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Analytical Methods

Automatic spot preparation and image processing of paper microzone-based assays for analysis of bioactive compounds in plant extracts

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ABSTRACT

The colorimetric determination of the concentration of phytochemicals in plant extract samples using a spotting automatic system, mobile phone camera and a computer with developed software for quantification is described. Method automation was achieved by using a robotic system for spotting. The instrument was set to disperse the appropriate aliquots of the reagents and sample on a Whatman paper sheet. Spots were photographed and analysed by ImageJ software or by applying the developed MatLab based algorithm. The developed assay was found to be effective, with a linear response at the concentration range of 0.03–0.25 g/L for polyphenols. The detection limit of the proposed method is sub 0.03 g/L. The paper microzone-based assays for flavonoids and amino acids/peptides were also developed and evaluated as applicable. Comparing the results with conventional PμZP methods demonstrates that both methods yield similar results. At the same time, the proposed method has an attractive advantage in analysis time and repeatability/reproducibility.

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1. Introduction

Plants have evolved the ability to synthesise chemical compounds that help them defend against attack from a wide variety of predators such as insects, fungi and herbivorous mammals. Some of these compounds, while toxic to plant predators, turn out to have beneficial effects when used to treat human diseases. Such secondary metabolites are highly varied in structure; many are aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Herbal medicine has already been of interest for centuries and at least twelve thousand compounds have been isolated so far, a number still estimated to be <10% of the total (Tapsell et al., 2006). This makes the study of plant extracts a cutting-edge research topic in analytical chemistry.

Contemporary high-tech methods of plant extracts analysis rely mostly on separation science, which is frequently hyphenated with mass-spectrometry (Allwood & Goodacre, 2010; Helmja et al., 2009). Despite the power of the chromatographic–spectroscopic methods, high throughput, rapid and cheap analytical methods are of interest that are portable and can be used *in situ* for screening purposes. Such devices could, for example, serve lateral flow strips (e.g. pregnancy tests). They are single-use point-of-care (POC) tests that have been widely adopted in both the developing and developed worlds. Although these lateral flow tests are reliable and inexpensive, they are restricted to simple operations, exhibit limited sensitivity, and are non-quantitative. Thus, there is currently considerable interest in developing new paper-based diagnostics to address these shortcomings by going beyond the conventional single-strip format. Most of these assays rely on colorimetry, which should not be perceived as a call to return to older, inefficient analytical methods. A novel, important point here is that the developer can take advantage of existing technology. Colorimetric assays can be prepared that take advantage of microfluidic technology and means of quantification such as cellular phones, as has been demonstrated by the pioneering works of Whitesides' group (Martinez, Phillips, Whitesides, & Carrilho, 2010). These tests are small, quick, simple, and inexpensive. A small drop of body fluid wicks through the corner or back of the paper and passes through channels to special testing zones. Substances in these zones react with specific chemicals in the sample to indicate different conditions; the results are shown by varying colours. Recently, it was demonstrated by Whitesides' group that simple telemedicine for developing regions can be constructed using cellular phone cameras and paper-based microfluidic devices, which they call *microfluidic paper-based analytical devices* (μPADs) (Martinez et al., 2008, 2010). Recent examples of such assays are μPADs in order to assess a worker's occupational exposure to metal-containing aerosols (Mentele, Cunningham, Koehler, Volckens, & Henry, 2012).

Paper microzone plates (PμZPs), (Apilux et al., 2010; Carrilho, Phillips, Vella, Martinez, & Whitesides, 2009; Dungchai, Chailapakul, & Henry, 2009) represent a slightly different approach

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to paper-based diagnostic devices and are even simpler than μPADs. PμZPs can be made by patterning a sheet of paper into an array of circular test zones. Like the wells in conventional plastic plates, each zone in the paper plate can be used to run an independent assay; the design of the plate facilitates parallel processing of large numbers of samples. A great advantage of μPADs and PμZPs is that they can be fabricated easily and the process does not require complex equipment or extensive training. This simplicity should enable scientists with limited experience to fabricate devices in order to experiment with and adapt μPADs/PμZPs independently. Finally, paper devices can be easily disposed of by burning.

So far, researchers have developed prototype μPAD/PμZP systems mostly for clinically relevant bioanalyses that use colorimetric assays and telemedicine. However, the applications of PμZP are definitely not limited to diagnostics for developing countries. PμZPs were recently used for the micro-determination of arsenic in aqueous samples by image scanning and computational quantification (Salman et al., 2012). Rapid tests should not be restricted to diagnose a human condition or its environment quality. As follows from the discussion above, the determination of key components in herbal extracts *in situ* could be important and valuable. One could envision a situation in which PμZPs tests are performed on a plantation to screen quality of a particular crop. Recently, we demonstrated (Vaher & Kaljurand, 2012) how red wine polyphenols, flavonoids and anthocyanins can be quantified at paper microzones via digital camera, and a remote computer with dedicated software (freeware ImageJ (http://rsbweb.nih.gov/ij/)).

