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Simple paper-strip colorimetric method utilizing dehydrogenase enzymes for analysis of food components

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Abstract

In this study, we present a simple chromatographic paper-based colorimetric assay for the analysis of selected food compounds. The biochemical principle utilises enzymes from the dehydrogenase family coupled with diaphorase in the presence of a tetrazolium dye, MTT, and NAD^+. The colored reaction product that developed on the surface of the chromatography paper could either be qualitatively analysed by the naked eye, that is, it could be used for screening analysis, or it could be recorded by a smartphone camera and evaluated in freeware ImageJ in order to obtain data for quantitative analysis. The general concept was demonstrated and optimised using (L)-glutamate dehydrogenase and the versatility of the proposed method was illustrated by using other enzymes from the dehydrogenase family. The linear range for (L)-glutamate was found to be 0.5–5.0 mmol L^{-1} using the digital image method. The limit of detection found by the naked eye was 0.05 mmol L^{-1} and 0.028 mmol L^{-1} by digital imaging. The paper assay stored in the freezer provided a reproducible response for at least 6 weeks. This research presents the successful modification of an MTT-cell viability test principle and its use in the fabrication of an inexpensive, easy to prepare, simple to use, portable and rapid-responding paper-based assay.

Keywords: paper assay; colorimetric detection; dehydrogenase enzyme; food analysis.

1. Introduction

Development of analytical methods utilising a paper platform dates back several decades [1]. The main advantages of paper flow tests are usually that they do not require an external auxiliary instrumentation such as pumps or power supplies, their low-cost character, ability to perform rapid tests, portability and simplicity. In particular, the fact that they do not necessitate external devices make them an attractive alternative to conventional microfluidics [2]. Most of the paper-strip methods presented in previous reports were designed for clinical diagnostic applications, for example, the transaminase test for point-of-care liver function testing [3], glucose [4] or the combination of glucose and uric acid in human serum [5], and so forth. Paper-based assays have also found applications in the direct measurement of alkaline phosphatase kinetics [6], antimicrobial susceptibility assays [7] and the detection of foodborne pathogens [8].

In this study, we focused on the development of a simple paper-based assay for the analysis of various food components. The paper is an inexpensive, easily modifiable and biocompatible material, allowing liquids to be transported by capillary forces [9]. The primary criteria for the development of our paper strips were defined as: i) low cost and easy to use, ii) simple fabrication procedure without using expensive machines and hazardous reagents, iii) functionality without using expensive external instruments and iv) a simple detection principle. For this purpose, the detection should, ideally, be realised by the naked eye for qualitative or semi-quantitative measurements, or by a smartphone camera for quantitative analysis. In particular, imaging principles performed by a smartphone camera are interesting and represent an emerging technology in the case of colorimetric paper strips [10], as it keeps the method simple, low cost and can help to improve accuracy. This is a particular advantage for analysis *in situ* in poorly equipped environments or in areas with limited financial and material resources, owing to the vast availability of mobile-phone technology to billions of users worldwide [11], and this number is still increasing.

Herein, we propose new applications of paper strips in food analysis based on colorimetric detection utilising an enzyme principle. The main principle of the well-known colorimetric assay for assessing cell viability performed in multi-well plates, the so called MTT test [12], was adapted, simplified and optimised for its use on chromatography paper. We tested various dehydrogenase enzymes to demonstrate the versatile application of the method for specifically analysing select food components. The color intensity of the purple reaction product was analysed both by the naked eye, that is, screening analysis[13], and using freeware ImageJ after taking a picture on a smartphone camera for quantitative analysis. The chromatography paper platform was successfully applied for the simple, rapid and low-cost flow-lateral analysis of various food components using

the same reaction principle. Moreover, the method did not require sophisticated approaches in terms of a paper pre-treatment, as is often the case in the fabrication of paper for total micro-analysis systems, also called lab-on-chips or microfluidic paper-based analytical devices [14].

2. Materials and methods

2.1 Materials

(L)-Malic acid, (L)-ascorbic acid and (L)-citric acid were obtained from Merck (Brazil). Alcohol dehydrogenase (ADH; 340 U mg^{-1}), (L)-lactate dehydrogenase (L-LDH) ammonium sulfate suspension (9.9 U μL^{-1}), (D)-lactate dehydrogenase (D-LDH; 123.9 U mg^{-1}), (L)-glutamate dehydrogenase (GDH; 14.7 U mg^{-1}), diaphorase (DP; 4.5 U mg^{-1}), gelatin from bovine skin type B, (L)-glutamic acid monosodium salt monohydrate (≥98%), (D)-fructose (≥99.0%), (L)-tartaric acid, thiazolyl blue tetrazolium bromide (MTT), sodium (D)-lactate (≥99.0%), sodium (L)-lactate (≥99.0%), (L)-malic acid, D-(+)-glucose monohydrate (≥99%), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NAD^+), sodium hydroxide, potassium hydroxide, methanol anhydrous (99.8%), propionic acid (≥99.5%) and poly(vinylpolypyrrolidone) (PVPP) were obtained from Sigma Aldrich (Brazil). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic, ethanol (≥99.8%) and silica gel blue (4/8 mm) were delivered by Synth (Brazil). Chromatography paper (Whatman™, grade 3 mm CHR) was purchased from Sigma (Brazil). Deionised water from a Millipore system (France) was used for the preparation of all aqueous solutions (conductivity 2.3 μS cm^{-1} and resistivity 18.2 mΩ cm).

Pictures were taken by a Samsung Galaxy S3 smartphone. Commercial food samples subjected to analysis were purchased from local food markets in Brazil.

