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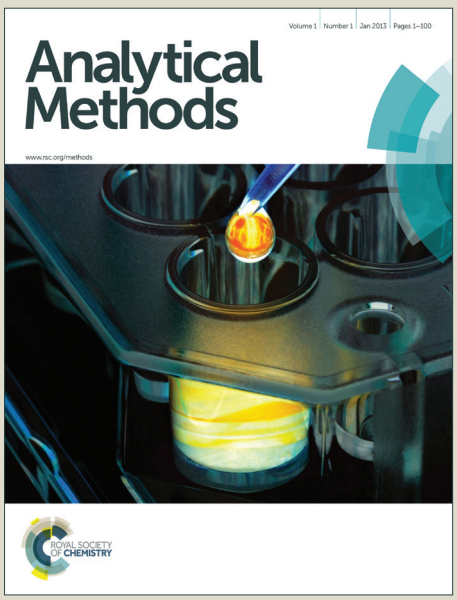

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A novel microfluidic device for estimating the total phenolic/antioxidant level in honey samples using a formaldehyde/potassium permanganate chemiluminescence system.

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Abstract

A microfluidic device has been investigated as a tool for the estimation of the total phenolic content/antioxidant level in honey using an acidic potassium permanganate chemiluminescence ($KMnO_4$-CL) detection system. Selected phenolic antioxidants, including quercetin, catechin, gallic acid, caffeic acid and ferulic acid, produced analytically useful chemiluminescence signals, with detection limits ranging between 2.4 nmol L^{-1} for gallic acid and 34.0 nmol L^{-1} for *o*-coumaric acid. The parameters that affect the chemiluminescence intensities of each antioxidant were carefully optimized, including chip geometry, volume and area of detection chip, pH, concentration of reagents used and flow rates. The effect of formaldehyde and other enhancers on CL signal intensity was extensively investigated.

The method was applied to honey samples. Nine different honey samples exhibited total phenolic/antioxidant levels of 41.2 to 765.4 mg kg^{-1} with respect to gallic acid. The Folin-Ciocalteu (FC) assay results were well correlated with the chemiluminescence results. The method was found to be selective, rapid and sensitive when used to estimate the total phenolic/antioxidant level, producing a good agreement with reported results for honey samples.

Keywords: Microfluidics, chemiluminescence, potassium permanganate, antioxidant, honey

Introduction

Honey is a natural food product well known for its high nutritional and medicinal value. In addition to sugars, honey also includes a wide range of minor constituents that possess antioxidant activity (AA), such as phenolic acids, flavonoids, ascorbic acid, catalase, peroxidase and carotenoids [1]. However, it has been reported that phenolic acids and flavonoids are the major components responsible for AA in honey samples [2]. Therefore, estimating the total phenolic/antioxidant level in honey samples is vital. The Folin–Ciocalteu (FC) assay is the most common assay used to estimate the total phenolic content in honey samples. Various other assays are also used to determine the AA, including the determination of antiradical scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$ assay) or the scavenging of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (TEAC assay) and the determination of the total antioxidant capacity using the phosphomolybdenum method or the ferric reducing antioxidant power (FRAP) assay [3]. However, these methods suffer from being slow and labour-intensive. Additionally, they are not very sensitive and have high detection limits. Chemiluminescence (CL) is the emission of light arising from a chemical reaction. It involves the production of light by species that can undergo highly energetic electron transfer reaction. CL reactions generally yield one of the reaction products in an electronic excited state producing light on returning to the ground state. The technique has attracted the attention of many researchers for the determination of total AA because of its sensitivity, convenience and simplicity. The CL systems that have been reported for evaluating antioxidants in food samples can be divided into two main categories. The first category is based on quenching the antioxidant scavenging activity of H_2O_2 and includes luminol and peroxyoxalate CL systems [4-6]. The

second category is based on the enhancement of CL signals due to the presence of phenolic OH groups. The most common CL system in this category is the permanganate CL system [7].

