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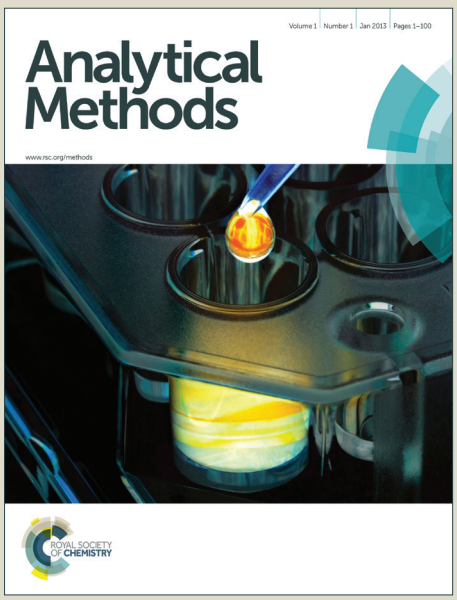

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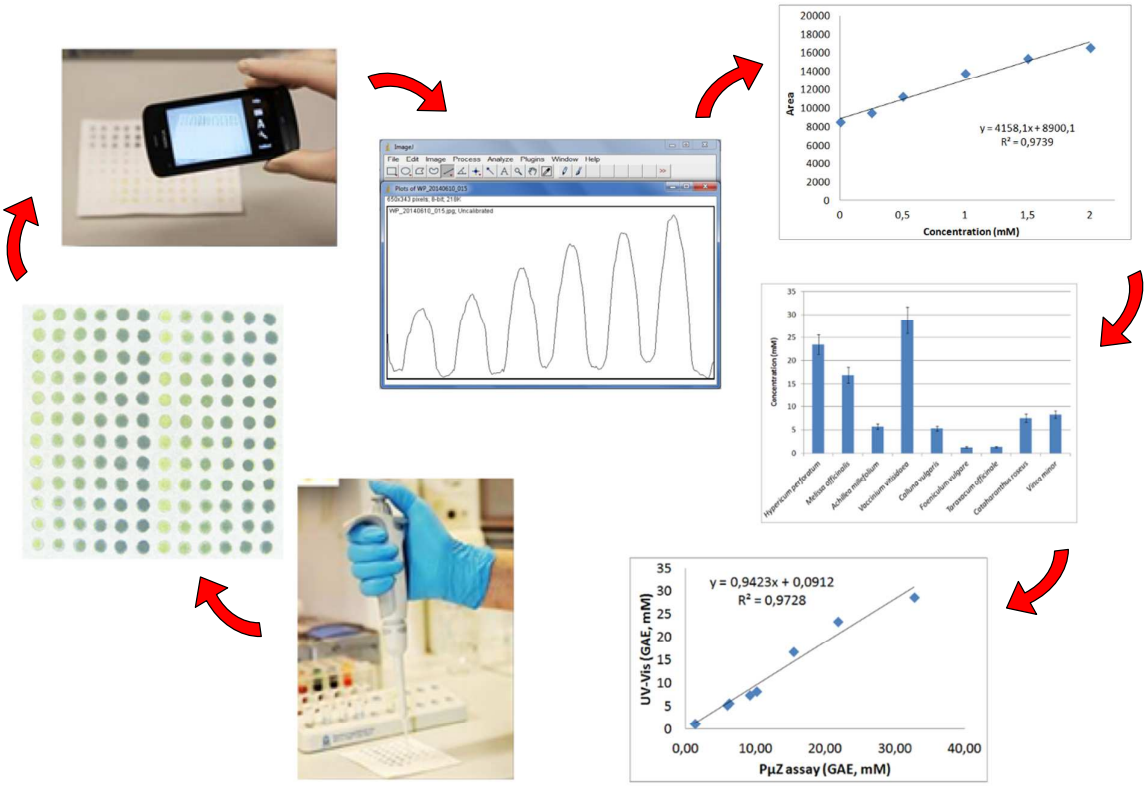
A rapid colorimetric paper microzone assay of total polyphenols in ionic liquid extracts was developed and validated.



