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Towards low-cost bioanalytical tools for sarcosine assays for cancer diagnostics

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Sarcosine is an amino acid that has been listed as a new indicator for prostate cancer. We present here two low-cost tools with the potential to detect this biomarker excreted in urine. The first one is a paper-based microfluidic device manufactured by wax printing technology in which an enzymatic assay is performed. The enzymatic assay comprises two coupled enzymatic reactions between sarcosine oxidase and horseradish peroxidase, with the ABTS redox indicator oxidized when sarcosine is present in the sample. This enzymatic assay on paper could be used as a preliminary screening method and presents a linear range up to 1 mmol L⁻¹, with a sensitivity of 21.2 A.U. (mmol L⁻¹)⁻¹; LOD = 0.21 mmol L⁻¹; LOQ = 0.61 mmol L⁻¹ and $r^2 = 0.8898$. The second bioanalytical approach consists of a capillary electrophoresis method with capacitively coupled contactless conductivity detection (CE-C⁴D). This method proved to be useful to separate sarcosine from 12 amino acids typically found in normal human urine using 10 mmol L⁻¹ of triethylamine (TEA) as the background electrolyte. This method could be used as a follow-up method after the initial screening and presented a stable baseline, LOD = 0.034 mmol L⁻¹ and LOQ = 0.069 mmol L⁻¹. Migration time reproducibility (RSD) for intraday and inter-day assays was smaller than 0.6% and 4.4%, respectively. Both tools were able to analyze this potential tumor marker for prostate cancer, with capabilities of miniaturization and low-cost, essential characteristics for point-of-care testing (POCT) technology. Paper-based devices are inexpensive and simple, making them ideal for screening tests and telemedicine, while separation by CE-C⁴D presents high sensitivity and throughput, useful characteristics for exploratory studies.

Introduction

Tumor biomarkers are molecules indicative of the existence of neoplastic processes^{1–3} and can be present in a variety of

biological matrices and fluids, such as plasma, urine, and saliva, among others.^{4–6} These markers are useful for cancer detection and appear as one of the main tools for the early identification of tumors and for the evaluation of the effectiveness of cancer treatments.^{1–3}

Sarcosine (*N*-methyl-glycine) is an amino acid derived from glycine due to oxidative methylation by the glycine-*N*-methyltransferase (GNMT) enzyme.⁷ The elevated sarcosine concentration is related to increased GNMT activity, and a reduction of sarcosine dehydrogenase (SARDH) and pipecolic acid oxidase (PIPOX) enzyme activities, which catalyze the oxidative demethylation of sarcosine to glycine.⁸ The increased GNMT and decreased SARDH and PIPOX activities were observed in prostate cancer (PCa) progression, especially in more advanced stages such as metastasis.⁸ Low levels of sarcosine are found in the urine of healthy individuals (~20 nmol L⁻¹); however, for patients with prostate cancer these levels can reach micromolar levels (or sub-micromolar when the patient is under treatment).⁹ Thus, sarcosine arises as a potential biomarker for early prostate cancer detection and can be used as the target analyte in screening tests,¹⁰ besides its excretion in urine, which also allows for a non-invasive diagnosis method.⁷

The most common techniques used for the determination of sarcosine are gas chromatography coupled with mass spectrometry (GC-MS),¹¹ liquid chromatography coupled with mass spectrometry (HPLC-MS)¹² or UV-vis detection (HPLC-UV) and capillary electrophoresis with laser-induced fluorescence detection (CE-LIF).¹³ Chromatographic separation techniques using MS as the detector have a high cost, whereas fluorescence detection techniques such as CE-LIF require an additional derivatization step.¹⁴

Capillary electrophoresis coupled with capacitively coupled contactless conductivity detection (CE-C⁴D) is an advantageous technique to analyze sarcosine in comparison with other analytical tools, since reagent and sample volume consumption is small and the analysis time is reduced, with the advantage of direct detection, which dispenses derivatization steps or chromophore addition in the background electrolyte.^{14,15} Moreover,

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CE- C^4D can be miniaturized in a microchip format,¹⁶ which brings portability to the system and allows its application as a point-of-care testing tool.^{17–20}