2.2 Paper-strip fabrication, reaction and the effect of gelatine

The chromatography paper (denoted as "paper") was cut into 3×3 or 6×6 cm^2 squares and visually divided into 1×1 cm^2 squares by drawing lines. The reaction principle is illustrated in Figure 1. To analyse (L)-glutamate, (L)-lactate, (D)-lactate or ethanol, a particular dehydrogenase (either GDH, L-LDH, D-LDH or ADH, respectively) was used in a coupled reaction involving diaphorase in the presence of NAD^+ and MTT. The optimised procedure for experiments using dehydrogenase enzymes was as follows. The enzyme reaction mixture (3 μL), that is, pH 8–9 phosphate buffer (P_B) containing gelatine (1.0% w/v), MTT (5.5 mmol L^{-1}), NAD^+ (5.0 mmol L^{-1}), selected dehydrogenase (0.25 U) and diaphorase (0.025 U), was pipetted onto the paper. The leftover enzyme mixture was stored in a freezer. The reaction was started by pipetting 3 μL of the standard

(0.5–10 mmol L^{-1}) or a sample solution (diluted by deionised water or adjusted to pH 7–9, if necessary), leading to the development of purple-colored circle. The color intensity was evaluated after 10 min, either by the naked eye or in ImageJ freeware after photos had been taken and imported from the smartphone to the computer for the quantitative analysis.

To investigate the effect of gelatine on the color distribution, the paper was immersed for 5 min into Petri dishes containing pH 8.0 P_B (4 mL) and different concentrations of gelatine (0.5–2.0% w/v). The standard solution was applied after the papers were completely dry and the quality of the purple reaction product was evaluated by the naked eye.

2.3 Evaluation of the color intensity

The color intensity of a purple circle was evaluated using freeware ImageJ in regular RGB mode without any additional settings. At first, the mean intensity of a selected homogeneous area was read using the "oval" tool (Figure 1 in the supplementary material). Afterwards, the same tool was used to read the mean intensity of a blank area, that is, the yellow (or white if no yellow was left around) unaffected area in direct proximity to a reaction spot. The selected area should be as representative as possible avoiding uneven shadows. The final mean value of the color intensity was obtained according the formula:

Final mean intensity = mean intensity of blank area (no reaction) – mean intensity of purple area (reaction)

This was necessary because, in a common laboratory or other *"in situ"* environment, the quality the photographs taken by a smartphone camera and a non-expert photographer vary from batch to batch, and the light discrepancies as well as the shadows across the whole surface of the paper can fluctuate. Therefore, this step corrected the influence of shadows on the color intensity of each particular reaction area.

2.4 Dipstick experiments

Dipsticks were fabricated by cutting the paper into strips of 3×0.5 cm^2. Afterwards, an orientation line was drawn 1 cm from an ending point. Then, the enzyme reaction mixture (3 μL) was pipetted in proximity of the orientation line, onto the shorter part of the paper. For qualitative analysis, the tip of the dipstick was gently immersed into the standard or the sample solution and taken out when the liquid achieved the orientation line. The color appearance was evaluated by the naked eye.

2.5 Pre-treatment of food sample

Solid food samples (spice mixtures and bouillon) were dissolved in water, centrifuged and the supernatant was removed and diluted to achieve an (L)-glutamate concentration within the 0.5–5.0 mmol L^{-1} range. Dark-colored samples, such as red wine, have to be decolorised in order to allow visual identification of a color reaction. Therefore, samples of red wines were repeatedly pre-treated with PVPP until the color removal was achieved. Samples with pH values below 7 were adjusted to within the pH range 7–9, either by addition of KOH solution or P_B.

2.6 Enzyme spectrophotometric assay for (L)-glutamate analysis

Enzyme assay kits were used for the comparative analysis of (L)-glutamate based on the same biochemical principle as described in the section above. The assays were performed as follows: pH 8.0 P_B (1969 µL) was pipetted into the cuvette followed by an enzyme mixture (25 µL) containing (L)-glutamate dehydrogenase (12.5 U), DP (0.25 U) and MTT (20 mmol L^{-1}). After this, NAD^+ solution (6 µL) was added to achieve a final concentration of 5.0 mmol L^{-1} in the cuvette, that is, in 2 mL of the reaction mixture. The reaction was initiated by addition of a standard (10–50 mmol L^{-1}) or a sample (6 µL), leading to the development of a purple color, which was measured by spectrophotometry after 30 min at a wavelength of 630 nm (cuvettes were kept at room temperature in a dark place to avoid the possible degradation of light-sensitive MTT).

2.7 CE-MS/MS analysis of (L)-glutamate

Capillary electrophoresis (CE) was performed by Agilent 7100 equipment connected to a 6430 triple-quadrupole mass spectrometer (MS/MS, Agilent Technologies, Santa Clara, CA, USA), which was coupled to an electrospray ionisation (ESI) source. Nitrogen was used as the carrier gas at a flow rate of 11 L min^{-1} and as the nebuliser gas with 11 psi at 160ºC. Multiple reaction monitoring (MRM) was employed in negative-ion mode to identify the transitions of *m/z* 146 → 128 for glutamate and *m/z* 160.9 → 116.9 for trichloroacetic acid (TCA) as an internal standard (IS). Moreover, a cell accelerator voltage of 4 V, a collision cell voltage of 8 V and a fragmentor voltage of 40 V were individually applied to monitor glutamate. Similarly, 3, 0 and 10 V also were employed for TCA, a capillary voltage was set to 4 kV, the dwell time was set to 100 ms and a 180 µmol L^{-1} TCA solution was used as the IS.