Costin *et al.* [7] utilized potassium permanganate CL system to monitor the total phenolic/total antioxidant level in wine samples. Later, Francis *et al.* [8] developed an antioxidant assay for fruit juices and teas utilizing the same CL system. They concluded that potassium permanganate CL system has great potential for the exploration of antioxidants in complex sample matrices. Herein, we evaluate a modification of this method for estimating the total phenolic/total antioxidant level in honey samples.

The method was modified and made suitable for application in a microfluidic platform rather than a flow injection system. Flow techniques consume large quantities of chemical reagents, which increase the cost of the analysis and has negative effects on the environment. Additionally, our objective is to make the device easily transportable; therefore, microfluidic platforms are ideal for our study.

Wang *et al.* [9] reported the use of thin-film organic photodiodes (OPD) in integrated on-chip peroxyoxalate chemiluminescence (PO-CL) for antioxidant capacity determination. Antioxidant standards were injected into a stream of PO-CL reagents, resulting in a CL emission decrease that was correlated with the AA. Amatatongchai *et al.* [10] developed an MF-CL system for measuring antioxidant capacity. The detection is based on a peroxyoxalate chemiluminescence (PO-CL) assay with 9,10-bis-(phenylethynyl)anthracene (BPEA) as a fluorescent probe and hydrogen peroxide as an oxidant. Similar detection limits were reported in both cases (on the order of μmol L^{-1}).

The peroxyoxalate CL system is based on the quenching of the CL signal, while the system proposed here is based on the enhancement of the CL signal. Methods based on enhancement of the CL signal usually provide higher sensitivity due to lower background signal compared to methods based on the quenching of the CL signal. The detection limits obtained using peroxyoxalate CL systems were relatively high, while the detection limit in the proposed permanganate/formaldehyde CL system in most of the cases is on the order of nmol L^{-1}.

The proposed method overcomes many of the above mentioned disadvantages of the standard methods currently in use. The method is rapid and the analysis can be completed within 10 min, including sample preparation steps. The method is not labour-intensive and can be easily automated.

Under optimized conditions, the detection limits vary from 2.4 nmol L^{-1} for gallic acid (GA) to 34.0 nmol L^{-1} for O-coumaric acid (OA).

We utilized the proposed system to estimate the total phenolic/total antioxidant level of nine samples of Omani honey. The results were compared with those obtained using FC and $DPPH^{\bullet}$ assays. To the best of our knowledge, this is the first report presenting the determination of the total phenolic/total antioxidant level in Omani honey.

Experimental

Materials

All reagents were of analytical grade, and dilutions were performed using deionized water (Millipore, MilliQ water system). Formaldehyde, formic acid, o-phosphoric acid, methanol, sodium dodecyl sulphate, CTAB, Tween 20, Tween 80, Triton X-100 and Folin Coicalteu reagent were obtained from Fluka (Buchs, Switzerland). Potassium permanganate, sodium polyphosphate, sodium hydroxide, potassium

permanganate and sodium thiosulphate were purchased from Aldrich (Gillingham, UK). Gallic acid, caffeic acid, catechin, o-coumaric acid, ferulic acid, uric acid, ascorbic acid, quercetin and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from BDH (Poole, England).

Preparation of solutions

The permanganate chemiluminescence reagent (0.5 mmol L^{-1}) was prepared by dissolving approximately 2.5 mmol L^{-1} $KMnO_4$ in deionized water with 1% w/v sodium polyphosphate. The pH of sodium polyphosphate was adjusted to pH 2.5 with *ortho*-phosphoric acid. Formaldehyde (2%) was prepared by diluting 541 µL in 10 mL of deionized water. Stock solutions (100 mg L^{-1}) of the antioxidants (caffeic acid, gallic acid, o-coumaric acid, ferulic acid and quercetin) were prepared daily by dissolving 10 mg of each antioxidant in 100 mL of 1 mmol L^{-1} NaOH solution separatly. Further dilutions were in deionized water. Honey samples were prepared by dissolving 2.5 g of honey in 100 mL of 1 mmol L^{-1} NaOH and filtering the sample.

