Talanta 133 (2015) 38–44

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Determination of polyphenolic compounds in *Cirsium palustre* (L.) extracts by high performance liquid chromatography with chemiluminescence detection

Edyta Nalewajko-Sieliwoniuk [a,*], Julita Malejko [a], Monika Mozolewska [a], Elżbieta Wołyniec [a], Jolanta Nazaruk [b]

[a] Department of Analytical Chemistry, Institute of Chemistry, University of Białystok, ul. Hurtowa 1, 15-399 Białystok, Poland
[b] Department of Pharmacognosy, Medical University of Białystok, ul. Mickiewicza 2a, 15-089 Białystok, Poland

ARTICLE INFO

Article history:
Received 16 December 2013
Received in revised form
2 May 2014
Accepted 12 May 2014
Available online 6 July 2014

Keywords:
Polyphenols
Cirsium palustre (L.)
High performance liquid chromatography
Manganese(IV)
Chemiluminescence
Antioxidant activity

ABSTRACT

The first method for the simultaneous determination of polyphenolic antioxidants in extracts from leaves of *Cirsium palustre* based on high performance liquid chromatography combined with flow injection chemiluminescence detection (HPLC-FI-CL) has been developed. The extracts were prepared by using methanol as extraction medium and two types of extraction methods (reflux and ultrasound assisted extraction). The post-column CL determination of polyphenols was based on their enhancing effect on the chemiluminescence intensity generated in manganese(IV)–hexametaphosphate–formaldehyde system in a phosphoric acid medium. Main antioxidants determined in *C. palustre* leaves were eriodictyol-7-*O*-glucoside, luteolin-7-*O*-glucoside and 6-hydroxyluteolin-7-*O*-glucoside belonging to flavonoids, and chlorogenic acid belonging to phenolic acids. Chromatographic separation was carried out on a C18 column with gradient elution by using a mobile phase containing 0.25% (v/v) phosphoric acid in water (solvent A) and 100% methanol (solvent B). Under the optimized conditions of chromatographic separation and CL detection the validation of the method was performed. The calibration curves showed good linearity in the concentration range from 0.5 to 40 μg mL^{-1}. The HPLC-FI-CL method was successfully applied to the determination of four polyphenolic compounds in methanolic extracts from leaves of *C. palustre*. The accuracy of the developed method was confirmed by the comparison of the results with those obtained by an HPLC-PDA method. The relative error of determination does not exceed 6.1%. However, the HPLC-FI-CL method is characterized by 40–65 times higher sensitivity compared to the HPLC-PDA method.

1. Introduction

Cirsium palustre (L.) Scop. is a biennial plant belonging to the Asteraceae family. As its name implies, marsh thistle is very common in damp ground including marshes. This plant is native to Europe and to western Asia [1]. Asiatic *Cirsium* species, especially *Cirsium japonicum*, have been traditionally used in oriental medicine as anti-hemorrhagic, diuretic, anticancer agents [2,3]. *Cirsium maackii* has been reported to have anti-inflammatory activity and European *Cirsium rivulare* pro-cognitive effect [4,5]. The activity of thistles in most cases depends on the presence of flavonoid compounds.

As it was showed in our previous studies [6,7] *C. palustre* is a valuable source of flavonoids. Eighteen compounds belonging to four various subclasses were isolated from flower heads and leaves of this plant. It was also found that methanolic extract from leaves of *C. palustre* has the highest total content of polyphenolic compounds among all examined *Cirsium* species [8]. The antioxidant activity of *C. palustre* leaves extracts was estimated spectrophotometrically by the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay and the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging method [9]. However, the contents of individual polyphenolic antioxidants in these extracts have not been determined yet.

Many batch and flow injection (FI) methods have been developed for the determination of the total polyphenolic content and antioxidant activity of plant and food samples [10,11]. In order to determine individual polyphenols in such complex matrices and identify which of them are the most likely to be responsible for the antioxidant activity of investigated samples, separation technique should be applied before their detection. The review of the literature shows that for the separation of polyphenols, mainly

* Corresponding author.
E-mail address: nedyta@uwb.edu.pl (E. Nalewajko-Sieliwoniuk).