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ARTICLE TYPE

Colorimetric Determination of Total Phenolic Content in Ionic Liquid Extracts by Paper Microzones and Digital Camera

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Abstract

In recent years, there has been a growing interest in the extraction of phenolic compounds from plants by using ionic liquid (IL) solutions. In this study, a colorimetric paper microzone assay was developed to analyse total phenolic content in five imidazolium based IL solutions with concentrations 50 – 100 mM. Nine herb methanol extracts and two herb IL extracts were used for validation. The method validation parameters were as follows: the linear range for gallic acid and catechin in BMImAc, $EMMImSO_4$, BMImCl, $BMImBF_4$ and $C_{12}MImCl$ aqueous solutions was between 0.25-2 mM, with the exception of catechin in $C_{12}MImCl$ aqueous solution where it remained between 0.25-1.5 mM. LOD and LOQ values were determined for gallic acid and catechin IL solutions, whose IL structure and concentration were varied. In case of all ionic liquids the determined LOD and LOQ values remained between 0.08-0.15 mM and 0.16-0.28 mM, respectively. The method was successfully validated against UV-Vis spectroscopy.

Introduction

Recent studies on the eating habits in different societies have shown that the diet rich in fruits, vegetables and grains is critically important for the prevention of cancer, diabetes, allergies, cardiovascular and inflammatory diseases in addition to bacterial and viral infections. These health promoting properties can be related to phytochemicals, particularly phenolic compounds, that are produced in plants as secondary metabolites.[1,2] Plant phenols are well-known due to their protective role against oxidative damage, through their redox properties, which can play an important role in adsorbing and neutralizing free radicals. Therefore, the interest in naturally occurring phenolic compounds has increased rapidly.[3-5]

Plant phenolic compounds are classified as simple phenolic compounds or polyphenols based on the number of phenol units present in the molecule, including phenolic acids, coumarins, lignins, lignans, tannins, flavonoids and flavanols in addition to simple phenols.[6,7] Characterization of plant phenols involves three main steps: sample preparation and extraction followed by classification and quantification by using spectrophotometry, gas chromatography (GC), high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) methods.[8-10]

The most common techniques used to extract bioactive compounds employ solvents, such as water, acetone, ethyl acetate, hexane, alcohols (methanol, ethanol, propanol) and their mixtures.[11-13] To improve determination selectivity, however, new extragents should be looked for. Recently, ionic liquids (ILs) are successfully used to extract bioactive compounds from plants.[14-17] ILs are salts with low melting point (below 100 °C), which is due to the inefficient packing of large irregular organic cations with smaller inorganic or organic anions.[18] ILs have been proposed as greener alternatives to volatile organic solvents because of their unique characteristics, such as negligible vapour pressure, good thermal stability, wide liquid range, good dissolving and extracting ability, excellent microwave-absorbing ability, designable structures, etc.[19] However, it is difficult to analyse phenolic compounds in ILs containing extracts. The simplest technique for quantification of extracted phenols is spectrophotometric assay. The most widely used methods for determination of total phenolic content (TPC) are Folin-Denis and Folin-Ciocalteu methods.[20] Both methods are based on the chemical reduction involving a reagent containing tungsten and molybdenum. During this reduction reaction, a blue coloured product with a broad light absorption spectrum (at around 760 nm) is formed. In case of both methods the reagents do not react specifically with only phenols but also with other substances like aromatic amines, sugars and ascorbic acid.[6, 21] The imidazolium cation containing ILs are widely used for extracting phenols.[5, 8] According to literature, imidazole is not expected to react with the Folin-Ciocalteu reagent.[22] However, tungsten (VI) and molybdenum (VI) are expected to form polyoxometalates (POMs) with imidazolium-based ILs.[23-25] POMs are a class of anionic metal-oxygen clusters built by the connection of $[MO]_x$ polyhedral of the early transition metals in their highest oxidation states.[15] The formed POMs will precipitate and therefore it is impossible to analyse ILs extracts with a spectrophotometer.

Paper has been used in spot-tests analysis of inorganic and organic substances since the 20th century. Formation of colored reaction products by mixing drops of a sample and a reagent gives qualitative information.[26] In the last decade, the paper-based analysis and paper-based microfluidic devices became of

great interest again.[27-30] Recently, for analysis of total phenolic content in wines and herb extracts using the Folin-Ciocalteu reaction, the assay in a filter paper sheet was developed, which is a simple and low-cost approach for this method.[31, 32]

The aim of the present investigation was to avoid limitations of classical spectrophotometry and develop a simple and fast method for evaluation of total phenolic content/antioxidant capacity in the ionic liquid extracts of herbs that could be carried out on the point of care in conditions where special instruments might not be available. The test quantifies the target analytes by color intensity of the reflected light which was quantified by a common digital camera.