The fused silica capillary (Agilent, Redmond, OR, USA) was 60 cm long with an internal diameter of 50 µm and an outer diameter of 360 µm. The capillary was pre-conditioned for 10 min by each of the following

solutions: 0.1 mol L^{-1} NaOH, deionised water and electrolyte (25 mmol L^{-1} propionic acid and 50 mmol L^{-1} NH_3 in deionised water). The samples were hydrodynamically injected at 100 mbar for 10 s. The electrophoretic run was performed at 25 kV and at a temperature of 25°C. The sheath liquid used for ESI was prepared by diluting the electrolyte 25× with methanol/water 1:1 (v/v).

3. Results and discussion

Generally, a dehydrogenase is an oxidoreductase enzyme that catalyses a transfer of electrons (hydride anions) from electron donor to electron acceptor (usually NAD^+/$NADP^+$) [15]. In this research, the application of various dehydrogenases for colorimetric detection on the paper platform was investigated. For this purpose, (L)-GDH, (L)-LDH, (D)-LDH and ADH we used in a coupled reaction involving DP for the colorimetric detection of particular analytes, according the principle illustrated in Figure 1. The method for all analytes was based on the same reaction principle, and only the enzyme in the first step of the reaction was changed. Therefore, the method was optimised with (L)-GDH, which was chosen for the model-method demonstration. Once optimised, the method was further applied to the experimental testing of other enzymes, and their application for qualitative analysis using dipsticks was assessed. Optimisation experiments of colored-spot development, described in Sections 3.1, 3.2 and partially in 3.3, were evaluated by the naked eye. For the quantitative analysis (see sections 3.2 and 3.3) the digital imaging was applied.

3.1 Paper pre-treatment and the effect of gelatine

Some studies demonstrated that pre-treating paper with the gelatine may improve the color intensity and homogeneity on the chromatography paper when employing enzyme colorimetric reactions [4, 16]. Therefore, we investigated the effect of paper pre-treatment in 0.5–2% (w/v) gelatine in pH 8.0 P_B. It was found that, once the paper was dried and the (L)-glutamate standard solution was applied, the color dispersion and intensity was not improved by the gelatine. Consequently, the paper was used for further studies as delivered without any pre-treatment, which is advantageous as it contributes to the method's simplicity.

The effect of gelatine in the enzyme mixture applied to the paper was investigated. In this case, a paper strip was not modified and used as delivered. The results showed that 1.0% (w/v) gelatine in the enzyme mixture slightly enhanced the color intensity. So, for further experiments, the enzyme mixture contained 1.0% gelatine for better reaction performance and illustration purposes; however, it was not essential for the method itself.

3.2 Optimal amount of enzyme units and reagents

The aim of these experiments was to find the lowest possible amount of reagents necessary for the preparation of the enzyme reaction mixture in order to maintain the highest possible color intensity. The number of (L)-GDH units was investigated within the range of 0.25–1.0, diaphorase in the 0.01–0.065 range and MTT concentrations were tested in the range of 2.5–10 mmol L^{-1} per loading. NAD^+ acted as a co-enzyme and was necessary for the reaction, but its increasing concentration did not affect the final color development. An NAD^+ concentration of 5.0–10.0 mmol L^{-1} was sufficient and consequently used in all experiments. It was found that the final and optimum composition contained in 3 μL of the enzyme mixture applied to the paper was: 5.5 mmol L^{-1} MTT, 5.0–10.0 mmol L^{-1} NAD^+, 0.25 U of a particular dehydrogenase [either (L)-glutamate or (L)/(D)-lactate] and 0.025 U DP. Afterwards, the standard or sample (3.0 μL) was applied and a colored spot was developed. There was no difference in color response within the pH range of P_B between values of 7 and 9. Bellow and above these values, the response decreased and it was particularly important to adjust the pH value of acidic samples, such as wines.

3.3 Analytical characteristics of the method

In the next stage, experiments comparing the reactions of dried and wet enzyme mixtures with the standards were performed (Figure 2). In the first case, the standard was applied to the dried enzyme mixture, whereas in the second case, it was applied shortly after the application of the enzyme mixture (before the spot on the paper had dried, i.e. within 10 min). It can be seen that, in the first scenario, the reaction area was smaller and the color was slightly more intense compared to the "wet" reaction. We assume that this was because of the accumulation of the color product on a smaller area. However, according to the naked eye, it was easier to distinguish between 2.5 and 5.0 mmol L^{-1} in the case of the "wet" reaction. Therefore, it can be concluded that, for qualitative analysis, both procedures can be used, but for semi-quantitative assessments, the "wet" reaction is more suitable. All experiments were performed within the temperature 20–30°C temperature range. In this case, the temperature did not have influence on the performance of the assay.

Figure 3 shows a sequence of calibration experiments within the 0.25–8.33 mmol L^{-1} range. The linearity was achieved within 0.5–5.0 mmol L^{-1} characterized by equation $y = 7.5x + 28.7$ with $R^2 = 0.9728$ (for raw data and calibration plot see Figure 2 and Tables 1-2 in the supplementary material). The quantitative data were acquired by using the digital image presented in Figure 3. The intra-batch calibration coefficient of variation of the mean color intensity calculated for four replicates of each calibration point was 7.3–9.9%, and

the inter-batch coefficient was 6.6–14.6% (n = 6). It is important to point out that calibration should be performed each measurement on the paper strip together with the samples.

