation includes protein unfolding and exposing the hydrophobic chains of the amino acids to polar solvent, which results in precipitation [35]. Therefore, it is possible that an addition of sodium chloride to TCA reagent would enhance these electrostatic interactions, leading to maximized precipitation and sedimentation. The original research [33,34] on the topic employed NaCl concentration in the range from 10 to 100 mmol L^{-1}. However, the concentration of proteins undergoing precipitation was several-fold lower than in serum samples. For that reason, 1.5 mol L^{-1} NaCl was initially tested.

The volumetric ratio of serum sample to TCA reagent was also optimized. The results presented in literature [26] confirm that the efficiency of deproteinization in human plasma is the same for the volumetric ratios in the range from 1:0.5 to 1:4 (sample:TCA). However, increasing the volume of the precipitation agent might allow for a faster formation of the supernatant, because the absolute amount of solution is bigger in relation to the amount of the precipitate. The results of these experiments are summarized in Table 1. The percentage values in Table 1 represent the percentage of the height of an Eppendorf tube filled in by the supernatant after vortexing. For instance, the value 15% means that the supernatant occupies 15% of a tube and 85% is occupied by the precipitated protein. In these experiments, the biggest percentage is the most desirable because it means that the biggest volume of the supernatant is formed.

1:3 serum to TCA ratio allowed for the formation of the largest volume of supernatant and therefore was used in further experiments. Next, the concentration of NaCl added to TCA reagent was optimized and the results are shown in the bottom part of Table 1. As can be noticed, the more NaCl is added, the faster phase separation is. Moreover, the precipitate sediments significantly faster when NaCl concentration increases, which translates to shortening the total analysis time. These results clearly indicate that 5 mol L^{-1} NaCl in the TCA reagent provides reasonably fast and effective separation of precipitated proteins from the supernatant containing the target analyte. The optimization process was finalized at 5 mol L^{-1} NaCl because larger amounts of NaCl did not dissolve in 10% (v/w) TCA.

Table 1
Optimization of serum to TCA volumetric ratio and NaCl concentration in the TCA reagents. The percentages in the table mean the percent of the height of an Eppendorf tube filled in by the supernatant formed after vortexing. The experiments reported in the upper part of the table were carried out with 10% (w/v) TCA with 1.5 mol L^{-1} NaCl.

serum:TCA volumetric ratio	5 min	10 min	15 min	20 min
1:1	0%	0%	0%	0%
1:2	0%	5%	5%	5%
1:3	4%	13%	17%	22%
1:4	7%	12%	15%	19%
NaCl concentration [mol·L^{-1}]	5 min	10 min	15 min	
0.0	4%	4%	13%	
0.5	4%	4%	13%	
1.5	4%	9%	17%	
3.0	9%	13%	22%	
5.0	22%	35%	43%	

The addition of excess of chloride ions to a sample, which is then subjected to a fluorometric assay, can be a questionable choice because halogens are known as effective fluorescence quenchers [36]. For that reason, the influence of sodium chloride concentration on the registered fluorescence intensity was established. Different concentrations of NaCl were added to TCA reagent and the peaks were registered for 100 μmol L^{-1} creatinine. The obtained results, shown in Fig. S7, suggest the opposite to the expected effect. The fluorescence intensity increases by 35% when NaCl concentration raises from 0 to 2 mol L^{-1}. When the concentration of NaCl increases even more, the fluorescence intensity decreases slightly, but is still more intense than in the absence of NaCl. In general, the increase of ionic strength decreases the stability constants of complexes which should, contrary to findings from this experiment, result in a decrease of fluorescence intensity. However, as stated in our previous study [29], the product detected by fluorescence is most likely an intermediate, which is transformed to a non-fluorescent end product. The lower the stability constant of the final product is, the higher concentration of the fluorescent intermediate can be accumulated. The decrease in fluorescence when NaCl concentration raises above 2 mol L^{-1} might be attributed to a combination of decrease by quenching and increase by ionic strength.