In this paper, the automatised colorimetric PμZP assay for herb quality testing is developed further. We address a number of issues with the aim of reducing manual operations with PμZPs as little as possible. First, we demonstrate that PμZPs can be prepared in large numbers by using robotic MALDI spotter, second, that an automatic image processing algorithm has been developed that enables us to estimate intensities of all PμZPs on the paper sheet simultaneously, contrary to the ImageJ, which enables us to calculate zone intensities one at the time. We will demonstrate that the results obtained by either software are concise and high precision. The developed algorithm also performs the principal component analysis of spots. Finally, the assays for polyphenols and flavonoids have been validated against manually performed and common assay based on spectrophotometry. The objects of study were herbal tea extract grown in a local plantation specialising in organic agriculture products.

2. Experimental

2.1. Materials

The gallic acid, (+)-catechin, ninhydrin (indane 1,2,3-trione hydrate), glutathione (GSH), Folin–Ciocalteu reagent (FCR, 2 M with respect to acid), aluminium chloride hexahydrate, sodium nitrite, sodium carbonate, sodium hydroxide, potassium chloride and methanol were obtained from Sigma. Dried samples of 43 herbs (flowers, leaves, seeds, roots) were obtained from a local manufacturer (Kubja Ürditalu, Raplamaa, Estonia) as a gift. The list of samples is given in Table 1. The cellulose chromatographic papers 1 Chr, 3MM Chr and filter paper FN16 were from Whatman. The paper thicknesses and flow rates were 0.18 mm, 0.34 mm, 0.22 mm and 130 mm/30 min, 130 mm/30 min, 80 mm/30 min, respectively.

2.2. Preparation of plant extract

The herb infusions were prepared as follows: 0.5 g of plant material was treated with 50 ml of water and extraction was carried out in accordance with the instructions printed on the package. For the alcoholic extracts the plant material was ground into powder. Then 0.5 g of plant sample was leached with 10 mL of 80% methanol (v/v) for 2 h at room temperature and extracting in an ultra-sonic bath at a temperature between 35 °C and 40 °C for 0.5 h. The extract was centrifuged for 10 min at 5000 rp/min. Extracts were stored as aliquots at −18 °C for a week and thawed immediately before analysis. No changes were noticed between frozen samples and those samples were analysed immediately after preparation.

2.3. Spectrophotometric measurements

2.3.1. Total phenolic quantification

The total polyphenol quantification in the herbal extracts was measured by the Folin–Ciocalteu method, following the procedure of Singleton (Singleton, Orthofer, & Lamuela-Raventós, 1999). In brief, phenolic groups are oxidised by phosphomolybdic and phosphotungstic acids in Folin–Ciocalteu reagent, forming a green–blue complex detectable at 765 nm. In the test, 200 μl of each extract solution of an appropriate concentration was mixed with 1 ml of Folin–Ciocalteu reagent (1:10 diluted with H_2O) and 800 μl of Na_2CO_3 (20%). The absorbance at 765 nm was measured after 2 h reaction at room temperature with a Cary 50 Bio UV–vis spectrophotometer (Varian). The aqueous gallic acid solution was freshly prepared in a series of concentrations (0.3–3 mM) and tested in parallel to establish the calibration curve. The total phenolic content of each herbal extract was calculated as gallic acid equivalent (g GAE/L).

2 μL of FCR was spotted onto Whatman Chr1, FN16, Chr MM3 chromatographic paper and then 2 μL solutions of different concentrations of gallic acid (in the range of 0.25–1.5 mM) were applied to the FCR spots for calibration. Finally, 2.5 μL of 20% sodium carbonate was added to each spot. Optimal volumes of analytes and samples were determined by experiment: the following reagent/sample must match the size of the spot generated by

Table 1
Comparison of the performance of ImageJ and MatLab algorithm and figures of merit of PμZP assay.

	Polyphenols	Flavonoids	Amino acids/peptides
Reflectance (MatLab viz ImageJ)	$R_R = (1.01 \pm 0.07)^* R_J - (0.01 \pm 0.13)$ ($R^2 = 0.97$)	$R_R = (0.99 \pm 0.07)^* R_J - (0.02 \pm 0.13)$ ($R^2 = 0.96$)	$R_R = (0.99 \pm 0.07)^* R_J - (0.02 \pm 0.10)$ ($R^2 = 0.97$)
Calibration	$R_R = (0.99 \pm 0.04)^* C_A + (0.02 \pm 0.03)$ ($R^2 = 0.98$).	$R_R = (0.47 \pm 0.04)^* C_A + (0.04 \pm 0.06)$ ($R^2 = 0.93$)	$R_R = (0.62 \pm 0.04)^* C_A + (0.01 \pm 0.18)$ ($R^2 = 0.97$).
LOD g/L	0.03	0.06	0.15
RSD (%)*	3.1	13.4	8.1

R_R; R_J – reflectances measured by MatLab algorithm and by ImageJ.
R^2 – square of the correlation coefficient of the corresponding line.
RSD – relative standard deviation.
LOD – limit of detection.
* Reproducibility, calculated on many sheets prepared for this work.

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