Other low-cost methods for detection of sarcosine include enzymatic assays, with either colorimetric or fluorometric detection.^{21,22} When these colorimetric tests migrate from batch processes to a paper-based format there is a decrease in the cost down to cents of dollars²³ due to the low-cost of materials and manufacture of these devices, and also the replacement of fluorescence readers and spectrophotometers with digital cameras or cell phones.^{24–26} Moreover, paper-based devices are portable, simple, inexpensive and user-friendly,²⁷ and arise as an alternative for the lack of resources to perform diagnostic tests in remote regions and rural areas.^{23–25,28}

We present here two methods that can provide a quick assessment of sarcosine; one, with the potential to be applied as a POCT tool for needy regions, bringing oncology bioanalyses where they are needed, and the other, as a companion method, that could be used to confirm diagnostics.

Materials and methods

Chemicals and instrumentation

Here we list the chemicals used and their respective suppliers: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS), sarcosine oxidase from *Bacillus* sp. (E.C 232-850-5), horseradish peroxidase (HRP) (E.C 232-668-6), L-glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-proline, L-phenylalanine, L-tyrosine, L-tryptophan, L-serine, L-threonine, L-cysteine, L-methionine, L-asparagine, L-glutamine, L-lysine, L-arginine, L-histidine, L-glutamic acid, and L-aspartic acid were purchased from Sigma-Aldrich (Saint Louis, MO, USA); triethylamine (TEA) was purchased from J.T. Baker (Phillipsburg, USA); NaCl, KCl, Na_2HPO_4 , and KH_2PO_4 were purchased from Synth (São Paulo, Brazil). All reagents were used as received.

Paper-based device fabrication

A paper-based microzone plate,²³ similar to an enzyme-linked immunosorbent assay (ELISA) microplate was designed using CorelDraw CS4 software, and printed on sheets of chromatographic papers Whatman No. 1 (letter size) using the Xerox Phaser 8560 wax printer. The printed wax patterns on the chromatographic paper were subjected to a thermal treatment on a heat press during 2 min at 150 °C, in order to melt the wax and let it permeate the entire thickness of the paper, creating the hydrophobic barriers, delimiting the microzones, where reactions took place.²⁸

Colorimetric assay for sarcosine detection

A volume of 5 μ L of a sarcosine oxidase (120 U mL^{-1}) solution diluted in PBS buffer (0.01 mol L^{-1} ; pH 8.3) was added on each spot on the paper-based microplate. After 20 min, with the spot dried, 5 μ L of a horseradish peroxidase solution (30 U mL^{-1}) diluted in PBS buffer (0.01 mol L^{-1} ; pH 6.0) were added on each spot on the microplate, and after 20 min, 5 μ L of chromophore solution were applied on the zones on the microplate. The

chromophore solution consisted of 25 mmol L^{-1} of the ABTS redox indicator diluted in PBS buffer (0.01 mol L^{-1} ; pH 6.0). This protocol was modified from well-established tests used for glucose detection on a paper platform.^{25,29} The enzyme concentration used (120 U mL^{-1}) was the same as that used for glucose oxidase in the glucose assay, since sarcosine oxidase exhibits the same level of activity as glucose oxidase, as described by the manufacturer (Sigma-Aldrich).

Sarcosine standards were prepared by dilution of a stock solution of 1 mol L^{-1} of sarcosine on PBS buffer (0.01 mol L^{-1} ; pH 6.0), in the following concentrations ranging from 20 mmol L^{-1} down to 0.1 mmol L^{-1} . Each sarcosine standard (5 μ L) was applied in triplicate to the microplate containing the enzymes and the chromophore. Blank measurements consisted on the application of 5 μ L of PBS buffer (0.01 mol L^{-1} ; pH 6.0) to the microzones containing the reagents. Furthermore, solutions of all twenty natural amino acids were prepared (4 mmol L^{-1}) on PBS buffer (0.01 mol L^{-1} ; pH 6.0), and 5 μ L of each amino acid solution were applied in triplicate in order to evaluate the specificity of the sarcosine oxidase enzyme. After 20 min the spots on the microzones were completely dry and the microplates were digitalized using a flatbed scanner. The average intensity of the color formed was obtained using the software Adobe Photoshop CS4, but other open source software can be used as well. The whole process, from the design of the microplate to the readout of the assay is represented in Fig. 1.

