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ORIGINAL ARTICLE

Phone camera detection of glucose blood level based on magnetic particles entrapped inside bubble wrap

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Abstract

OBJECTIVES: Glucose is an important diagnostic biochemical marker of diabetes but also for organophosphates, carbamates, acetaminophens or salicylates poisoning. Hence, innovation of accurate and fast detection assay is still one of priorities in biomedical research.
METHODS: Glucose sensor based on magnetic particles (MPs) with immobilized enzymes glucose oxidase (GOx) and horseradish peroxidase (HRP) was developed and the GOx catalyzed reaction was visualized by a smart-phone-integrated camera.
RESULTS: Exponential decay concentration curve with correlation coefficient 0.997 and with limit of detection 0.4 mmol/l was achieved. Interfering and matrix substances were measured due to possibility of assay influencing and no effect of the tested substances was observed. Spiked plasma samples were also measured and no influence of plasma matrix on the assay was proved.
CONCLUSIONS: The presented assay showed complying results with reference method (standard spectrophotometry based on enzymes glucose oxidase and peroxidase inside plastic cuvettes) with linear dependence and correlation coefficient 0.999 in concentration range between 0 and 4 mmol/l. On the grounds of measured results, method was considered as highly specific, accurate and fast assay for detection of glucose.

INTRODUCTION

Toxins, drugs and all xenobiotics entering into living organism affect biochemical constitutions of body fluids as the result of organism effort to establish the homeostasis. So scanning of biochemical markers reveals the origin of toxins and makes the diagnosis faster which causes early initiation of treatment preventing life-threatening conditions (Shockcor & Holmes 2002; Pohanka *et al.* 2012). Glucose is a saccharide molecule which plays crucial role in human metabolism. It is essential source of energy for brain and heart and it is also necessary for muscles and other tissues and organs. For physiological functioning of organs reliant on glucose, tight range of glucose blood level is necessary. In normal conditions, metabolic pathways control glucose blood level and strictly regulate it (Cryer 2007; Giugliano *et al.* 2008; Martinkova & Pohanka 2015). Unfortunately, during pathological conditions such as diseases (diabetes mellitus, metabolic syndrome

etc.), long-lasting stress, hospitalization and poisoning (carbamates, organophosphates, salicylates, morphine, dopamine, acetaminophen etc.) blood glucose level is deflected out of physiological limits and hyper- or hypoglycemia can occur. This condition can be life-threatening or even lethal under some circumstances. In the light of this fact, glycemia is one of important biochemical markers and it is commonly measured at patients not only suspected of poisoning (Sabzghabaee *et al.* 2011; Pohanka *et al.* 2012; Sonneville *et al.* 2015).

In principle, glucose colorimetric assay is based on the first glucose biosensor innovated by Clark and Lyons in 1960s. Their electrochemical biosensor determined glucose using enzyme glucose oxidase (GOx) and molecular oxygen from ambient air. Glucose was transformed to gluconolactone and hydrogen peroxide and redox processes were detected by electrode (Clark and Lyons 1962; Martinkova & Pohanka 2015). Lately, reaction was broadened by enzyme peroxidase (HRP) which utilized increasing hydrogen peroxide to coloration of colorless chromogenic substrate (Morin & Prox 1973). Our intention was to construct simple portable devices for a fast glucose assay usable at any situation. Currently used portable device for glucose measurement – glucose meters are not sufficient because of some reasons. They are not accurate enough, the standard deviation of glucose meters measurement is around 15% when validation performed to standard laboratory methods. Moreover, many substances (maltose, acetaminophen, ascorbate, dopamine etc.), body conditions (hematocrit, pH, oxygenation, hyperlipidemia etc.) and sample handling states (hemolysis, anticoagulants or volume of sample) may interfere with the measurement (Tonyushkina & Nichols 2009).