Apparatus

Serpentine (depth 150 µm, width 150 µm, volume 13 µL), teardrop (depth 150 µm, width 200 µm, volume 2 µL) (TD), 32 splits (depth 150 µm, width 200 µm, volume 3 µL) (SF) and spiral (depth 150 µm, width 200 µm, volume 3 µL) microfluidic chips, Fluidic Connect 4515 chip holders and fused silica capillaries were obtained from Micronit (Netherlands), and syringe pumps were obtained from Basi Bee (USA). The detector was a Hamamatsu H7155-2 photomultiplier tube, (PMT) (Japan) effective detection area is 8 mm. The acidity was measured using a Hanna HI18314 pH meter (Romania).

Microfluidic setup for antioxidant analysis

Figure 1 presents a schematic of the microfluidic device used in this study. Using Fluidic Connect 4515 chip holders and silica capillaries, chip 4 (serpentine) was connected to the syringe pump with two syringes (one for permanganate and one for formaldehyde solution). Chip 4 was connected to chip 3 (SF) via a silica capillary (150 µm ID, 20 cm long). The PMT was placed on top of chip 3. Its position was optimized to obtain the strongest CL signal. Stopped flow experiment was carried out using quartz cell, CL reagents and 100 ppm GA.

Determination of total phenolic content using the Folin-Ciocalteu assay

The determination of the total phenolic content was carried out as described by [11]. In contrast, the prepared honey samples were used without dilution. A 0.5-mL volume of each standard (10 - 100 mg L^{-1}) or sample solution was added to 2.5 mL of water and 0.5 mL of Folin-Ciocalteu reagent and incubated for 5 min. Next, 20 mL of sodium carbonate (w/v) was added. The reaction mixture was then left to stand for 1 hr at room temperature before measuring the absorbance at 785 nm. The total antioxidant content was determined based on the absorbance obtained with respect to GA as a standard.

DPPH radical scavenging method

The radical scavenging activity of honey samples was tested based on the scavenging of the DPPH free radical. The sample (0.5 mL) (500 mg mL^{-1} in methanol) was mixed with 3.5 mL DPPH (6×10^{-5} M in methanol) in a test tube. The tube was incubated at room temperature in the dark for 2 hr and then centrifuged for 10 min at 4200 rpm. Absorbance of the DPPH control was noted and subtracted from those of the samples

to obtain the scavenging activity of the honey samples using the equation $((Abs_{control}-Abs_{sample})/Abs_{control}) \times 100\%$.

In all experiments, each honey sample was analysed twice and the average of two analysis was used in all calculations.

Results and Discussion

Three phenolic acids and one polyphenolic compound were selected for the initial optimization study: gallic acid (GA), caffeic acid (CA), o-coumaric acid (OA) and quercetin (QR). Initially, QR was not soluble in water. However, all four analytes were highly soluble in 1 mmol L^{-1} sodium hydroxide. Potassium permanganate solution (0.3 mmol L^{-1}) and 2 µL of the analyte were injected directly into the TD chip at flow rates of 20 µL min^{-1} and 30 µL min^{-1}, respectively. The TD chip was placed directly under the PMT tube in a black box. Using this protocol, a weak CL signal was observed for all analytes. We then optimized the potassium permanganate and polyphosphate concentrations. The optimum concentrations were found to be 0.5 mmol L^{-1} and 1% for potassium permanganate and polyphosphate, respectively. According to literature, the pH strongly affects CL signal intensity in potassium permanganate CL system [7]. Figure 2 shows the effect of the pH on the CL signal intensity. For all analytes, the maximum signal was obtained at pH 2.5-3.0. Therefore, pH 2.5 was selected as the optimum pH and used for the rest of this study. The CL signal intensity required further enhancement to minimize the detection limits. The high sensitivity reported in the literature using flow injection analysis may be due to high sample and reagent volumes used [7]. In this work, however, the sample volume used is more than 35 times less than that used in the flow injection system. Additionally, the mixing occurs at much lower flow rates in microfluidics (70

μL min^{-1}) than in flow injection analysis (2600 μL min^{-1}) [7]. It is also worth noting here that the residence time in microfluidics is very short.