Instrumentation

CE analyses were carried out using the P/ACE MDQ capillary electrophoresis equipment (Beckman Coulter, Fullerton, CA) adapted with a C^4D detector. A bare fused-silica capillary (with 360 μ m outer diameter (OD) and 50 μ m inner diameter (ID)) with a total length of 50 cm (35 cm to the detection window, effective length) was purchased from Polymicro Technologies (Phoenix, AZ). An Ohm's plot for each buffer tested in the CE separations defined the maximum applied voltage during the separation, avoiding excessive Joule heating in the capillary. Samples were injected by applying a positive 0.5 psi pressure to the sample vial for 6 s, resulting in a sample plug of 7.13 nL (~1% of the capillary volume until the detection window). The bare fused-silica capillary was conditioned each day by rinsing it with ultrapure water (2 min), followed by rinsing with a basic solution of sodium hydroxide 100 mmol L^{-1} (5 min), followed by a second rinse with ultrapure water (3 min), and finally rinsing with the running buffer (3 min). A pressure of 20 psi was applied to the system in all rinsing steps.

Detection system (C^4D)

The detection system used in conjunction with the CE equipment was a C^4D commercial system (model ER225) with the ET120 headstage (EDAQ, Sydney, Australia). The C^4D detector was optimized for every running buffer solution using the C^4D profiler software, which automatically varied the frequency and amplitude of the signal. The capillary was filled with BGE by applying a pressure of 20 psi for 1.5 min and the optimization software was started. The 12 amino acids and the sarcosine were

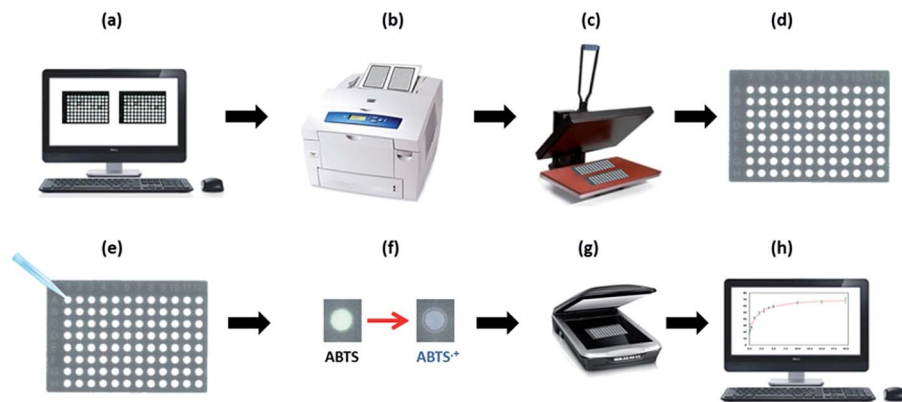


Fig. 1 Steps involved in the production of paper microzone plates and analysis of an enzymatic paper-based assay. (a) Definition of the design of the microdevice. (b) Wax printing on the paper surface. (c) Melting and permeation of the wax on paper. (d) Microplate finalized. (e) Addition of the enzyme solutions. (f) Development of the enzymatic reactions. (g) Scanning of the microplate. (h) Image analysis and diagnostics.

detected using a frequency of 1100 kHz, an amplitude of 1.8 V peak to peak, and the signal gain mode “on”.

Results and discussion

Paper-based colorimetric assay for sarcosine detection

Paper-based microfluidic devices (μ PADs) using the wax printing technology have a cost of cents of dollars.²⁸ The low-cost and versatility attributed to μ PADs are ideal characteristics for screening assays, which can be applied for sarcosine analysis. Although instrumental techniques are commonplace to amino acid analysis, we present here, for the first time, a paper-based assay using enzymes and a redox indicator for quantitative sarcosine analysis. The colorimetric assay for sarcosine detection is based on two coupled enzymatic reactions.