Materials and methods

Seven different herbs were obtained from a local manufacturer (Kubja Ürditalu, Raplamaa, Estonia): ST.-Johns wort (*Hypericum perforatum*), balm leaves (*Melissa officinalis*), milfoil flower (*Achillea millefolium*), cowberry leaves (*Vaccinium vitisidaea*), heather flower (*Calluna vulgaris*), fennel seeds (*Foeniculum vulgare*), and dandelion root (*Taraxacum officinale*). *Catharanthus roseus* for quantitative analysis was obtained from Herbalveda (Northolt Middlesex, UK) and *Vinca minor* was collected in Võrumaa (South Estonia). Lime fruit, used to evaluate matrix effect, was purchased from a local store.

For sample preparation, ILs 1-butyl-3-methylimidazolium acetate, (BMImAc, 99%), 1-ethyl-2,3-dimethylimidazolium ethyl sulphate, ($EMMImEtSO_4$, 95%), 1-butyl-3-methylimidazolium chloride, (BMImCl, 98%), 1-butyl-3-methylimidazolium tetrafluoroborate, ($BMImBF_4$, pure) and 1-dodecyl-3-methylimidazolium chloride, ($C_{12}MImCl$, 98%) were purchased from Sigma-Aldrich. Five polyphenols – gallic acid, (±)-catechin, rutin, naringin, and ferulic acid were purchased from Sigma-Aldrich. The Folin-Ciocalteu reagent (FCR), sodium carbonate and methanol were also purchased from Sigma-Aldrich. All chemicals were used as received. The filter paper sheets (grade 1 CHR) for paper microzone (PμZ) assay were purchased from Whatman.

Preparation of samples

Stock solutions of gallic acid and (±)-catechin were prepared in methanol at a concentration of 30 mM and stored at +4 °C. To prepare aqueous solutions, ionic liquids $EMMImSO_4$, $BMImBF_4$ and $C_{12}MImCl$ were used at a concentration of 100 mM, and BMImCl as well as BMImAc at a concentration of 200 mM. The solutions were stored at room temperature.

The herb methanolic extracts were prepared as follows: 0.5 g of finely pounded plant material was leached with 10 ml of 80 % (v/v) methanol for 2 h at room temperature and then in an ultra-sonic bath at 40 °C for 0.5 h. After extraction, the solutions were centrifuged for 10 min at 5000 rp/min and the centrifugate was stored at -18 °C.

Air-dried *Catharanthus roseus* and *Vinca minor* were finely pounded, 0.5 g of plant material was mixed with 5 ml of a 0.5 M EMImCl or BMImCl water solution, stored at room temperature for 60 min and then placed in the ultra-sonic bath for extraction at 40 °C for 30 min. After extraction, the solutions were centrifugated for 10 min at 5000 rp/min and stored at -18 °C.

Lime juice was squeezed from fresh fruit, centrifuged for 10 min at 5000 rp/min and stored at -18 °C. The lime juice was diluted in different ILs for 30 times to evaluate matrix effect for PμZ assay.

Infrared spectroscopy

An infrared (IR) spectrum was recorded with a Bruker Tensor 27 FTIR spectrometer in the scanning range of 400-4000 cm^{-1}, with a resolution of 4 cm^{-1} and 24 scans were averaged for the spectrum. The sample was mixed with KBr in a weight ratio of 1:100, pounded into flour by hand by using a mortar and a pestle, and pressed to pellet.

Spectrophotometric assay

The Folin-Ciocalteu method was used to measure TPC, following the procedure of Singleton.[20] For explanation, the phosphomolybdic and phosphotungstic acid in the Folin-Ciocalteu reagent will oxidize phenolic groups, forming a green-blue complex, absorbing UV light at 765 nm. While performing the procedure, 20 µl of sample at appropriate concentration was mixed with 1.58 ml water, then 100 µl of the Folin-Ciocalteu reagent was added and mixed well. After 5 min 300 µl of sodium carbonate (20%) was added, the solution was mixed well and stored at room temperature for 2 h. The absorbance was measured at 765 nm with a Cary 50 Bio UV-Vis spectrophotometer (Varian). The calibration was performed with gallic acid at a concentration of 0.3-3 mM, and for each sample gallic acid equivalents (g GEA/L) were calculated using linear regression in Microsoft Excel.