The influence of protein concentration on measured fluorescence intensity was also established. Binary mixtures of 100 μmol L^{-1} creatinine with BSA in various concentrations ranging from 0 to 70 g L^{-1} were subjected to flow deproteinization and subsequent fluorometric creatinine determination. The results are depicted in Fig. S8. For protein concentration above 50 g L^{-1} the magnitude of fluorescence decrease in relation to fluorescence registered for pure creatinine standard depends on the protein concentration in the sample. This might be considered to be a limitation of the proposed solution. However, a similar conclusion arises from the work done by Crowell et al. [33], which leads to a conclusion that this dependence is probably attributed to the deproteinization method itself, not to the way it is conducted. Moreover, the results reported in Section 3.5 prove that varying concentration of protein in the sample does not affect accurate creatinine quantification in real samples.

3.4. Analytical performance

To establish the analytical performance of the developed MCFA system with on-line deproteinization, calibration curves in the range from 0 to 500 μmol L^{-1} of creatinine were registered in the following conditions: (i) creatinine standards diluted in the flow reactor with TCA-NaCl reagent in 1:3 ratio; (ii) creatinine standards with 50 g L^{-1} BSA deproteinized in the flow reactor; (iii) creatinine standards with 50 g L^{-1} BSA diluted in the flow reactor with distilled water. 50 g L^{-1} BSA was chosen as an average content of albumins in human serum. The calibration dependencies are shown in Fig. 4 and the analytical parameters of the obtained curves, as well as limits of detection and quantification, are summarized in Table 2. In Fig. 4 condition (i) is called creatinine, condition (ii) – creatinine + 50 g L^{-1} BSA deproteinized and condition (iii) is referred to as creatinine + 50 g L^{-1} BSA.

First of all, it is clear that the deproteinization process in the flow reactor is not complete. The slope of the calibration curve registered using creatinine standards with BSA subjected to deproteinization compared to the curve registered using pure creatinine standards is 36% lower. This indicates that some residual protein

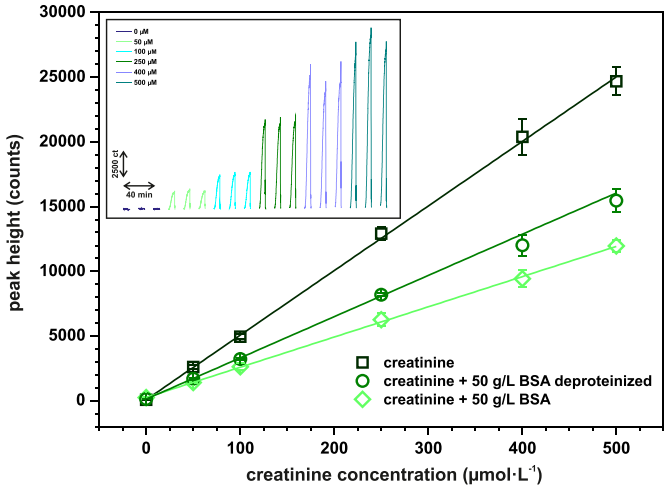

Fig. 4. Calibration curves registered with pure creatinine standards (squares), deproteinized creatinine standards with 50 g L^{-1} BSA (circles) and creatinine standards with 50 g L^{-1} BSA not subjected to deproteinization in the flow reactor (diamonds). Insert: Recording of the calibration process from 0 to 500 μmol L^{-1} creatinine with 50 g L^{-1} BSA subjected to deproteinization. The spaces between peaks are due to the fact that the flow through detector was stopped during protein precipitation and sedimentation in the reactor.

remains in the supernatant. Another indicator of such process is a larger intercept of the calibration curves registered in the presence of BSA. The more protein is in the sample, the bigger the intercept is due to light scattering. On the other hand, it is possible that some fraction of creatinine is in the precipitated protein phase instead of in the supernatant therefore the overall signal is lower when the sample is subjected to deproteinization. However, when comparing the slopes and the analytical parameters of calibration curves registered with and without deproteinization (32 versus 23 counts·L·μmol^{-1}, which gives 39% sensitivity rise for deproteinized standards), it is evident that deproteinization using flow reactor improves the method's sensitivity and limit of detection quantification of creatinine. The precision of fluorometric creatinine determination for 100 μmol L^{-1} of creatinine with 50 g L^{-1} BSA subjected to on-line deproteinization was 2.4% (n = 3).