Sarcosine oxidase (SOX) catalyzes the demethylation of sarcosine in the presence of oxygen and water, which yields glycine amino acid, formaldehyde, and hydrogen peroxide as products (eqn (1)). In the subsequent reaction, horseradish peroxidase catalyzes the reduction of the hydrogen peroxide to water, oxidizing the redox indicator – in this case, ABTS to $\text{ABTS}^{+\cdot}$ (eqn (2)).²⁹ The redox indicator in its oxidized form presents a distinct blue-green coloration, which is detected (Fig. 1f).

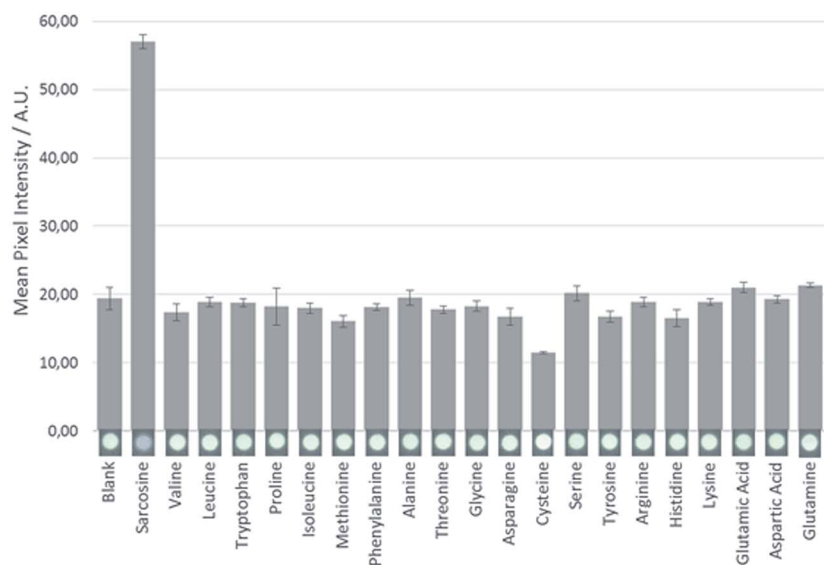
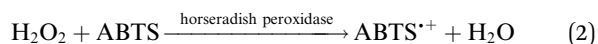
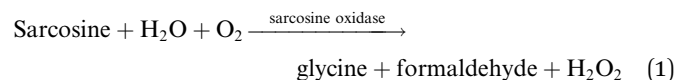


Fig. 2 Evaluation of sarcosine oxidase specificity towards sarcosine (5 μ L of 120 U mL^{-1}) against all twenty natural amino acids (4 mmol L^{-1}) on PBS buffer (0.01 mol L^{-1} ; pH 6.0) on the microzone paper plates. Each bar represents the average of triplicate measurements, and the error bars represent the 95% confidence interval of Student's *t*-distribution. The image at the bottom of each bar shows a representative image of the colorimetric assay.

Digitalized colorimetric assays were analyzed by Adobe Photoshop software using the histogram tool in the RGB channels. The analytical signal in those tests is the mean pixel intensity of the color developed in each zone in the microplate. The mean pixel intensity in the RGB color model (eqn (3)) ranges from 255 (white) to 0 (black), which translates smaller response signals to more colored (highly concentrated samples) in these colorimetric assays. In order to obtain an analytical curve with a positive correlation coefficient the scale was inverted by subtracting the obtained signal (S_{obt}) from 255, to obtain a corrected signal (S_{corr}) (eqn (4)). It is worth mentioning that the use of the grayscale (eqn (3)), in this assay, has demonstrated superior results than the use of the separated channels in the RGB color model analysis.

$$S_{\text{obt}} = \frac{R + G + B}{3} \quad (3)$$

$$S_{\text{corr}} = 255 - S_{\text{obt}} \quad (4)$$

In order to evaluate the assay specificity to sarcosine, all 20 natural amino acids were used as substrates to the sarcosine oxidase enzyme (Fig. 2), once in biological matrices where sarcosine can be found, those biomolecules could be present as

well, with emphasis to glycine, which is the substrate of sarcosine biosynthesis.⁷

As can be seen in Fig. 2, the signals obtained with amino acids other than sarcosine as substrates for the sarcosine oxidase enzyme are comparable with the blank signal, so there was no measurable activity of sarcosine oxidase towards natural amino acids as substrates. The signal obtained with sarcosine as the substrate for SOX is ~ 3 times the signal of blank measurements, which shows the specificity of the enzyme towards the substrate and the specificity of the assay itself, which also suggests that any natural amino acid present in urine or plasma will not result in a false-positive outcome in this colorimetric assay.