Paper microzone assay

To measure the TPC in ionic liquid extracts, the Folin-Ciocalteu method was performed in cellulose chromatographic paper 1 Chr with a thickness of 0.18 mm and at a flow rate 130mm/30min, the paper was obtained from Whatman. The paper size was varied according to number of samples. For example one square centimetre space for one spot. In the procedures, 2 µl of FCR was spotted onto the filter paper sheet, after drying 2 µl of the IL solution with different gallic acid or (±)-catechin concentrations in the range of 0.25-2 mM, the pure IL solution for blank value, or an appropriately diluted sample was applied to the FCR spot. Finally, 2.5 µl of 20% sodium carbonate solution was added to each spot from the other side of the paper sheet. This was essential for the solutions where $C_{12}MImCl$ was used because these solutions made the paper surface slightly hydrophobic, and by adding the solution from the back of the paper a better diffusion of sodium carbonate was achieved. Optimal amounts of reagents and samples were investigated in a previous study.[23]

Calculations

The formed green-blue dots were photographed from the front side of the paper with a cell phone camera (8 MP). To obtain a reliable picture, the camera lens must be exactly parallel with the paper sheet and the paper must be lighted as evenly as possible.

The photo was imported to a personal computer and the freeware image processing program ImageJ (http://rsbweb.nih.gov/ij/) was used to quantify the colour intensity that was reflected from the spot for further calculations of analyte concentrations. Particularly, the command “Analzye/Gels” was used to calculate the intensities of the spot colour. Microsoft Excel software was used for regression analysis. Linear as well as polynomial

regression was used for calculations.

Results and discussion

Polyoxometalate formation

The Folin-Ciocalteu method with a spectrophotometric assay was used for testing TPC in IL extracts. In the first step where FCR was added to the ionic liquid solution, precipitation occurred. Due to the formation of the precipitate the spectrophtometrical analysis proved impossible. To investigate the precipitate formed, the following procedure was carried out.

The mixture of FCR and IL was centrifuged for 10 minutes at 5000 rp/min, the solution was removed, the precipitate was air-dried and the IR spectrum was recorded (Figure 1).

The formed compound had characteristic peaks at 3145, 3105, 1571, 1465, 1167 and 625 cm^{-1}. These peaks were attributed to the imidazole ring ν(C-H), the imidazole ν(ring), the imidazole H-C-C and the H-C-N bending, the imidazole C_2-N_1-C_5 bending, respectively. In the region of 700—1100 cm^{-1}, four characteristic peaks at 954, 911, 785 and 745 cm^{-1} corresponded to ν(Mo—O_a), ν(Mo—O_b—Mo) and ν(Mo—O_c—Mo) bonds vibrations of the polyoxoanion.[16]

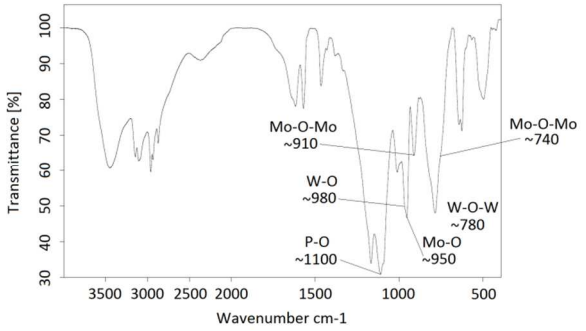

Fig. 1. IR spectrum of the formed complex.

According to the recorded spectrum and literature, the formed complex has Mo-O, W-O and P-O bonds, similarly to polyoxomolybdate and polyoxowolframate ionic liquids.[15, 16]

Paper microzone assay of TPC in IL solutions

By mixing an IL solution with a phenolic compound and the Folin-Ciocalteu reagent (FCR), a colourful solution with a colourful precipitate was obtained. The IL solutions with various amounts of the phenolic compound were spotted onto the FCR dots that were located on the filter paper sheet. The formed polyoxometalate complex precipitated onto the FCR dot and in case of all dots the complex was spread evenly. The colour intensity of the received dot was directly related to the concentration of the added phenolic compound and the amount of the formed precipitate depended on the IL concentration. The formed precipitate on paper should not have a significant influence on the measurement of TPC while the concentration of IL and the volume of FCR are constant during the analysis; thereby the amount of the formed precipitate is constant. The data received for further calculations corresponded to the area of the peak that was proportional to the colour intensity of one dot.