The limit of detection (LOD) of the proposed method was investigated experimentally by applying the standard solution step-by-step onto separate areas until the colored reaction product was no longer detectable by the naked eye. This is also important for qualitative analysis, when there is a need to detect low levels of an analyte. Figure 4 shows the response of the assay to various concentrations in order to experimentally determine the LOD for (L)-glutamate. The LOD was found to be 0.05 mmol L^{-1}. Using the digital imaging[17] the LOD was found to be 0.028 mmol L^{-1}. LOD was estimated by following formula: 3 x S_b/S, where S_b is the standard deviation of the blank and S the slope of the analytical curve. The LOD acquired by the instrumental approach was as expected lower than the one found by the naked eye (the data is presented in Table 3 and Figure 3 in the supplementary material). The limit of quantification calculated by 10 x S_b/S was 0.092 mmol L^{-1}.

For the screening analysis[18], the series of experiments were performed as illustrated in Figure 5. The color spot obtained for the sample was visually compared with the calibration spots and the closest color intensity was identified (as in the case of pH test strips). The difference between 5.0 and 10 mmol L^{-1} was still distinguishable, which makes the paper assay suitable for the estimation of an approximate concentration in a sample. Interestingly, in this particular experiment, the coefficient of determination was 0.9913 when the calibration was analysed in ImageJ freeware. This suggests that the calibration has to be made on each individual paper strip, as light conditions, color distribution and picture quality vary from case to case. However, this is a minor issue considering the low cost and simplicity of the method.

3.4 Interferences and real-sample analysis

Before real-sample analysis, various common food components were tested for interferences. For each reaction of an enzyme mixture containing different dehydrogenases, the standard (0.5 mmol L^{-1}) was applied for visual comparison, which acted as a control. Consequently, (L)-ascorbic acid, (L)-citric acid, (L)-lactate, (D)-glucose, (D)-fructose, sucrose, (L)-tartaric acid, (L)-malic acid, (L)-glutamate, acetic acid and 15% (v/v) ethanol were applied. The concentration of the tested solutions was 50 mmol L^{-1}, except for ethanol, in which case a 15% (v/v) solution was tested. It was found that even 100× higher concentrations compared to the target analyte standard did not cause any formation of a colored product. However, as might be expected, the exception was (L)-ascorbic acid, which directly interfered with MTT, owing to its enediol group [19]. In this case, a false signal was observed with an intensity equal to that of the analyte. Therefore, we do not recommend using this method

for the analysis of samples containing >0.05 mmol L^{-1} (L)-ascorbate (i.e. above the LOD of the method determined by the naked eye).

Color interference from red wines can be avoided by using PVPP as a pre-treatment, as proposed in section 2.5.

To verify the applicability of this method in the analysis of food samples, the assay was used to determine the level of (L)-glutamate in commercial samples, namely, in the powder mixtures of spices and bouillon. Table 1 summarises the results obtained by the proposed method, spectrophotometry and CE-MS/MS. It can be seen that satisfactory correlation was achieved considering the diverse character of the methods.

Table 1. Comparison of the analyses of food samples by paper-based assays, spectrophotometry and capillary electrophoresis (CE-MS/MS). Results are expressed in mmol L^{-1} ± S. D. (n = 3, α = 0.05).

Sample	(L)-glutamate		
	Paper strip	Spectrophotometry	CE-MS/MS
Spice mix	30 ± 2	25.51 ± 0.03	26 ± 3
Bouillon	29.0 ± 0.3	33.95 ± 0.02	27 ± 3

3.5 Dipsticks

Dipstick experiments were performed in order to demonstrate another practical application of the proposed method as well as to show that the method works well with other dehydrogenase enzymes and can be presented as a general concept. Figure 6 illustrates various qualitative determinations of ethanol, (L)-lactate and (D)-lactate. Paper dipsticks were impregnated with the enzyme reaction mixture on a small area and immersed into either a standard solution or a real sample. The results confirmed that the reaction also occurs with other dehydrogenase enzymes, and the dipstick arrangement can easily be prepared and applied for rapid screening analysis. The dipsticks presented in this work were able to qualitatively analyse the sample within a few seconds. This may be interesting for isomer distinction, for example, screening analysis of (D)-lactate can be used as an indicator of bacterial spoilage of vacuum-packed meat [20] or wine [21]. Method optimisation, especially a detailed study of the interferences, should be performed as well as separate studies of the optimal amount of enzyme units and the analytical characteristics of the method for each particular dehydrogenase, as these can vary due to the different nature of commercial products.

3.6 Storage stability

In order to investigate the operational stability of the paper strip, the enzyme mixture prepared initially was applied onto the chromatography paper. The standard solution of the same concentration (5.0 mmol L^{-1}) was applied every third or fourth day to the pre-prepared enzyme spot and the color intensity was compared with that obtained from the new reaction performed independently as a reference. The storage stability was monitored for the paper strip stored in both a freezer and a desiccator (room temperature). In both cases, the paper was protected by an aluminium foil. It was found that the enzyme mixture deposited on the paper assay and stored in a desiccator had darkened within the next few days, probably owing to the degradation of MTT. On the contrary, the enzyme mixture on the paper assay that was stored in the freezer had kept its characteristic yellow color and did not lose any response ability after at least 6 weeks at –18 °C.

3.7 Comparison with the literature

Table 2 compares the presented concept with other paper assays utilising enzymes (not solely, in some cases) in colorimetric reactions that have previously been reported.

Table 2 Comparison of selected paper-based colorimetric assays using enzymes for the target reaction.