The current work is focused on construction of an innovated glucose assay which may replace glucose meters due to its better properties such as accuracy and specificity using the well-known reaction for glucose determination together with currently emerging materials. Magnetic particles (MPs) are known for their ability to immobilize enzymes and another biomolecules onto their surface and they are extensively used in today biosensors construction (Khun *et al.* 2012; Wang *et al.* 2012; Baretella *et al.* 2013; Martinkova *et al.* 2016). Also interest on bubble wrap as liquid samples container or cuvette replacing reaction container and phone camera or digital camera as detection devices have been arising (Lu *et al.* 2009; Zhu *et al.* 2012; Bwambok *et al.* 2014; Yetisen *et al.* 2014). In the device construction, MPs were selected as carriers of enzymes HRP and GOx necessary for colorimetric reaction while bubble wrap served as reaction container and camera phone for detection of solution color intensity. Use of bubble wrap and camera phone makes our method available and affordable, MPs should provide its accuracy and colorimetric reaction using two specific enzymes should provide the specificity of the assay.

MATERIALS AND METHOD

Chemicals

All chemicals apart from plasma samples were obtained from commercial sources in at least analytical purity and used without further modification apart from solution preparation. Iron oxide magnetic micro particles carboxy-functionalized (20 mg/ml), GOx from *Aspergillus niger*, type VII (2.0 mg/ml), peroxidase from horseradish type VI (4.0 mg/ml), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 15 mg/ml), o-PD, glucose, sucrose (4 mmol/l), sorbitol (4 mmol/l), deoxyribose (4 mmol/l), maltose (4 mmol/l), fructose (4 mmol/l), reduced glutathione (4.5 μmol/l), trolox (50 μg/ml), urea (20 mmol/l), bovine serum albumin (80 mg/ml) and phosphate buffered saline (10 mmol/l, pH 7.4) were purchased from Sigma Aldrich (Saint Louis, Missouri, USA). Ascorbic acid (20 μg/ml) and sodium acetate trihydrate were bought from Penta (Prague, Czech Republic). Acrylonitrile butadiene styrene filament for 3D printer was gained from Prusa (Prague, Czech Republic). Phosphate buffer was prepared by dissolution of one tablet in 200 ml of demineralized water. Sodium acetate buffer (50 mmol/l, pH 5.5) for preparation of o-PD solution and enzymes immobilization onto MPs was made from sodium acetate trihydrate. Solution of o-PD was prepared by mixing of 3.5 mg of o-PD powder with 50 ml of sodium acetate buffer. Demineralized water was prepared by reverse osmosis process using device Aqua Osmotic 2 (Aqua Osmotic, Tisnov, Czech Republic). Animal blood samples were obtained post mortem from mice after sacrificing of the animal in Faculty of Military Health Sciences, University of Defense (Hradec Kralove, Czech Republic) vivarium facility. Animal manipulation and sacrificing was permitted and supervised by ethical committee in the Faculty of Military Health Sciences. In total 10 blood samples were collected to heparinized tubes and plasma was received by centrifugation at 3,000 RPM for 10 minutes.

Apparatus

Detection of color change of measured solution was performed by 5 Mpx camera of smartphone Sony Xperia MT27i using operation system Android 2.3.7., device version number 6.0.B.3.184 (Tokyo, Japan). Thermo Shaker PRT-60 HL from Biosan (Riga, Latvia) was used for stirring of mixture during preparation of MPs-GOx-HRP using 800 revolutions per minute. CyberScan pH 6000 from Eutech was used for adjustment of buffer solutions pH.

Data processing

All samples were measured in pentaplicate under standard ambient temperature and pressure conditions. Color intensity of solutions containing different glucose concentration was detected by transforming of color in the pictures into numeric data using RGB image pro-

cessing GIMP 2.8.14 (GNU type of software). Color intensity of three points in each bubble was randomly chosen so fifteen numeric data from every glucose concentration was gained and averaged. Measured data were processed using Origin 9.1 software (OriginLab Corporation, Northampton, Massachusetts, USA). Signal vs. noise equal to three criterion (S/N=3) was used for limit of detection calculation.