The results obtained in this experiment pose an important question – how to calibrate the developed system to quantify creatinine in real samples. In the Authors' opinion, there are two possible scenarios – either performing the calibration using pure creatinine standards and then applying a mathematical correction to the obtained calibration curve or performing the calibration using creatinine standards enriched with BSA subjected to deproteinization in the flow system. Although the first option would allow faster calibration (no need to wait for the precipitate to sediment), the second option accounts for interday variability in deproteinization efficiency. For that reason deproteinized creatinine standards with 50 g L^{-1} BSA were employed for the MCFA system calibration. On the other hand, standard addition method (SAM) could be used to analyze real samples and eliminate the matrix effect. However, this measurement mode does not comply clinical analysis requirements, that call for calibration curve method.

A comparison between the developed flow system and the ones reported in the literature is presented in Table 3. Only papers in which the Authors demonstrated application in serum samples were selected for the comparison. An advantage of fluorometric detection, namely its excellent sensitivity, is revealed in this comparison, because the developed MCFA system offers one of the lowest limits of quantification. Unfortunately, the comparison exposes the main disadvantage of the presented system – a low sample throughput of 3 h^{-1}. The sampling rate is an order of magnitude lower than that of the other listed flow methods. However, it is important to highlight that this result could be improved by applying the following changes: (i) multiplying the number of deproteinizing reactors; (ii) speeding up the pumping rate in the cleaning procedure; (iii) replacing P6, P7 and P8 for pumps with larger dispense volume (pumps with up to 60 μL dispense volume are available from Bio-Chem Fluidics.

3.5. Real sensing scenarios

The developed flow system with on-line deproteinizing reactor was tested in real analytical scenarios using control sera and their mixtures, indicated in Supplementary Table S1. Creatinine in these samples was quantified with the fluorometric assay in the flow system, but the deproteinization was carried out in two ways: (i) manually, according to the protocol described in Section 2.5; (ii) in the flow reactor according to the established optimal conditions. The supernatants from the first deproteinizating procedure were injected directly to part A (shown in Fig. 2, top) of the MCFA system. For the first deproteinization procedure, the system was calibrated with pure creatinine standards, whereas for flow deproteinization, it was calibrated with creatinine standards enriched with 50 g L^{-1} BSA.

The obtained results are shown in Fig. 5. The correlation between creatinine concentration determined in serum subjected to flow deproteinization and serum subjected to conventional deproteinization is y = (0.91 ± 0.09) x + (37 ± 28) with Pearson's r 0.979 and is depicted in Fig. 5A. A two-tailed paired Student's t-test with 95% confidence interval and 6° of freedom was used to evaluate the statistical agreement between two deproteinization procedures. The calculated t_{exp} is 1.081 and is lower than the critical t value for this test equal 2.447, indicating that the results obtained using developed deproteinizing flow reactor are in agreement with these obtained using a well-established deproteinization procedure. The Bland-Altman plot, shown in Fig. 5B, confirms the agreement between the two methods. It is worth highlighting that both deproteinization methods allowed to obtain results within the range declared by the serum manufacturer, as shown in Table S1.

Table 2
The regression parameters as well as limits of detection and quantification for three calibrating conditions shown in Fig. 4. Limits of detection and quantification were calculated as concentrations corresponding to blank signal +3 and + 10 standard deviations of 10 blank measurements, respectively.

calibrating conditions	regression equation	R^2	limit of detection [μmol·L^{-1}]	limit of quantification [μmol·L^{-1}]
creatinine	y = 50 x + 78.3	0.9994	0.68	2.3
creatinine + 50 g L^{-1} BSA with deproteinization	y = 32 x + 128	0.9994	1.6	5.3
creatinine + 50 g L^{-1} BSA without deproteinization	y = 23 x + 254	0.9998	5.3	17

Table 3
A comparison between the developed flow system and other flow systems presented in the literature for creatinine determination in serum samples.

Method	Flow technique	Sample pretreatment	Linear range[a] [μmol·L^{-1}]	Precision	Sample throughput [h^{-1}]	Ref.
kinetic Jaffé	MCFA	dilution if creatinine concentration exceeds 300 μmol L^{-1}	up to 250	<1%	15–40	[6]
single-point Jaffé	MSFA	off-line deproteinization with TCA	up to 177	3.6%	n.d.	[5]
photometric detection of ammonium ion after creatinine conversion with CIH	FIA	on-line enzymatic conversion or stream splitting for removal/correction for endogenous ammonia	up to 1000	3%	60	[11]
chemiluminescence detection of hydrogen peroxide after creatinine enzymatic converstion with CNH, CRH and SAO	FIA	none, but correction for endogenous creatine is required	up to 1000	2.4%	n.d.	[24]
potentiometric with sensing membrane with creatinine-tungstophosphate	FIA	off-line deproteinization with ethanol, supernatant evaporation and dissolution of the residues	3.5–10000	0.7%	40 + at least 10 min for each sample deproteinization	[37]
potentiometric detection of ammonium ion after creatinine conversion with CIH	CF	on-line predialysis for removal of endogenous ammonia	25–5000	<3%	30	[14]
fluorometric with 3,5-dinitrobenzoic acid	MCFA	on-line deproteinization with TCA	5.3–500	2.4%	3	this work