Firstly, different ILs such as BMImAc, $EMMImSO_4$, BMImCl, C_{12}MImCl and $BMImBF_4$ at a concentration of 100 mM were used to test the effect of the IL cation and anion on TPC. Colour intensities of the formed dots were measured at different gallic acid concentrations. Gallic acid was chosen for standard phenolic compound for two reasons: high stability and wide recognition as standard compound to measure TPC.

Secondly, the influence of IL concentration was studied using gallic acid as standard phenolic compound in concentrations of 0.25, 0.5, 1, 1.5 and 2 mM in BMImAc solutions with different concentrations, such as 50, 100, 150 and 200 mM.

All the obtained results (Figure 2) were used to calculate relative standard deviations (RSD). Errors remained between 4.34-9.96% and the biggest difference in areas occurred at the first calibration point where the standard solution had not been added (blank value).

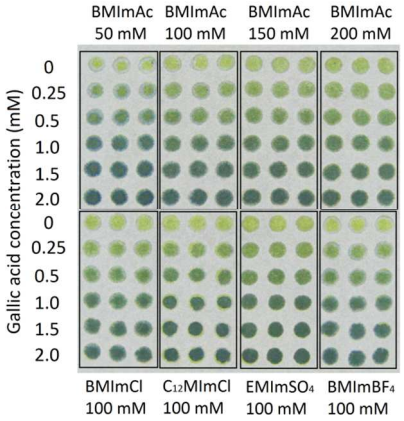

Fig. 2. Paper microzone assay for TPC.

Validation of paper microzone assay of TPC in IL solutions

Linearity

The reflection versus concentration is, in general, a nonlinear function. However, in this work, the linear approximation in the concentration range between 0.25-2 mM proved to be suitable in most cases. According to the results obtained, the only exception was 100 mM C_{12}MImCl catechin solutions whose concentrations between 0.25-1.5 mM proved to be suitable for calibration. The obtained data is shown in Table 1.

Tabel 1. Statistical data for the regression equations of the calibration curve for gallic acid or catechin in different ILs, correlation coefficients and LOD, (n=3).

Phenolic compound	IL	IL concentration (mM)	Equation	R^2	LOD (mM)	LOQ (mM)	Linear range (mM)
GA	BMImAc	50	y = (3236±159)x + (2339±475)	0.904	0.11	0.20	0.25-2.0
CA	BMImAc	50	y = (14502±907)x + (12036±2707)	0.945	0.14	0.25	0.25-2.0
GA	BMImAc	100	y = (3260±133)x + (2442±149)	0.959	0.09	0.17	0.25-2.0
CA	BMImAc	100	y = (6325±437)x + (11986±1304)	0.953	0.15	0.27	0.25-2.0
GA	BMImAc	150	y = (3089±115)x + (2386±342)	0.935	0.08	0.15	0.25-2.0
CA	BMImAc	150	y = (6185±437)x + (11173±1303)	0.933	0.15	0.28	0.25-2.0
GA	BMImAc	200	y = (2874±121)x + (2532±362)	0.974	0.09	0.18	0.25-2.0

CA	BMImAc	200	y = (5504±449)x + (9975±1340)	0.937	0.14	0.25	0.25-2.0
GA	$EMMImSO_4$	100	y = (2859±109)x + (2510±325)	0.996	0.08	0.16	0.25-2.0
CA	$EMMImSO_4$	100	y = (1157±52)x + (1219±154)	0.972	0.10	0.19	0.25-2.0
GA	$BMImBF_4$	100	y = (2778±127)x + (2310±379)	0.942	0.10	0.19	0.25-2.0
CA	$BMImBF_4$	100	y = (4033±277)x + (5070±826)	0.952	0.14	0.27	0.25-2.0
GA	$C_{12}MImCl$	100	y = (2505±157)x + (3047±467)	0.993	0.13	0.25	0.25-2.0
CA	$C_{12}MImCl$	100	y = (1673±127)x + (1668±264)	0.975	0.12	0.23	0.25-1.5
GA	BMImCl	100	y = (2504±130)x + (2675±389)	0.962	0.11	0.21	0.25-2.0
CA	BMImCl	100	y = (1751±87)x + (4117±260)	0.969	0.11	0.20	0.25-2.0

Limits of detection and quantification

Several different approaches for determining the detection limit (LOD) and quantification limit (LOQ) have been advanced over the years. In this study, calibration-design-dependent estimation was used. This approach is based on the use of the confidence limit function, the horizontal intersection with the regression line defines the LOD and the horizontal intersection with the lower confidence limit function of the regression line defines the LOQ.[33, 34] According to the results obtained, the LOD for gallic acid and catechin in different ILs was between 0.08-0.15 mM and LOQ was between 0.16-0.28 mM. Higher LOD and LOQ values were achieved for catechin solutions compared to gallic acid.