Immobilization of HRP and GOx onto magnetic particles

Iron MPs carboxy-functionalized was used for immobilization of both enzymes (HRP and GOx) needed for glucose colorimetric assay. Immobilization of both enzymes was done by protocol described previously (Martinkova *et al.* 2016). Briefly, MPs in amount of 400 ml washed three times with 1 ml of SAB were mixed together with 3 mg of EDC dissolved in 200 µl of SAB and shaken for 15 minutes. After activation of carboxy groups on the MPs surface, MPs were washed three times with 1 ml of SAB. HRP in the amount of 200 µl and GOx in the amount of 100 µl were added into washed MPs and mixture was shaken for 2.5 hours. After this incubation, mixture of MPs was washed three times with 1 ml of PBS and three times with 1 ml of SAB. Finally, washed MPs were dissolved in100 µl of SAB. This way prepared MPs-POx-GOx particles were injected into bubbles of bubble wrap in amount of 15 µl, where they were drying for 24 hours.

3D printing of dark box for photography

Optimization of glucose assay with camera phone detection is reliant on stable light conditions. For that purpose, shooting box was made by 3D printer using fused deposition modeling technique (Singh *et al.* 2015) from 2.9±0.5 mm black filament of acrylonitrile butadiene styrene with 20% of infill. The printed object was proposed in 123D Design software (Autodesk; San Rafael, CA, USA) and printed in Prusa i3 (Prusa Research; Prague, Czech Republic) device. The size of the box was set to be 20 cm high, 15 cm wide and 10 cm deep. On the upper side of box 2×2 cm square for camera lens was cut out and on the lateral side 2.5×2.5 cm square for external light source was cut-out. The box was printed bottomless enabling easy insertion of bubble wrap into box.

Concentration curve

Measurement of glucose concentration curve was inspired by work of Morin and Prox (1973) when the well-known reaction of glucose catalyzed by enzymes GOx was applied. Hydrogen peroxide created during reaction is subsequently used for reaction with chromogenic substrate catalyzed by enzyme HRP. This chromogenic substrate (o-PD in our case) changes its color depending on amount hydrogen peroxide and also on glucose concentration. Glucose standard solutions in concentration range from 0 to 18 mmol/l (0, 1, 2, 4, 8, 12 and 18 mmol/l) were prepared and diluted five times with o-PD solution. Made solutions in the amount of 150 µl were injected into bubbles with entrapped and dried MPs-HRP-GOx and incubated for 12 minutes. After incubation, the pictures of bubbles were taken by phone camera from stable distance 20 cm in 3D printed box enabling stable light condition due to only external light source (Figure 1). Photos were processed by computer software Gimp for gaining numeric data replying the color intensity of bubbles measured in blue color channel. Average numeric data was plotted into concentration curve graph.

Interferences and matrix effect

MPs and bubble wrap was prepared the same way for interferences and matrix effect assays as was prepared for concentration curve measurement. Possibly interfering substances – saccharides such as fructose, deoxyribose, maltose, sucrose and sorbitol in concentration 4 mmol/l was used for this measurement. Glucose (4 mmol/l) and water were used as substrates for assay reaction as positive and negative control respectively. Process of measurement and data processing was the same as chosen for concentration curve assay described above in chapter Concentration curve.