In the literature, several chemicals are reported to enhance the potassium permanganate CL system [12]. We tested some of these enhancers and compared the obtained CL signal with CL signals obtained without an enhancer. The enhancers were mixed online with potassium permanganate solution using two chips (chips 1 and 4 in Figure 1). In this study several enhancer were tested such as sodium thiosulphate, formic acid and formaldehyde. When sodium thiosulphate and formic acid were used separately as enhancers, some improvement in the CL signal was observed, but a remarkable improvement in the CL signal intensity was observed when formaldehyde was used as an enhancer. The CL signal intensity improved by a factor of more than ten for CA and GA, a factor of nine for OA and a factor of 27 for QR (Figure 3). It is clear that the using formaldehyde greatly enhances the CL signal. Formaldehyde in known to be a toxic substance however, a minute amount of dilute solution (2 %) is used in the proposed method.

It has been demonstrated that in potassium permanganate CL systems, the light emitting specie is possibly the excited state Mn(II)*. Mn(VII) oxidizes phenolic acids and formaldehyde possibly increases the rate of the oxidation. This results in increasing generation of radical intermediate that react with Mn(III) to produce Mn(II)*. [13] The observed red emission (734±5nm) is due to the relaxation of Mn(II)* from an excited state ($^4T_1$ to $^6A_1$ transition).[14]

Instrumental setup of the detection window was also optimized as it plays an important role in improving sensitivity of the proposed method. Generally, for a fast CL reaction like $KMnO_4$ system, if the mixing process is slow, most of the reagents will react during the mixing process and sensitivity will drop down. However, if

mixing is completed before significant reaction has occurred, sensitivity will improve considerably. Another factor that may affect the sensitivity is detection area and volume of the flow cell. Both factors improve the sensitivity of the CL method. This is because number of light emitting species increases as area of detection or volume of the flow cell increases.

To study the effect of flow cell volume and detection area, three different detection chips were evaluated. These are TD, spiral and SF chip. The shape, volume, detection area and mixing mechanisms of these chips are tabulated in table 1.

When spiral chip was used, the signal intensity was half that obtained using TD chip. This is probably because the latter, despite having a small volume of only 2 µL, is a very efficient mixer (see table 1 for the mixing mechanisms of this chip). We then decided to replace the spiral chip with an SF chip (chip 3 in Figure 1). This has resulted in improving of CL signal intensity by a factor of 2.5 relative to TD chip and a factor of five relative to spiral chip. It worth noting here, that improvement in CL signal intensity obtained by using SF chip compared to TD chips cannot be explained based on the improvement in the mixing efficiency alone. Possibly, the increase in the detection area and the volume of chip have played an important role. TD chips are expected to produce the highest mixing efficiency, as the mixing in TD is based on three mixing mechanisms, while SF chips uses two mixing mechanisms as described in table 1. (see supplement 1 for further information).

Finally, the sample and CL reagent flow rates optimized. The optimized conditions are listed in Table 2.

Analytical appraisal

Using optimum conditions (Table 2), the calibration curves for GA, CA, CA, ferulic acid (FA), QR and catechin (CC) were obtained. These curves were established with a

series of standard solutions containing each analyte separately. Eight solutions with different concentrations of each analyte were used, and non-linear regression curves were obtained for all of the analytes. In CL method none linear response is commonly observed especially when a calibration curve is obtained for a wide range of concentrations. This is due to the fact that the proportion of analyte to CL reagent can affect the kinetics of the reaction [7]. The limits of detection, limits of quantification, concentration range and calibration equations are listed in Table 3. A definition of detection limit in this work was based on analyte concentration that gives a response signal three times that of the standard deviation of a blank signal, while the limit of quantifications was calculated based on analyte concentration that gives a response signal ten times that of the standard deviation of a blank signal. Only 2 µL of the sample was injected, for which the detected amount of each analyte varied from 4.8×10^{-14} to 68.0×10^{-14} mol. The LOD values are comparable with that reported earlier using a flow injection system despite the fact that the sample volume being only 2 µL in the proposed method while that in flow injection method was 70 µL [7]. Excellent reproducibility was obtained, with an RSD of less than 2%. The stability of the antioxidant samples was tested, and it was found that these samples are stable for at least 2 hr when placed in ambient conditions.