Robustness

The robustness of the photographed picture was studied. 100 mM BMImAc gallic acid solutions at a concentration of 0.25, 0.5, 1, 1.5 and 2 mM were used to test the robustness. The paper sheet with standard solutions was photographed in 0.5, 24 and 72 h after analysis and under two different light conditions (with direct lighting and light from behind the paper). The received data were used to calculate RSD and according to the results, the colourful dots formed were stable for at least 72 h and light conditions had a negligible effect on the final result. The lower RSD values were detected in the case of the higher concentration of gallic acid (Table 2).

Tabel 2. Robustness of achieving data from the recorded pictures at different times and in different light conditions

Concentration (mM)	RSD (%)
0.25	10.96
0.5	8.23
1.0	3.82
1.5	1.65
2.0	0.91

Precision

Repeatability (intra-day) and reproducibility (inter-day) were calculated using gallic acid and catechin (Table 3) in 100 mM BMImAc solutions at a concentration of 0.25 to 2.0 mM. The contents of phenols were calculated using the linear and polynomial regressions. According to the results, the most reliable data is obtained using the polynomial regression.

Tabel 3. Repeatability and reproducibility for different gallic acid and catechin concentrations in 100 mM BMImAc solutions.

		Intra-day		Inter-day	
Concentration (mM)	Phenolic compound	Linear regression (RSD %)	Polynomial regression (RSD %)	Linear regression (RSD %)	Polynomial regression (RSD %)
0.25	GA	15.59	11.30	13.07	12.86
0.25	CA	15.96	5.18	15.83	4.91
0.5	GA	4.35	1.72	16.65	6.73
0.5	CA	23.26	0.14	21.66	2.15
1.0	GA	9.71	4.85	15.78	8.63
1.0	CA	11.48	4.15	9.34	8.39
1.5	GA	0.88	3.03	3.95	1.40
1.5	CA	2.52	2.95	0.19	1.91
2.0	GA	2.11	0.67	1.57	13.60
2.0	CA	6.90	0.46	9.30	3.14

Matrix effect of IL solutions

The matrix effect could manifest itself as a contribution of IL to the reflection intensity. The possible matrix effect of the *Achillea millefolium* methanol extract (TPC = 6.17 GAE, mM) and lime juice (TPC = 2.10 GAE, mM) was evaluated, the results are shown in Table 4. One filter paper sheet contained triplicate data of the standard compound in 100 mM IL solutions at a concentration of 0.25, 0.5, 1, 1.5 and 2 mM and the triplicate of the extract diluted in the same IL solutions (1:30). The *Achillea millefolium* methanol extract was diluted in $BMImBF_4$ and catechin was used as standard phenolic compound. For lime juice, two ILs – BMImAc and $BMImBF_4$ were used and gallic acid served as standard phenolic compound.

Tabel 4. Matrix effect evaluation for PμZ assay using lime juice and *Achillea millefolium* methanol extract with gallic acid or catechin as standard phenolic compound (PC), respectively (n = 6).

Extract	IL	PC added (mM)	TPC found (mM)	Recovery (%)
Lime	BMImAc	0.25	0.26	102.1
		0.5	0.47	93.1
		1.0	0.91	91.1
		1.5	1.33	88.9
		2.0	1.88	94.2
Lime	$BMImBF_4$	0.25	0.22	89.8
		0.5	0.44	88.1
		1.0	0.97	96.9
		1.5	1.47	97.7
		2.0	1.96	98.0
Achillea millefolium	$BMImBF_4$	0.25	0.32	128.5
		0.5	0.54	108.4
		1.0	0.95	94.9
		1.5	1.43	95.0
		2.0	1.79	89.6

As the recoveries are between 90-100%, it can be concluded that the matrix effect of IL solutions on the assay is not significant.