Matrix effect was measured using standard substances naturally occurred in plasma, which may affect the assay results. Ascorbic acid, reduced glutathione, trolox as the water soluble derivative of vitamin E, bovine serum albumin and urea were chosen as possibly affecting substances and prepared in double of their physiological concentration occurring in plasma. Substances were mixed together with 8 mmol/l glucose standard solution in ratio 1:1 and applied in the same way like reaction substrates. Water and 4 mmol/l glucose were used as reaction substrates as well representing negative and positive control respectively. All

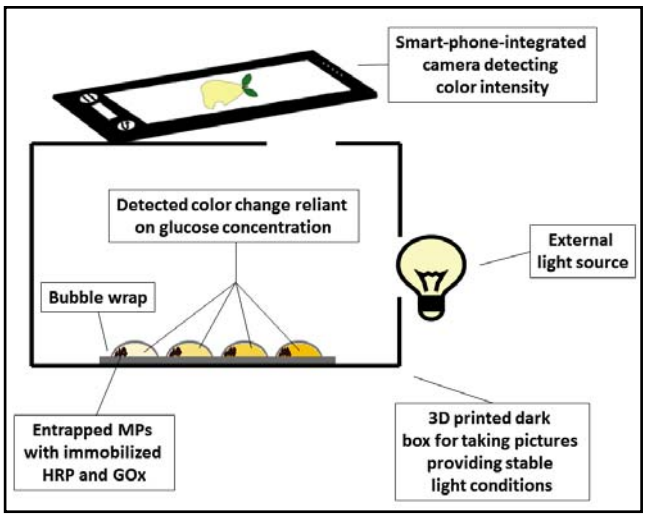

Fig. 1. Diagram of performed assay. Bubble wrap with entrapped MPs-GOx-POx was used as reaction container where change of color intensity after biochemical reaction was detected by smart-phone-integrated camera inside dark box.

prepared substrates were measured according to procedure described above in chapter Concentration curve as well as data gaining and processing.

Average results of both interfering and affecting substances were compared with results of positive and negative controls and plotted into graph.

Spiked plasma samples

In order to reveal all the effects of plasma on assay, spiked plasma samples were measured the same way as glucose standard solutions in chapter Concentration curve. Plasma samples from mice were mixed together with glucose standard samples in concentration range from 0 to 36 mmol/l (0, 2, 4, 8, 16, 24 and 36 mmol/l) in ratio 1:1. This way prepared samples were injected together with o-PD solution into bubbles with 24 hours dried MPs prepared according to procedure described in chapter Immobilization of HRP and GOx onto magnetic particles. After incubation time, images of bubble wrap were taken.

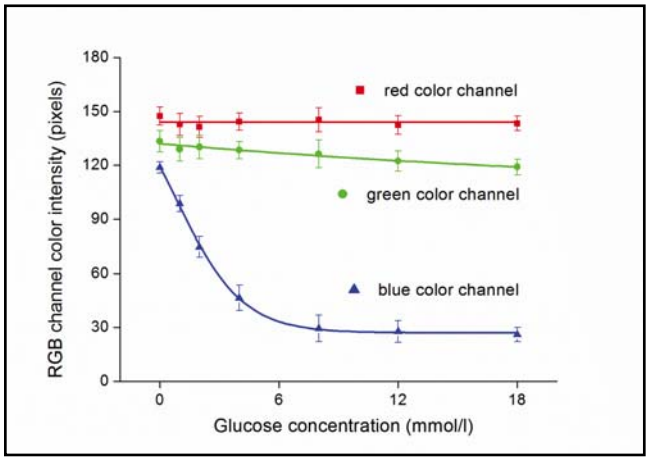

Fig. 2. Glucose concentration curves measured in red, green and blue color channels. MPs-GOx-HRP transformed colorless substrate o-PD inside bubbles of bubble wrap to yellow-orange solution and color intensity of solution was detected by smartphone integrated camera. Error bars indicate standard deviation for n=5.