http://dx.doi.org/10.1016/j.talanta.2014.05.062
0039-9140/

high performance liquid chromatography (HPLC) was applied. The most popular post-column detection of polyphenolic antioxidants is spectrophotometry based on free radical discoloration reactions (with DPPH radical or ABTS radical cation) and a reaction with Folin–Ciocalteu reagent [12,13]. Less common type of post-column assay of polyphenolic compounds is chemiluminescence (CL) [14]. HPLC coupled with CL detection combine the benefits of high efficiency of separation offered by HPLC and high sensitivity of CL systems. For the post-column addition of CL reagents the flow injection systems are employed. Most of the HPLC-FI-CL methods were used only to screen for antioxidants in plant and food samples. These screening methods, in most cases, are based on the inhibition of chemiluminescence generated in the luminol-based systems [15,16] and on the enhancement of acidic potassium permanganate chemiluminescence [17,18]. Only in a few studies quantification of individual polyphenolic compounds by using HPLC-FI-CL methods was carried out. These methods are based on the enhancing effect of phenolic compounds on chemiluminescence generated in the acidic cerium(IV)-rhodamine 6G [19] and acidic cerium(IV)-Tween 20 systems [20]. They were applied to the determination of phenols in red wine and apple juice, respectively. The HPLC-FI-CL method for the determination of flavonoids in tea based on the on-line photochemical degradation and subsequent tris(2,2′-bipyridine)ruthenium(III) chemiluminescence detection has also been developed [21]. Trace amounts of rutin and quercetin in Chinese herbal medicine plants were determined by the HPLC method with post-column luminol-potassium ferricyanide chemiluminescence detection [22]. Gold nanoparticle-catalyzed luminol chemiluminescence detector coupled to HPLC instrumentation was used for the determination of phenolic compounds in red wine [23].

A problem often observed in HPLC-FI-CL systems with gradient elution is unacceptable chemiluminescence baseline drift due to the change in the concentration of organic modifier, pH, ionic strength of the mobile phase. The second problem in HPLC-FI-CL systems is the quenching of chemiluminescence by organic solvents used as the constituents of mobile phases. These problems usually occur when alkaline solution of luminol is used as a post-column CL reagent [24]. Therefore, searching for new CL detection systems, more compatible with chromatographic conditions, is a very important task. Up till now, only a small number of analytical applications of post-column manganese(IV)-based chemiluminescence detection has been reported. Brown et al. [25] and Smith et al. [26] demonstrated the possibility of using chemiluminescence of acidic manganese(IV)–formaldehyde system as a post-column detection of alkaloids, indoles and some analytes of forensic interest. However, these HPLC-FI-CL methods have not been applied to the analysis of real samples. McDermott et al. [27] used post-column manganese(IV)–formaldehyde CL detection for determination of two key biomarkers of oxidative stress, glutathione (GSH) and glutathione disulfide (GSSG), in whole blood. In this work, as a post-column detection of polyphenols in *C. palustre* leaves extracts we employed manganese(IV)-based chemiluminescence, but in the presence of two sensitizers: formaldehyde and sodium hexametaphosphate. These CL reagents were previously used by us for the determination of the total polyphenolic content in *C. palustre* extracts using flow injection analysis without chromatographic separation step [9]. We observed that weak chemiluminescence generated after mixing acidic colloidal manganese(IV), sodium hexametaphosphate and formaldehyde was strongly enhanced by plant polyphenols. It was found that *C. palustre* leaves are rich in polyphenolic antioxidants and the highest total contents of these compounds were determined in methanolic extracts. Therefore, we decided to conduct further investigation on the determination of individual polyphenols in methanolic extracts from leaves of *C. palustre* in order to find out which polyphenolic compounds are the most responsible for the activity of these extracts.

The aim of this study was the elaboration of the first HPLC-FI-CL method that would allow the quantification of polyphenolic constituents of *C. palustre* leaves. Polyphenols were determined in methanolic extracts prepared by two extraction modes: reflux (RE) and ultrasound assisted extraction (UAE). To verify the accuracy of the proposed method, the results were compared with those obtained by the HPLC-PDA method.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade unless otherwise stated and solutions were prepared by using water purified in a Milli-Q Plus water purification system (Millipore S.A., Molsheim, France). The following standard polyphenolic compounds were used: eriodictyol 7-*O*-glucoside and luteolin 7-*O*-glucoside from Extrasynthese (Genay Cedex, France), chlorogenic acid from Sigma-Aldrich (Steinheim, Germany) and 6-hydroxyluteolin 7-*O*-glucoside isolated from *C. palustre* [7]. The stock solutions (1000 μg mL^{-1}) of investigated polyphenols were prepared in HPLC gradient grade methanol and kept in the dark. The working solutions were prepared daily by an appropriate dilution of the stock solutions with water. HPLC gradient grade methanol and acetonitrile were from Sigma-Aldrich (Steinheim, Germany). HPLC grade phosphoric and trifluoroacetic acids were supplied from Sigma-Aldrich (Steinheim, Germany), acetic acid was from POCH (Gliwice, Poland).