Abbreviations: MCFA – multicommutated flow analysis, MSFA – monosegmented flow analysis, FIA – flow injection analysis, CF – continuous flow, CIH – creatinine iminohydrolase (EC 3.5.4.21). CRN – creatininase (EC 3.5.2.10), CRH – creatinase (EC 3.5.3.3), SAO – sarcosine oxidase (EC 1.5.3.1), TCA – trichloroacetic acid, n.r. – not reported.
[a] The linear range spans from the reported limit of quantification to the concentration reported as the end of the linear range.

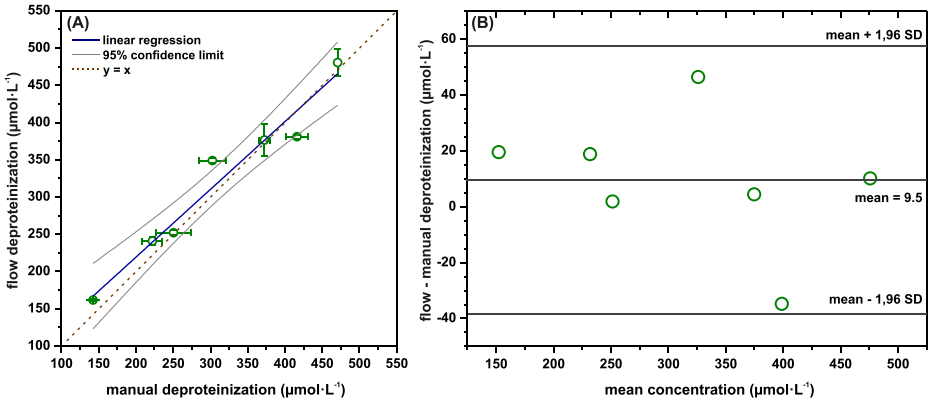

Fig. 5. (A) Correlation and (B) Bland-Altman plot of the creatinine determined in serum samples subjected to flow deproteinization and conventional, manual deproteinization procedure.

4. Conclusions

Although creatinine is a very frequently determined analyte and various flow systems were designed for its quantification, fluorometric creatinine determination is scarcely reported. In fact, this is the first non-enzymatic fluorometric flow system for the quantification of creatinine in serum samples.

The application of on-line sample deproteinization in the flow system is a major step in flow analysis due to a large number of analytical methods suffering from protein interference. This is the first example of chemical deproteinization in flow system reported in the literature to the authors' best knowledge. The fact that the deproteinization efficiency in the developed reactor is not 100% leaves room for improvement of the analytical procedure. On the other hand, on-line chemical deproteinization with a 3D-printed reactor allows to decrease the cost of the analysis in comparison to on-line deproteinization with porous membranes. Additionally, it simplifies the maintenance of the system, as there is no problem of membrane fouling.

A drawback of the proposed solution might be that the flow deproteinization elongates the total analysis time. However, by multiplying the number of flow reactors, the time needed for sample deproteinization would be similar, or even shorter, than in conventional procedure with TCA precipitation. Moreover, the flow reactor allows for an attractive possibility of performing calibration using only one creatinine standard, diluted on-line in the reactor [38]. Lastly, it is important to highlight that for complete validation of the developed flow system it would be necessary to analyze standard reference materials or to conduct a creatinine recovery study using spiked sera samples.

CRediT authorship contribution statement

Iga Malicka: Software, Investigation, Validation, Formal analysis, Writing – review & editing. **Izabela Lewińska:** Conceptualization, Methodology, Writing – original draft, Supervision. **Łukasz Tymecki:** Conceptualization, Resources, Writing – review & editing, Visualization, 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.2021.339246.

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