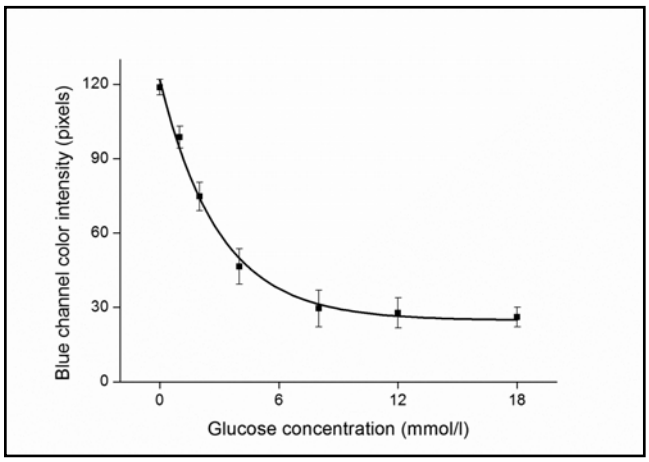

Fig. 3. Glucose concentration curve measured in blue color channel using MPs-GOx-HRP and substrate o-PD inside bubbles of bubble wrap. Error bars indicate standard deviation for n=5.

Comparison of reference and innovated method

Color intensities acquired by data processing during spiked plasma assay were compared with results measured according to article published by Morin and Prox (1973) with minor modification: o-PD was used as chromogen instead of reduced sodium p-diphenylamine sulfonate.

RESULTS AND DISCUSSION

Concentration curve

Data from blue channel of RGB color system was processed and glucose concentration curve was constructed (Figure 2). Blue color channel of RGB was chosen as the most accurate one for gaining the information about color intensity of bubbles depending on the glucose concentration. The exponential decay curve of blue color channel showed in the Figure 3 replies increasing glucose concentration with correlation coefficient 0.997 and limit of detection (S/N=3) was set to be 0.4 mmol/l. Concentration curve had linear dependence in concentration range from 0 and 4 mmol/l with slope –17.3. The principle of our work was inspired by study of Bwambok and coauthor (2014) where determination of glucose inside bubble wrap using different chromogen (potassium iodide in PBS) and the same enzymes (GOx, HRP) was performed by digital camera or scanner. Comparing with our article the blue channel for numeric expression of color intensity was also chosen as the one with the best properties and the decreasing direction of the concentration curve was as well showed in Bwambok and coauthors (2014) work. Concerning limit of detection, the value 0.4 mmol/l is more than satisfying due to physiologically occurring concentration of glucose in blood stream. Even if the blood glucose level is lowered as a result of poisoning or other pathological conditions the glucose concentration below 2 mmol/l causes brain damage and brain death so too low glucose blood level is not viable (Cryer 2007). Limit of detection can be further improved by prolongation of reaction time if necessary, on the other hand, the reverse inhibition of reaction by increasing hydrogen peroxide could be observed due to damaging of GOx structure so the prolongation of reaction time is not demanded (Harris *et al.* 2013).

Interferences and matrix effect

Selectivity of our method was proved by testing interfering substances as assay substrates instead of glucose. Color intensities of positive control (4 mmol/l glucose) and negative control (water) were compared with color intensities of interfering substances – sugars in the same concentration as glucose (4 mmol/l). As shown

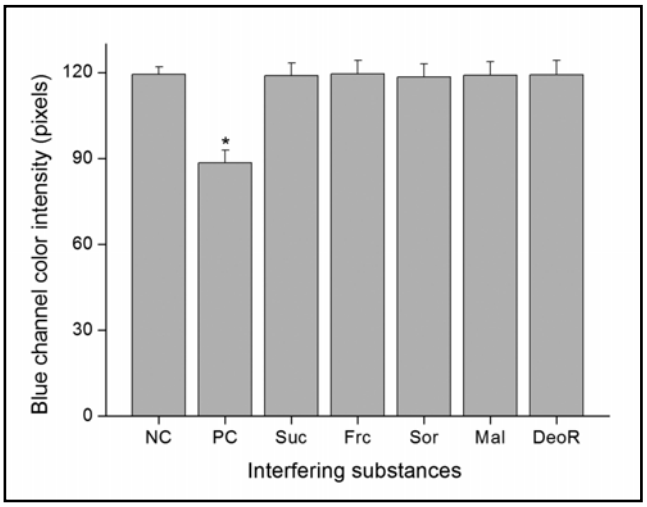

Fig. 4. Comparison of blue channel color intensities of potentially interfering substances (Suc – sucrose; Frc – fructose; Sor – sorbitol; Mal – maltose; DeoR - deoxyribose) with color intensity of glucose (PC – positive control) and water (NC – negative control). Results significantly different from NC are marked by the symbol * above column. Error bars indicate standard deviation for n=5.