The analytical curve for the sarcosine assay is represented in Fig. 3a. The ABTS redox indicator responds to analyte concentrations as low as 0.3 mmol L^{-1} ; however, this assay presents a small linear range (below 3.0 mmol L^{-1}) and signal saturation occurs above 10 mmol L^{-1} (a *t*-test was performed comparing the upper and the lower limits, in order to identify the signal saturation concentration). Due to this reason, a growth and saturation curve (eqn (5)) was fitted to describe the behavior of the signal, as reported by Chaplan *et al.*²⁹

$$Y = \frac{S_{\text{max}}[\text{conc}]}{K + [\text{conc}]} \quad (5)$$

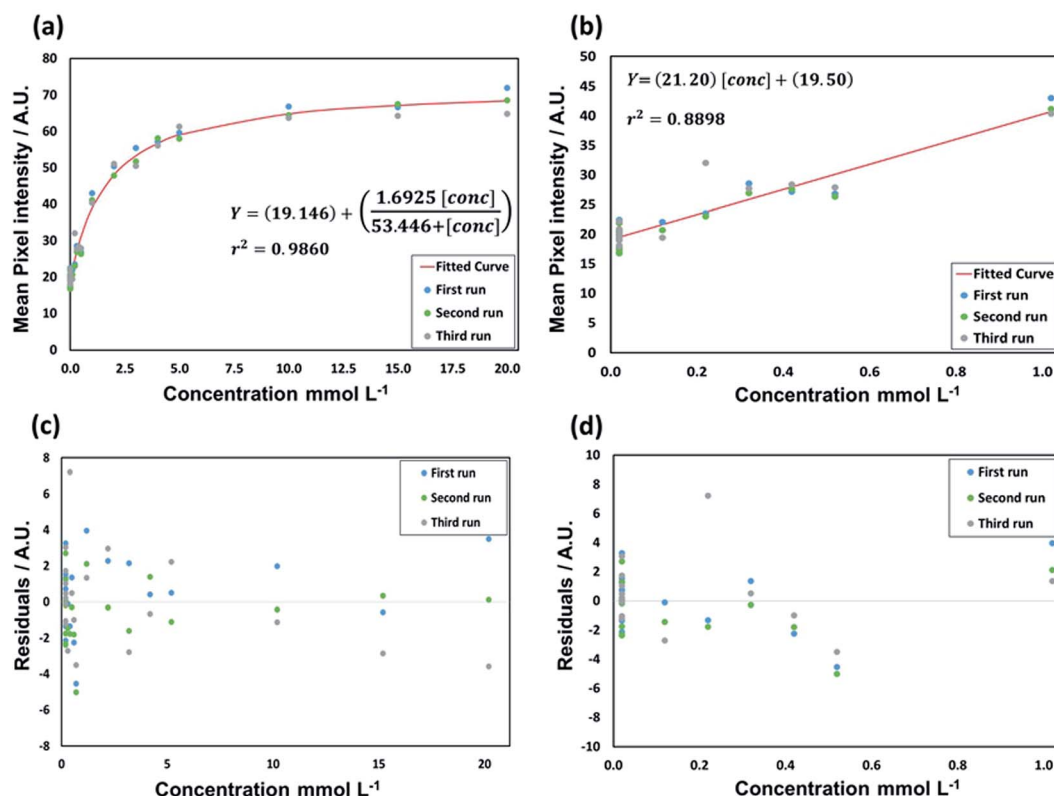


Fig. 3 Analytical parameters obtained for sarcosine analysis in paper-based enzymatic assay. (a) Analytical curve for the sarcosine oxidase assay with fitting of the data with a saturation model. (b) Linear regression plot for the linear region of the sarcosine oxidase assay (0 to 1 mmol L^{-1}). (c) Residual distribution plot for the growth and saturation model for the sarcosine oxidase assay. There is no apparent trend in the distribution of the residuals. (d) Residual distribution plot for the linear region of the sarcosine oxidase assay. There is no apparent trend in the distribution of the residuals.

where Y is the analytical signal, $S_{\max}$ is the maximum analytical signal, $[\text{conc}]$ is the concentration of the analyte, and K is a constant.