Analyte	Utilised enzymes	LOD	Real samples	Shelf-life stability	Ref.
(L)-glutamate	glutamate dehydrogenase	[a]0.05 mmol L^{-1} [b]0.028 mmol L^{-1}	wines, instant soups	6 weeks at -18 °C	our work
glucose and uric acid	glucose oxidase and urate oxidase	0.213 and 0.287 mmol L^{-1}	human serum	60 days at -20 °C	5
neurotoxins paraoxon and aflatoxin B1	acetylcholinesterase	100 and 30 nmol L^{-1}	solutions of neurotoxins	2 months at 4 °C	22
serum transaminases ALT and AST	Peroxidase	53 and 84 U L^{-1}	human blood or serum	11 weeks at 25 °C	3
glucose	glucose oxidase and peroxidase	0.3 mmol L^{-1}	human serum	60 days at -20 °C	4
glucose	glucose oxidase and peroxidase	1 mmol L^{-1}	none	3 months at 3 and 25 °C	16
glucose	glucose oxidase and peroxidase	2.8 mmol L^{-1}	artificial urine	not provided	23
foodborne patogens	β-galactosidase; β-galactopyranoside; phosphatidylinositol specific phospholipase C	[c]0.01; 0.23 and 0.12 µg mL^{-1}	samples of "bologna"	not provided	8
phenylalanine	phenylalanine dehydrogenase	0.5 mg dL^{-1}	plasma	not provided	24

[a] by naked eye; [b] by digital imaging; [c] defined as the lowest detectable amount of enzyme that can be distinguished from the control.

4. Conclusion

In this paper, a biochemical principle conventionally used for cell viability tests was adapted and applied for the fabrication of a paper-based assay. The method can utilise enzymes from the dehydrogenase family coupled with diaphorase in the presence of tetrazolium dye MTT and NAD^+ in order to develop a color-reaction product on a chromatography paper platform. This concept was tested with four different dehydrogenases and was demonstrated to be applicable for the analysis of food samples. The concept presented herein can be understood as a general platform, and other dehydrogenases may be substituted for the specific analysis of various food components. Like many methods, this one has certain drawbacks, specifically, interference with (L)-ascorbic acid and it is necessary to adjust the pH of acidic samples prior to analysis. The main advantages of this method are the fact that there is no need to pre-treat the paper, only very low volumes of sample and reagents are required for the analysis and there is, consequently, very little waste, which is ideal for green chemistry purposes, a rapid time response and the simplicity of the whole assay.

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Figure captions

Figure 1. Schematic illustration of the colorimetric reaction principle used in this work. The enzyme reacts specifically with a particular analyte in the sample in the presence of co-enzyme NAD^+ to form reduced NADH + H^+. The formed NADH + H^+ subsequently react with an electron-accepting dye, in our case yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is catalysed by the diaphorase enzyme, forming a purple color that can be detected either by the naked eye or a smartphone camera.

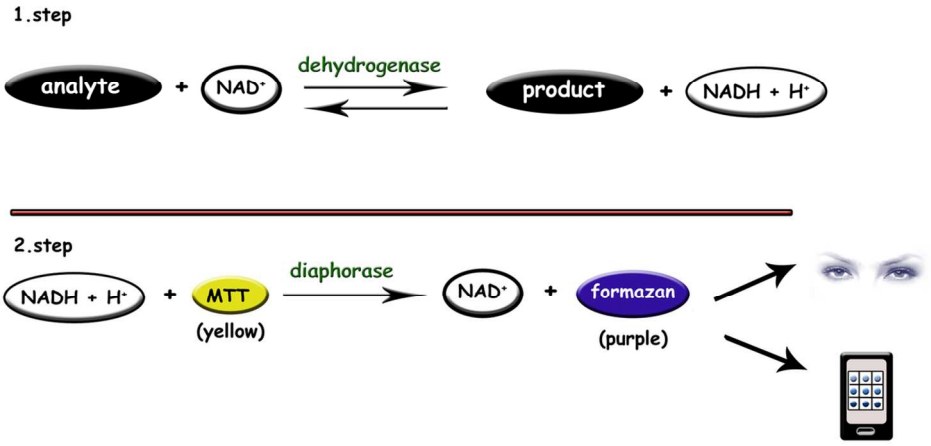
Figure 2. Comparison of reactions when the standard mixture was applied: [A] - after the enzyme mixture was completely dried and [B] - when the standard mixture was applied within 10 min, i.e., the enzyme mixture and paper were still wet. The numbers represent the glutamate concentration for both reactions [A] and [B].

Figure 3. Assay performed in triplicate for (L)-glutamate determination at concentrations of 0.25–8.33 mmol L^{-1}.

Figure 4. Experimental determination of the detection limit. The numbers represent glutamate concentrations expressed in mmol L^{-1}.

Figure 5. Assay with (L)-glutamate concentrations of 0.5–10 mmol L^{-1}, providing color spots that are clearly distinguishable by the naked eyes. In the second line are samples obtained by dissolving a spice mixture and bouillon in water, which were consequently diluted and analysed.

Figure 6. Qualitative determination of ethanol, (L)-lactate and (D)-lactate. **E** – Dipstick with the enzyme mixture before reaction. **B** – Blank reaction with deionised water. **1** – 10 mmol L^{-1} of a particular standard solution. **2** – 50 mmol L^{-1} of a particular standard solution. **Ethanol 3** – sample of white wine. **Ethanol 4** – sample of red wine. **(L)–lactate 3** – sample of olive brine. **(L)–lactate 4** – sample of red wine. **(D)–lactate 3** – sample of olive brine spiked with 10 mmol L^{-1} (D)–lactate. **(D)–lactate 4** – sample of red wine spiked with 10 mmol L^{-1} (D)–lactate.