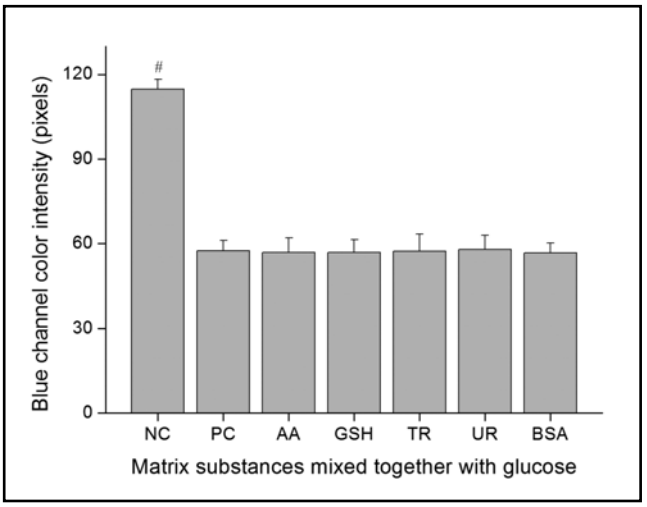

Fig. 5. Graph of effect of substances (AA – ascorbic acid; GSH – reduced glutathione; TR – trolox; UR – urea; BSA – bovine serum albumin) naturally occurred in plasma on glucose determination. Color intensity of substances mixed together with glucose were compared with glucose alone (PC – positive control) and water (NC – negative control). Results significantly different from PC are marked by the symbol # above column. Error bars indicate standard deviation for n=5.

in Figure 4, color intensity of sugars such as fructose, maltose, sucrose, sorbitol and deoxyribose in blue color channel are not significantly different from color intensity of negative control and they are significantly different from positive control. Glucose has the lowest blue channel numeric data, which means (according to decreasing direction of concentration curve) that the glucose has the highest response of detection device. So, the other sugars are not able to be substrates for enzyme GOx and the reaction is highly selective for glucose. Other sugars cannot affect the assay. Our results comply with results measured by Ho and coauthors (2014), whose also tested chosen sugars (sucrose, fructose, maltose etc.) as possibly interfering substances and they proved no influence of the sugars on GOx or glucose determination.

Matrix effect measurement should reveal the influence of matrix substances on glucose assay. Blue channel color intensities of negative control (water) and positive control (4 mmol/l glucose) were compared with color intensities of matrix substances (in concentration twice higher than their physiological value) mixed together with 8 mmol/l glucose in ration 1:1. In Figure 5, color intensities of all analytes are shown. Color intensities of matrix substances mixed together with glucose are not significantly different from positive control on the other hand they are significantly lower than negative control. That means that tested matrix substances do not affect the transformation of glucose on gluconolactone by GOx and so the glucose assay. Our findings correlated with the recent works such as studies of Turkmen and coauthors (2014) and of Homma and coauthors (2014).

Spiked plasma samples

Spiked plasma samples were measured due to possibility of influencing the assay by plasma matrix. Mice plasma samples were spiked by glucose standards in range from 0 to 36 mmol/l in 1:1 ratio so the concentration of glucose in measured samples were from 0 to 18 mmol/l. Color intensities of spiked plasma curve are showed in Figure 6. Exponential decay concentration curve have correlation coefficient 0.999 and limit of detection was equal to 0.43 mmol/l. Slope of straight line in concentration range from 0 to 4 mmol/l has slope equal to –18.9. Because of the very similar slope and shape of standard concentration and spiked plasma curve we are able to say, that minimal effect of plasma matrix was observed.