This model fits the data well; as it can be observed in the residual plot (Fig. 3c) and by an F -test performed for this dataset (data not shown). However, some issues are related to this approach. It is necessary to attribute a physical meaning to the constant K . Chaplan *et al.*²⁹ observed that K was not affected by the lighting conditions during test digitalization, but there is still a lack of understanding about this constant and which factors affect it. Another problem related to this approach is the forced zero-point condition, where all the points of the curve are subtracted from the blank measurement. This approach is problematic because information regarding the dispersion of the blank measurements and the measurement of the standard itself is lost. As long as paper is an anisotropic material,³⁰ the paper signal presents an intrinsic variation spot by spot. When subtracting an average blank value from a standard value, the errors associated with the blank dispersion are not taken into account (because an average value was used), which could lead to virtually better results, due to the non-propagation of errors in this procedure. Also, this colorimetric assay is heteroscedastic in nature, so the direct subtraction of standard measurements from a blank average value should be avoided in order not to overestimate method capabilities.³¹

When a non-linear model is fitted to the dataset the figures of merit of the analytical method are affected. For example, the sensitivity of the method is not constant throughout the entire range of the analytical curve and it becomes range dependent. One common approach is to find the dynamic linear range of the calibration curve and using these concentration values to estimate the figures of merit of the method.

The dynamic linear range for the sarcosine oxidase assay is represented in Fig. 3b. This range is confirmed to be linear by an F -test (data not shown) and by the analysis of the residual plot (Fig. 3d). When considering the dynamic linear range, the figures of merit for this method are a sensitivity of 21.2 A.U. mmol L^{-1} ; limit of detection (LOD) = 0.21 mmol L^{-1} ; limit of quantitation (LOQ) = 0.61 mmol L^{-1} ; $r^2 = 0.8898$.

As stated by Cernei *et al.*,⁹ sarcosine is present in the urine of patients with prostate cancer at concentrations of $10^{-6} \text{ mol L}^{-1}$, which would require an improvement of ~ 2 orders of magnitude on the LOD and LOQ of the present test. This requirement, however, does not hinder the application of paper-based microfluidic devices on the detection of such small amounts of analytes. As presented by Wong *et al.*,³² a tuberculosis biomarker was concentrated 20-fold in an artificial urine sample, demonstrating the feasibility of the method. Moreover, Han *et al.*³³ reported a biomolecule preconcentrator on a paper-based device using the ion concentration polarization (ICP) effect, achieving preconcentration factors up to 1000-fold for fluorescent dyes and BSA, which could reach the clinical levels required for sarcosine urinalysis.⁹ So as a next step, we envision the development of a unique functional device that incorporates a pre-concentrator before the testing zones, which will reach the concentration limits required for this kind of bioanalysis. Other useful approaches to improve the figures of merit of the analytical

method would be the combination of redox indicators,³⁴ or using a diffuse reflectance spectrophotometer as the detector.³¹ Although we propose this bioassay as an inexpensive screening tool, our paper-based microfluidic device technology does not yet attend the sensitivity requirements for sarcosine analysis. Certainly, a more instrumental approach such as capillary electrophoresis can be used to overcome this issue. In this regard, CE can be miniaturized using low-cost materials, such as a polyester-toner (PeT),³⁵ and we further investigated CE- C^4D as a companion low-cost bioanalytical tool for sarcosine analysis.