112x60mm (300 x 300 DPI)

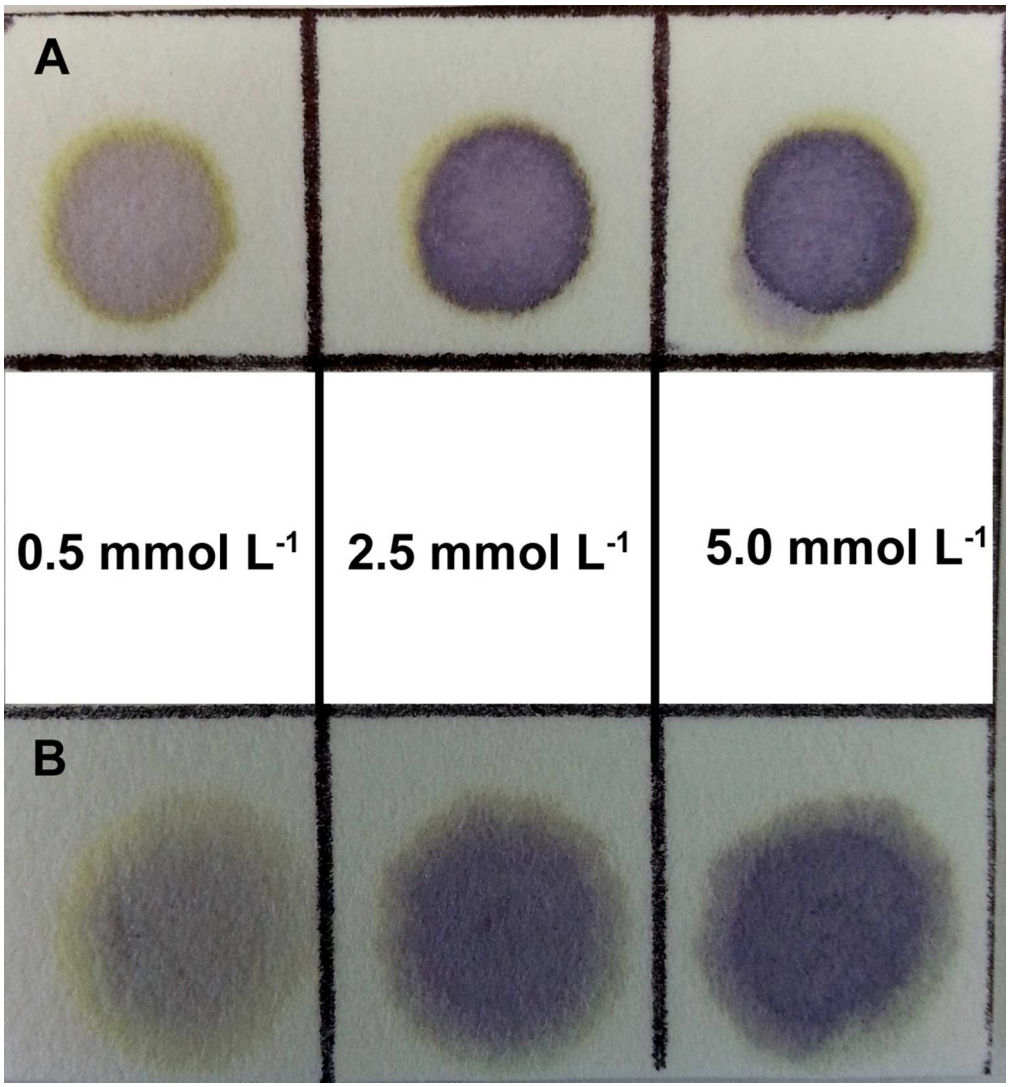


Figure 2. Comparison of reactions when the standard mixture was applied: [A] - after the enzyme mixture was completely dried and [B] - when the standard mixture was applied within 10 min, i.e., the enzyme mixture and paper were still wet. The numbers represent the glutamate concentration for both reactions [A] and [B].
99x107mm (300 x 300 DPI)

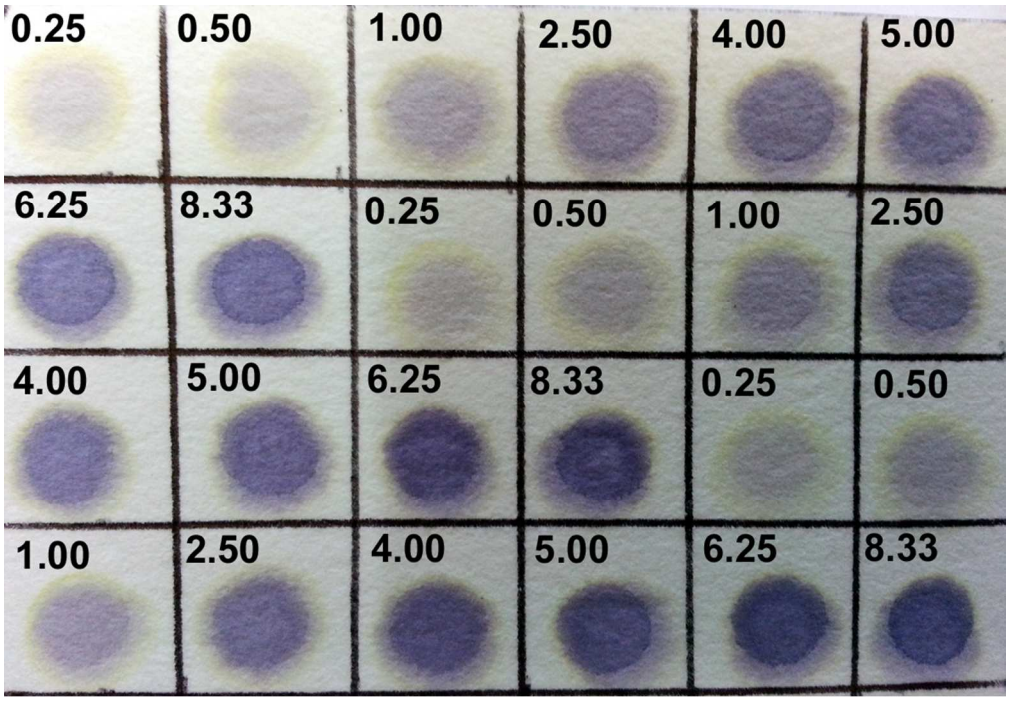


Figure 3. Assay performed in triplicate for (L)-glutamate determination at concentrations of 0.25–8.33 mmol L-1.
99x69mm (300 x 300 DPI)