Comparison of the obtained results with UV-Vis assay

Herb methanol extracts were diluted as required in distilled water for the spectrophotometric assay and in 200 mM BMImAc for the paper microzone assay, in order to obtain 100 mM IL concentration in sample. Each sample was analysed in triplicate and the obtained data were compared using relative standard deviations (Table 5).

Tabel 5 Comparison of obtained TPC in herb methanol extracts by using UV-Vis and PμZ assay

#	Plant material	UV-Vis (GAE, mM)	PμZ assay (GAE, mM)	RSD (%)
1	*Hypericum perforatum*	21.79	23.46	5.22
2	*Melissa officinalis*	15.39	16.86	6.45
3	*Achillea millefolium*	6.17	5.72	5.38
4	*Vaccinium vitisidaea*	32.70	28.79	9.01
5	*Calluna vulgaris*	5.86	5.30	7.07
6	*Foeniculum vulgare*	1.24	1.30	3.37
7	*Taraxacum officinale*	1.31	1.34	1.72
8	*Cataharanthus roseus*	9.14	7.52	13.6
9	*Vinca minor*	10.12	8.27	14.2

In herbal extract analysis the F-test and t-test were performed using Microsoft Excel Data Analysis. According to the F-test results, the variances of the two populations equal $F < F_{crit}$ ($0.45 < 4.28$), and according to the t-test results, the means are the same – $t_{stat} < t_{crit}$ ($0.04 < 2.18$) and $p > 0.05$.

Quantification of *Catharanthus roseus* and *Vinca minor* IL extracts

C. roseus and *V. minor* IL extracts were used for quantitative analysis. Gallic acid standard solutions (0.25-2 mM) in 100 mM BMImCl were used for calibration. The extracts were diluted as required to calculate TPC (Table 6).

Tabel 6. TPC in *Catharanthus roseus* and *Vinca minor* IL extracts.

Plant material	IL used	TPC (GAE, mM)	RSD (%)
Catharanthus roseus	EMImCl	7.27	2.78
Catharanthus roseus	BMImCl	7.14	0.72
Vinca minor	EMImCl	6.70	2.45
Vinca minor	BMImCl	6.61	5.16

According to the calculated values, *C. roseus* extracts contained phenolics 12.86-13.09 mg of GAE/g. This concentration agrees well with the results obtained by V. Kumar *et al.*, whoanalysed *C. roseus* from different regions of Rajasthan, India, and found it to contain phenolics 7.99-23.12 mg of GAE/g.

Gallic acid equivalents for different phenolic compounds

The antioxidant capacity of herb extracts is closely related to the content of phenolic compounds and their chemical structure. According to literature the phenolic content of plant extracts can be used as antioxidant indicator.[35, 36] We tested the developed colourimetric paper microzone assay for evaluating the antioxidant capacity of different classes of phenolic compounds. The calculated GAE values are presented in Table 7. Methanol stock solutions (10 mM) of standard compounds were diluted in 200 mM BMImCl aqueous solution as required to achieve a sample with 1 mM analyte concentration in 100 mM IL. According to literature, trolox equivalent antioxidant capacity (TEAC) values for the compounds used decrease in the order rutin > gallic acid > catechin > ferulic acid > naringin. Total antioxidant capacity was calculated using Ce(IV)-based reducing capacity assay.[37] Literature data was used for a linear correlation with calculated GAE values and the results correlated well, R^2=0.912.

Tabel 7. Comparison of calculated gallic acid equivalents (GAE) for ferulic acid, naringin, catechin and rutin with literature data.

Phenolic compound	Class	GAE (mM)	Ce(IV) assay$_{TEAC}$**Error! Bookmark not defined.**
Gallic acid	Phenolic acids	1.00	3.27
Ferulic acid	Phenolic acids	0.59	2.18
Naringin	Flavanon glycoside	0.40	1.93
Catechin	Flavanol	0.82	2.47
Rutin	Flavonol glycosides	1.12	3.84

Conclusions

The colorimetric paper microzone assay can be used in cases when the traditional spectrometric assay is not possible (e.g. due to the appearance of precipitates). The principle of the assay was verified in the analysis of herbal IL extracts. When the formation of deposits of complexes of the polyoxometalate-imidazol ionic liquid prevents spectrophotometric determination of analytes, the PuZ assay proved to be reliable, robust, with decent LOD and free from interferences. Moreover, the assay requires no instrumentation, being thus available at the point of care and, finally, the assay is benign and has all features of green analytical chemistry.[38]

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Notes and references

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