Separation condition optimization

An essential feature of BGE solution in CE is the low conductivity in order to minimize Joule heating on the capillary besides providing high sensitivity towards C^4D detection.³⁶ Another important characteristic of the background electrolyte is the pH, since the degree of ionization of the analytes and the amount of negatively charged silanol groups of the fused-silica capillary are the function of BGE pH.³⁷

Sarcosine presents two ionizable hydrogens, one from the carboxylic acid group (pK_a 2.06) and the other from the

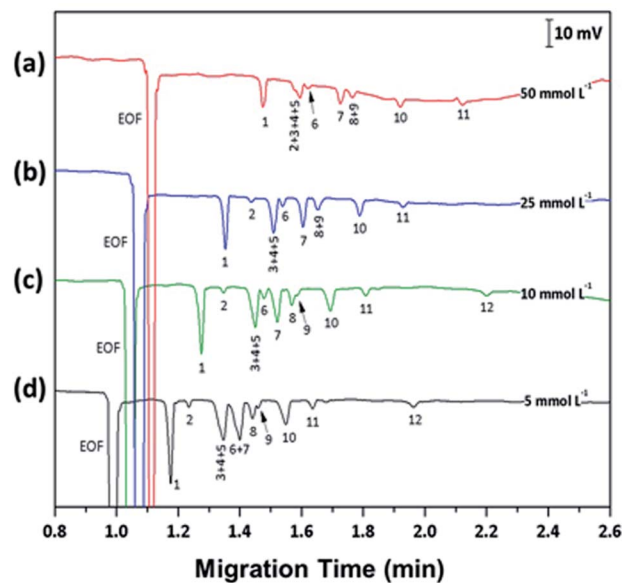


Fig. 4 Selection of the background electrolyte for CE- C^4D analysis for sarcosine in the presence of 12 amino acids, typically found in normal human urine.³⁹ BGE was TEA as indicated in the figure. Capillary: 360 μm and 50 μm (OD and ID, respectively). The total length was 50 cm and effective length of the capillary was 35 cm. The injection was carried out by applying a pressure of 0.5 psi for 6 s. A frequency of 1100 kHz, an amplitude of 1.8 V peak to peak, the amplitude was set to 100%, and the headstage gain was on. Peak identification: (1) lysine ($240 \mu\text{mol L}^{-1}$); (2) proline ($20 \mu\text{mol L}^{-1}$); (3) leucine ($100 \mu\text{mol L}^{-1}$); (4) isoleucine ($20 \mu\text{mol L}^{-1}$); (5) phenylalanine ($40 \mu\text{mol L}^{-1}$); (6) methionine ($30 \mu\text{mol L}^{-1}$); (7) sarcosine ($100 \mu\text{mol L}^{-1}$); (8) alanine ($50 \mu\text{mol L}^{-1}$); (9) serine ($20 \mu\text{mol L}^{-1}$); (10) glycine ($90 \mu\text{mol L}^{-1}$); (11) tyrosine ($30 \mu\text{mol L}^{-1}$); (12) glutamic acid ($10 \mu\text{mol L}^{-1}$). Arginine ($110 \mu\text{mol L}^{-1}$) co-migrated with the EOF marker as its net charge was approximately zero (a pI of 10.92). (a) TEA 50 mmol L^{-1} . (b) TEA 25 mmol L^{-1} . (c) TEA 10 mmol L^{-1} and (d) TEA 5 mmol L^{-1} .

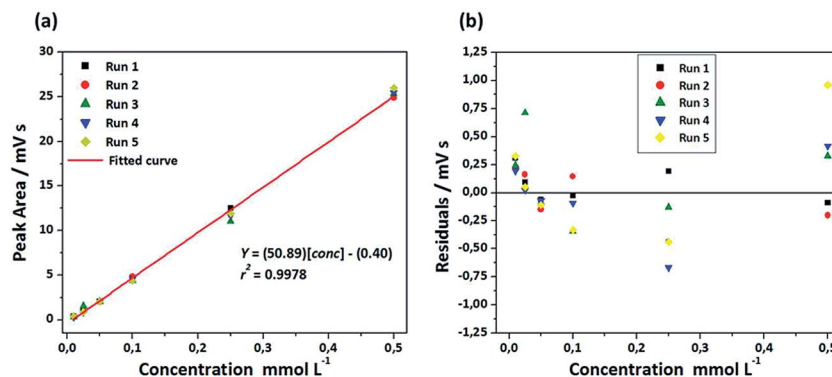


Fig. 5 Analytical data for sarcosine determination by CE-C⁴D. (a) Analytical curve for sarcosine. (b) Residual distribution plot for the linear regression of the sarcosine analytical curve. There is no apparent trend in the distribution of the residuals.