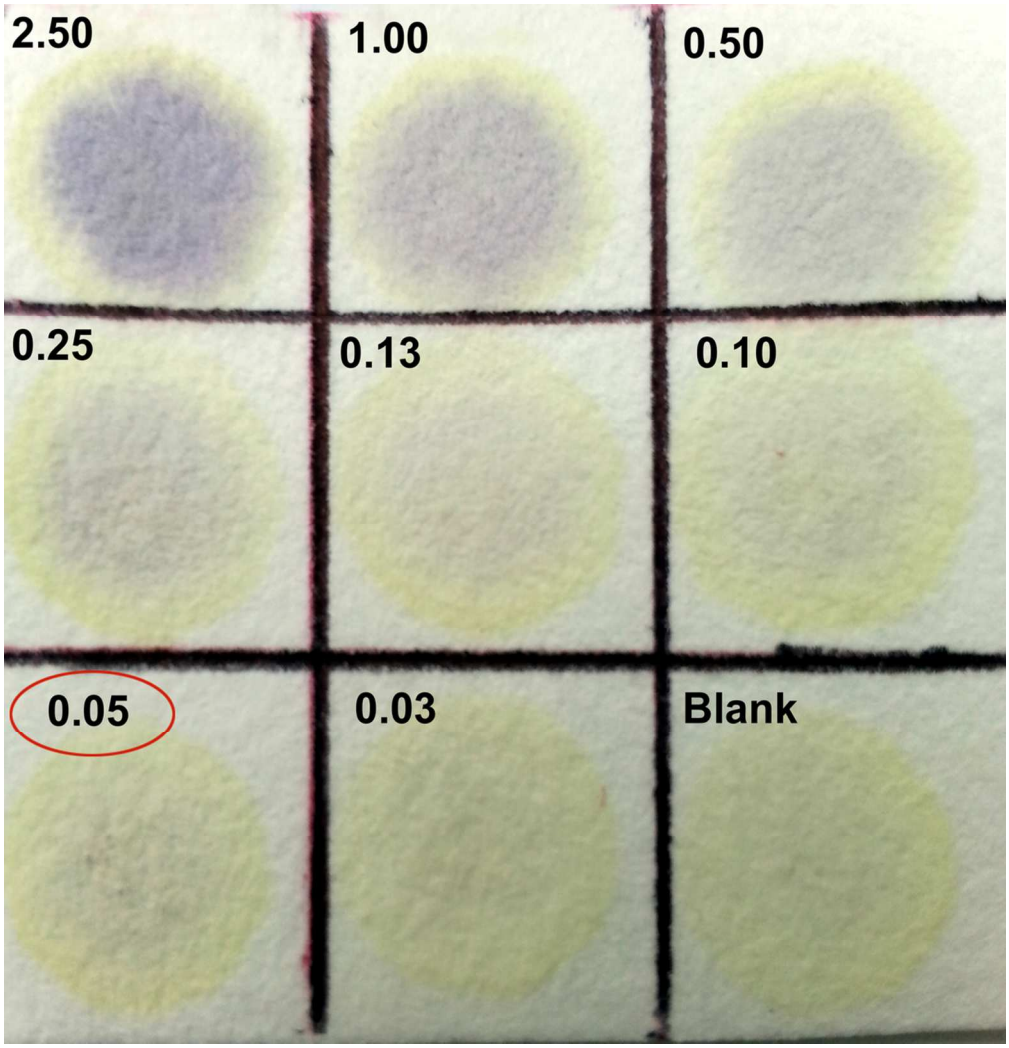


Figure 4. Experimental determination of the detection limit. The numbers represent glutamate concentrations expressed in mmol L-1.
103x107mm (300 x 300 DPI)

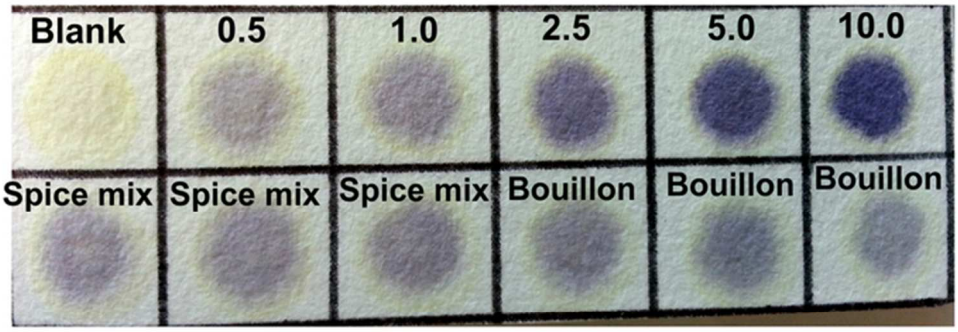


Figure 5. Assay with (L)-glutamate concentrations of 0.5–10 mmol L-1, providing colour spots that are clearly distinguishable by the naked eyes. In the second line are samples obtained by dissolving a spice mixture and bouillon in water, which were consequently diluted and analysed.
59x29mm (300 x 300 DPI)