Interference studies

To apply the suggested method to the determination of total antioxidant content in honey, the interference of major components present in honey was investigated by adding these components to a solution containing 100 µg L^{-1} of each antioxidant. A foreign species was considered not to interfere if it contributes a relative error less than 5% during the determination of 100 µg L^{-1} of each antioxidant. The tolerable

molar concentration ratios of foreign species to every antioxidant tested at the 5% level were over 2000 for glucose and sucrose and 100 for bovine serum albumin.

Applications

Nine honey samples were analysed by the developed CL method. The nine samples can be divided into three types, known locally as *azhar*, *seder* and *sumur*. Three different samples from each type were analysed twice. The average total phenolic/antioxidant level as GA and the standard deviation (SD) are shown in Table 3. The result clearly shows that the total phenolic/antioxidant level of *sumur* is higher than that of the other two types, while that of *azhar* honey was lowest. The SD values in all cases were below 5%. The total phenolic/antioxidant level of these nine samples was also determined using FC assay (Table 4). The results showed a very strong correlation between the FC assay and the new developed method. The correlation factor between these two methods was greater than 0.995, as presented in Figure 4. The total phenolic/antioxidant levels found here generally fall within the broad range reported in the literature [14].

The $DPPH^{\bullet}$ assay was also carried out for these nine samples. This assay measures the AA of a sample (Table 4). A direct correlation was observed between the AA of a sample and the total phenolic/antioxidant level of the honey sample. The CL methods showed that total phenolic/antioxidant level of sumur honey is much higher than that of the other two types. The AA of *sumur* samples was highest when measured using the $DPPH^{\bullet}$ assay. The lowest total phenolic/antioxidant level was observed for *azhar* samples using the $DPPH^{\bullet}$ assay.

It has been reported that there is a correlation between the colour of honey and the total phenolic/antioxidant level. This correlation was also observed here; *sumur* honey has the darkest brown- yellow colour, followed by *seder* and then *azhar* honey [15].

Conclusion

A novel microfluidics device has been successfully developed for estimating total phenolic/antioxidant level in honey samples using potassium permanganate/formaldehyde CL system. The developed system is sensitive, simple and portable. The high sensitivity is due to the novel SF chip design and the use of formaldehyde as an enhancer. The total analysis time is approximately 10 min, including sample preparation. This method was successfully used to estimate the total phenolic/antioxidant level in nine Omani honey samples. The results showed that *sumur* honey is a much richer antioxidant source than *seder* and *azhar* honey.

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Table 1: The shape, the volume, the detection area and the mixing mechanism of the chips used.

Chip type	Shape	Mixing mechanism	Volume of detection window (µL)	Detection area (mm^2)
Teardrop	Teardrop shaped mixing units in three layers.	1- The chaotic flows 2- The induction of secondary flow as result of centrifugal forces as the solution travels through the folding 3- The increase of the contact surface of the fluid at the interfacial area and causes diffusion to occur quickly	2	13.3
Spiral	Spiral chip in two layers	Only the second mixing mechanism of the teardrop chip mentioned above.	3	30
SF	Spiral chip with 32 flow split units in two layers.	Second and third mixing mechanisms of the teardrop chip mentioned above.	3	30

Table 2: Factors included in the optimisation of the CL reaction and the optimal conditions.