protonated amine group (pK_a 10.35), which translate to a zwitterion with a pI of 6.2.³⁸ In order to improve the separation of this amino acid a high pH BGE was chosen, composed of triethylamine (TEA), pK_a 10.8, in the following concentrations: 5 mmol L⁻¹ (pH 10.9); 10 mmol L⁻¹ (pH 11.3); 25 mmol L⁻¹ (pH 11.5) and 50 mmol L⁻¹ (pH 11.7). The electropherograms for the analysis of sarcosine in a distilled water solution containing 12 amino acids commonly found in human urine are shown in Fig. 4. In a higher pH BGE the amine and carboxylic groups of sarcosine are deprotonated and the compounds present a net negative charge, which is necessary for electrophoretic mobility and C⁴D detection. The high pH of BGE also completely deprotonates the silanol groups of the fused-silica capillary, leading to a high and stable electroosmotic flow (EOF), and consequently to a short analysis time.

For the lowest concentration of BGE (5 mmol L⁻¹) (Fig. 4d) a co-migration between methionine and sarcosine occurred, which did not happen for increasing concentrations of BGE. When TEA concentration was increased to 10 mmol L⁻¹ there was a complete separation of methionine and sarcosine peaks and a stable baseline was obtained. When comparing BGE concentrations of 10 mmol L⁻¹ (Fig. 4c), 25 mmol L⁻¹ (Fig. 4b) and 50 mmol L⁻¹ (Fig. 4a), we noticed that the lower concentration (10 mmol L⁻¹) of BGE separated a greater number of amino acids and maintained the size of the peak of sarcosine, while for higher concentrations an instability in the baseline and a decrease in resolution were observed.

The BGE 10 mmol L⁻¹ TEA was used for the determination of the reproducibility intra- and inter-day of the proposed method. The analytical curve obtained for sarcosine is represented in Fig. 2. A linear regression was performed in this dataset, and no trend was observed in the residual plot (Fig. 5b), which corroborates with the model choice. The figures of merit for this method are: LOD = 0.034 mmol L⁻¹; LOQ = 0.069 mmol L⁻¹; $r^2 = 0.9978$. A fresh amino acid mix was prepared each day by dilution of the stock solutions. The sample was consecutively injected 10 times (each day) for 3 consecutive days. Migration time reproducibility (relative standard deviation – RSD) intraday and inter-day was less than 0.6% and 4.4%, respectively, which shows good method stability.

Conclusions

Capillary electrophoresis proved to be a suitable method for sarcosine analysis, due to the reproducibility, sensitivity, speed of analysis and low consumption of the sample and reagents. In fact, the total analysis time was less than 2.5 min, and this method proved useful to separate sarcosine from 12 amino acids typically found in normal human urine by optimization of BGE concentration (10 mmol L⁻¹ of TEA), being possible to evaluate the electrophoretic profile of this potential tumor biomarker in a complex sample. This method allowed for rapid and direct detection of amino acids without the need for derivatization steps, and future research involves system miniaturization using low-cost materials, such as polyester toner devices (PT), for point-of-care applications.

Paper-based devices meet the requirements of point-of-care testing (POCT) technology due to advantageous characteristics such as: (i) portability; (ii) low cost; (iii) great versatility to support a variety of assays; and (iv) applicability in less resourceful regions on screening campaigns with skilled personnel or independently, by telemedicine. By using sarcosine oxidase, peroxidase and a redox indicator (ABTS) a colorimetric method was proposed for the first time, and the intensity of the generated color was proportional to sarcosine concentration. This paper-based enzymatic assay was able to detect a potential tumor marker for prostate cancer – sarcosine – with great specificity, with no activity towards all twenty natural amino acids that could be concomitant in urine. Even though the methods hereby reported did not meet the physiological sarcosine level requirements, several modifications are compatible with our systems, attending to the aforementioned requirements. These modifications can include, but are not limited to: addition of a preconcentrator,³³ change in detection methods *e.g.*, use of a diffuse reflectance spectrophotometer³¹ or using a combination of redox indicators.³⁴ This assay has the great capability to be manufactured and applied locally, meeting the requirements of POCT, which can improve the health monitoring of the population.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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