Comparison of standard and the new method

Color intensities of spiked plasma samples (in concentration range from 0 to 16 mmol/l) measured according to method innovated by Morin and Prox (1973) and measured according to our innovated method were mutually compared (Figure 7). The innovated method had linear dependency with reference method in concentration range from 0 to 4 mmol/l with correlation coefficient 0.999. In this extent, the novel method has better sensitivity than reference assay. On the other hand, in concentration range from 8 to 16 mmol/l, reference method has better sensitivity than novel method. It is possibly caused by inhibition of GOx by higher concentration of hydrogen peroxide increasing during reaction in higher glucose concentration (Harris 2013). Moreover, advantages of the innovated method

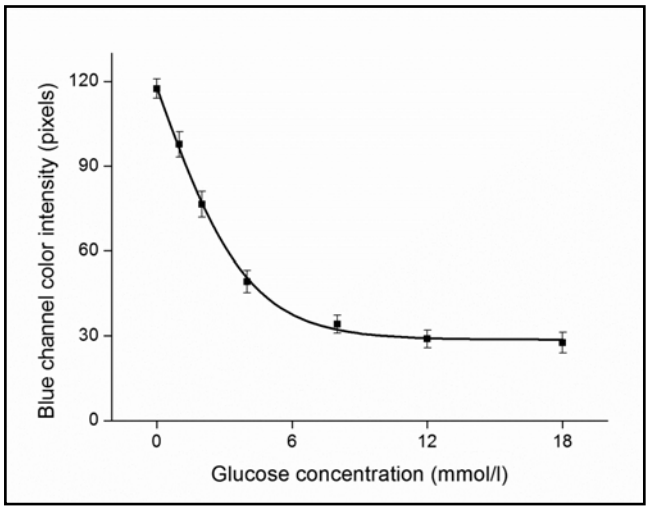

Fig. 6. Concentration curve of spiked plasma samples with exponential decay direction replying increasing glucose level. Error bars indicate standard deviation for n=5.

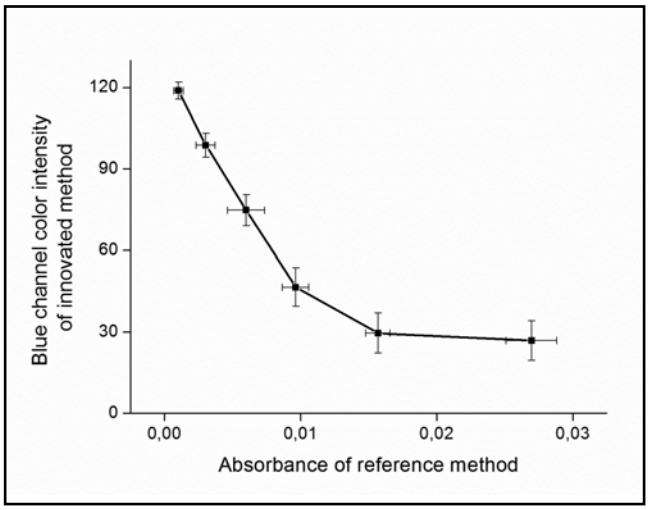

Fig. 7. Comparison of reference and novel method measured using spiked plasma samples in concentration range from 0 to 16 mmol/l.

lie in a small amount of needed chemicals and analytes, available detection device or simple handling.

CONCLUSION

Colorimetric sensor for glucose determination using enzymes GOx and HRP immobilized onto surface of MPs and inside bubble wrap as reaction container was performed by phone camera. Optimization of this method came from previous works (Morin & Prox 1973; Martinkova *et al.* 2016 and Bwambok *et al.* 2014) but the connection of well-known principles has brought the simple, fast, specific and affordable assay with adequate concentration measuring range and limit of detection with no known interfering or assay affection substrates and with better properties than reference method.

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