Variable	Range	Optimum using Microfluidics
$KMnO_4$ (mmol L^{-1})	0.1-1.0	0.5
Formaldehyde (%)	0-6	2
$KMnO_4$ / Formaldehyde flow rate ($\mu L min^{-1}$)	5-100	20
Bufer (Sodium polyphosphate, %)	0.8-1.2	1.0
pH	1.5-3.5	2.5
Antioxidant flow rate ($\mu L min^{-1}$)	10.0-90.0	30.0
Antioxidant target volume (μL)	1.0-5.0	2.0

Table 3. Linear range, calibration equations, limits of detection, limits of quantification and detected amount of various phenolic acids and polyphenols.

Antioxidant	Range (mol L^{-1})	Equation	R^2	LOD (mol L^{-1})	LOQ (mol L^{-1})	Detected amount (mole)
Caffeic Acid	5.6×10^{-8} - 2.8×10^{-6}	$y = -8083.4x^2 + 93085x + 20962$	0.9972	4.3×10^{-9}	1.4×10^{-8}	8.6×10^{-14}
Catechin	3.4×10^{-8} - 1.7×10^{-6}	$y = -2302.4x^2 + 26685x + 4927.2$	0.9903	8.3×10^{-9}	2.7×10^{-8}	16.0×10^{-14}
Ferulic Acid	5.1×10^{-8} - 2.6×10^{-6}	$y = -726.28x^2 + 9858.5x + 3790.2$	0.9919	30.0×10^{-9}	9.9×10^{-8}	60.0×10^{-14}
Gallic Acid	5.9×10^{-8} - 2.9×10^{-6}	$y = -3137.6x^2 + 51293x + 11363$	0.9955	2.4×10^{-9}	7.9×10^{-9}	4.8×10^{-14}
o-Coumaric Acid	6.9×10^{-8} - 3.0×10^{-6}	$y = -569.02x^2 + 9351.2x + 491.26$	0.9989	34.0×10^{-9}	1.1×10^{-7}	68.0×10^{-14}
Quercitin	3.3×10^{-8} - 1.6×10^{-6}	$y = 428.77x^2 + 7663.7x + 1210.7$	0.9948	12.0×10^{-9}	4.1×10^{-8}	25.0×10^{-14}

x is the concentration of the antioxidant in mol L^{-1}, y is the CL signal intensity

Table 4: The average total phenolic/antioxidant level as GA and the standard deviation (SD) of various honey samples evaluated using the proposed method, FC method and DPPH* assay.

Sample	Total phenolic in honey (mg kg^{-1} with respect to GA) using the proposed method	SD the proposed method	Total phenolic in honey (mg kg^{-1} with respect to GA) using FC	SD FC	AA% of honey sample using DPPH$^{\bullet}$ assay	SD AA%
Azhar 1	53.9	6.5%	54.6	7.1%	17.8	0.9%
Azhar 2	41.2	0.8%	43.6	8.9%	15.4	0.5%
Azhar 3	70.7	4.4%	68.2	11.1%	27.9	0.6%
Sudur 1	82.4	1.3%	83.7	6.0%	28.4	0.3%
Sudur 2	112.4	0.1%	115.1	3.4%	33.0	0.2%
Sudur 3	98.4	1.2%	98.6	3.9%	31.6	0.7%
Sumur 1	642.8	0.4%	659.7	0.6%	93.8	0.1%
Sumur 2	631.2	3.5%	651.0	6.3%	92.8	0.1%
Sumur 3	765.4	1.3%	749.2	3.5%	92.3	0.1%