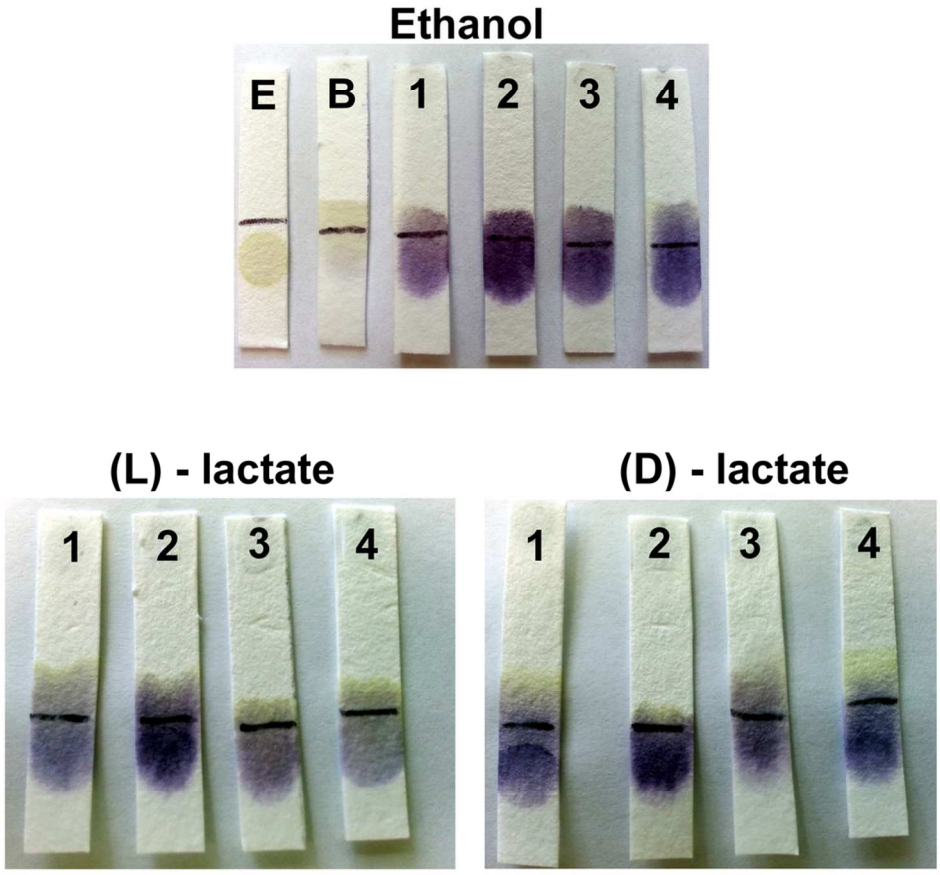
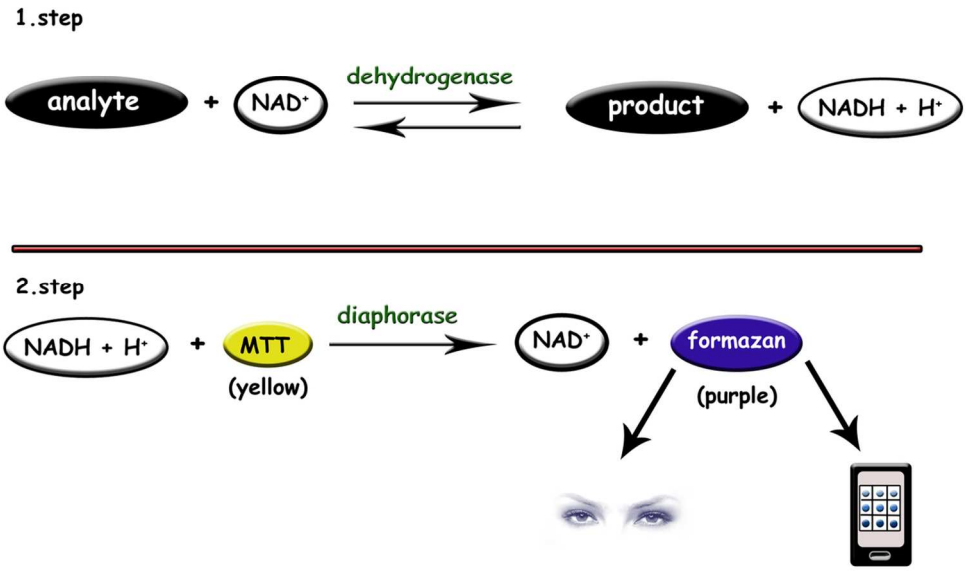


Figure 6. Qualitative determination of ethanol, (L)-lactate and (D)-lactate. E – Dipstick with the enzyme mixture before reaction. B – Blank reaction with deionised water. 1 – 10 mmol L-1 of a particular standard solution. 2 – 50 mmol L-1 of a particular standard solution. Ethanol 3 – sample of white wine. Ethanol 4 – sample of red wine. (L)–lactate 3 – sample of olive brine. (L)–lactate 4 – sample of red wine. (D)–lactate 3 – sample of olive brine spiked with 10 mmol L-1 (D)–lactate. (D)–lactate 4 – sample of red wine spiked with 10 mmol L-1 (D)–lactate.
99x99mm (300 x 300 DPI)



112x70mm (300 x 300 DPI)