Captions

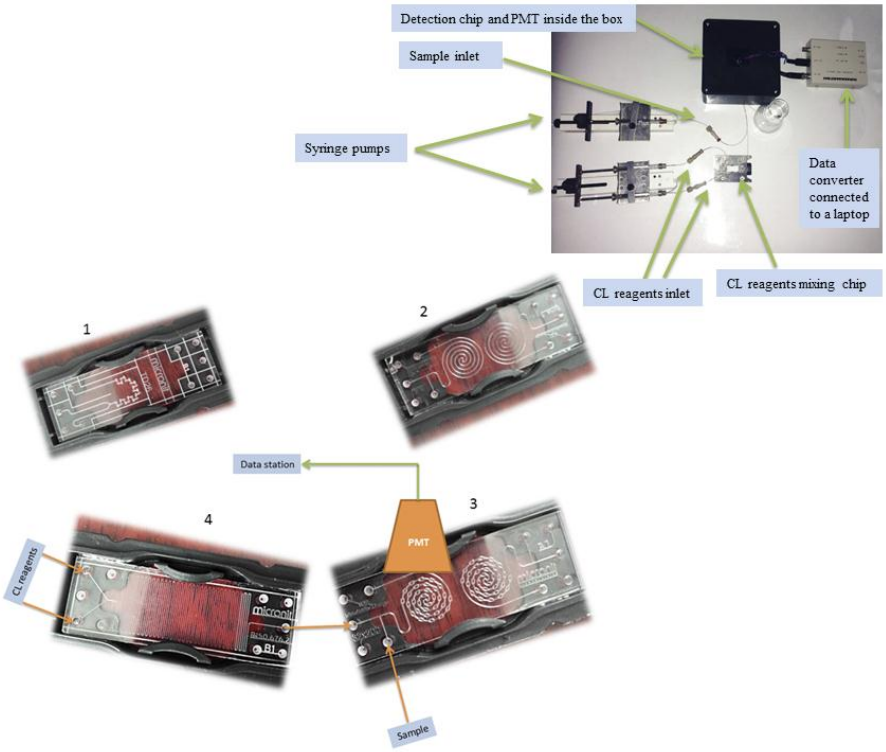

Fig. 1: TD chip, 2: spiral chip, 3: SF chip, 4: serpentine chip. In the final instrumental setup, SF and serpentine chips were used. Inset picture is for the instrumental setup.

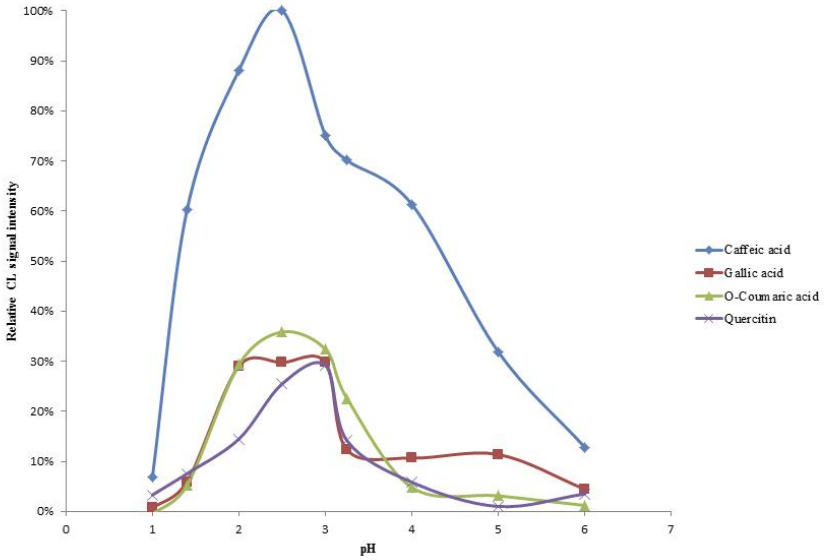

Fig. 2: Effect of pH on the CL signal intensity of the antioxidants.

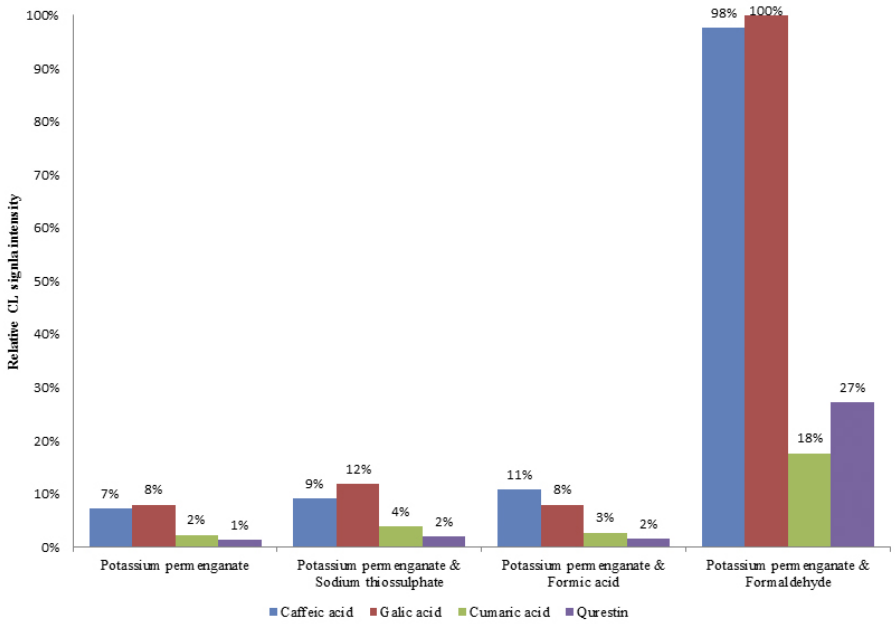

Fig. 3: Effect of the various enhancers on the CL signal intensity of the antioxidants.

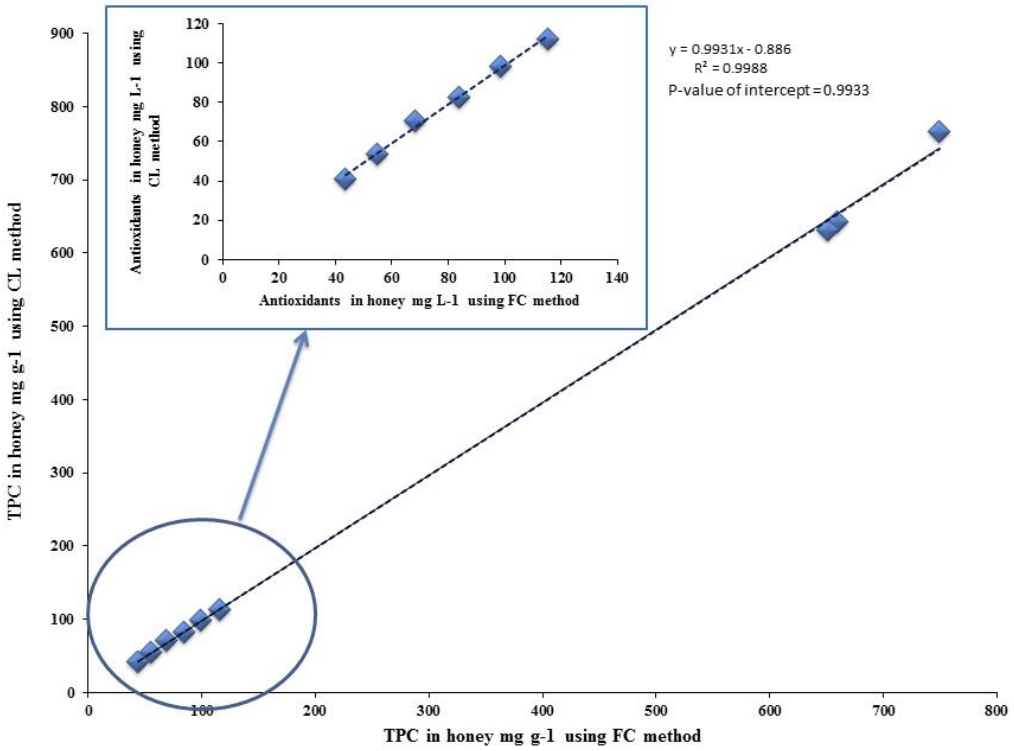

Fig. 4: Total phenolic contents in the honey samples determined using the proposed CL method and the FC method.