Formaldehyde was provided by POCH (Gliwice, Poland). The working solution of this reagent was prepared daily in water to reach a concentration of 0.8 mol L^{-1}. A colloidal manganese(IV) solution was prepared according to Jáky and Zrinyi method [28], which has been slightly modified. Manganese dioxide was formed during the reaction of sodium formate (Sigma-Aldrich, Steinheim, Germany) with potassium permanganate (POCH, Gliwice, Poland) in the solution of pH 6.8 adjusted with sodium hydroxide (POCH, Gliwice, Poland). The precipitate was collected on a glass Büchner funnel (with sintered disc porosity grade G-4) and then rinsed three times with water. 0.4 g of wet manganese dioxide was dissolved in 1 L of 4.5 mol L^{-1} phosphoric acid (POCH, Gliwice, Poland). The mixture was placed in an ultrasonic bath at a temperature of 25 °C for 24 h. Such prepared solution was kept in the dark at an ambient temperature and used after four days to ensure the highest sensitivity of the chemiluminescent measurements [29]. The concentration of colloidal manganese(IV) solution determined by iodometric titration was 1.7×10^{-3} mol L^{-1}. In such prepared acidic solution of manganese(IV) an appropriate amount of sodium hexametaphosphate ($Na_6(PO_3)_6$) (Sigma-Aldrich, Steinheim, Germany) was dissolved.

2.2. Plant material and extract preparation

Leaves of *C. palustre* were collected in June 2012 near Białystok, situated in the North-East of Poland. The voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, Medical University of Bialystok (Poland). After air-drying, the leaves were powdered with a pestle and a mortar and passed through a 1.6 mm sieve.

Plant material (5 g) was extracted: (a) with 100 mL methanol under reflux for 1 h, (b) with 100 mL methanol at 40 °C in an ultrasonic bath for 30 min. After filtration, the extracts were evaporated to dryness under a vacuum.

Extraction efficiencies calculated as percentage weight of the starting plant material (% w/w) were: 16.0% and 16.2% for extracts obtained by reflux and ultrasound assisted extraction, respectively.

After evaporation of solvent, 5 mg of residues from extracts were dissolved in 5 mL of methanol. Before analysis, the extracts were diluted with water, to adjust the concentration of polyphenols to the linear calibration range, and filtered through a 0.45 μm PVDF syringe filter.

2.3. Apparatus and procedure

The Varian 920-LC analytical high performance liquid chromatograph with photodiode array (PDA) detector (Varian, Inc., Australia) was used throughout the study. Chromatographic separations were carried out on XBridge BEH Shield RP18 column (5 μm, 4.6 mm × 150 mm) with XBridge BEH Shield RP18 Sentry Guard Cartridge (5 μm, 4.6 mm × 20 mm) (Waters, USA). A mobile phase containing 0.25% (v/v) phosphoric acid in water (solvent A) and 100% methanol (solvent B) was applied. The elution was performed using the following linear gradient profile: solvent B increased in 25 min from 30 to 60%, in 5 min from 60 to 90%, and was held for the following 5 min. After each injection a re-equilibration period of 5 min followed. The flow rate of the mobile phase was 1.0 mL min^{-1} and the injection volume was 50 μL. All chromatographic separations were performed at 25 °C. The wavelengths used for the quantitative determination of chlorogenic acid, eriodictyol 7-*O*-glucoside, 6-hydroxyluteolin 7-*O*-glucoside and luteolin 7-*O*-glucoside by the HPLC-PDA method were 326, 283, 346 and 346 nm, respectively.

The configuration of the HPLC-FI-CL system used in this work is illustrated in Fig. 1. The post-column addition of the CL reagents to HPLC eluate was carried out using the flow injection system. It was composed of a peristaltic pump (Gilson Minipuls 3, France) delivering of 0.8 mol L^{-1} formaldehyde solution and 1.7×10^{-3} mol L^{-1} manganese(IV) solution (prepared in 4.5 mol L^{-1} H_3PO_4 and containing 4% (w/v) sodium hexametaphosphate), PTFE tubing (0.8 mm i.d.), Perspex T-piece merging both streams (T_1), and a CL detector (KSP, Poland). A flow cell (FC) was a flat spiral of PTFE tubing (1.0 mm i.d., 25 cm length) located directly in front of the window of a photomultiplier tube (Carls Zeiss, Jena, Germany). The distance between the mixing point of all solutions (T_2) and the flow cell was 5 cm. The flow rate of the CL reagents was set at 3.6 mL min^{-1}. The photomultiplier was operated at 1100 V. Detector response was recorded on a computer with KSP software.

2.4. Statistical analysis

Data were analyzed with Microsoft Office Excel software (version 2007, Microsoft Corp, USA). Values were expressed as mean ± standard deviation. *F*-test was used to assess the significance of variances of two sets of the results (obtained by the HPLC-FI-CL and by the HPLC-PDA methods). The significance of the mean difference of the two sets of results was evaluated by

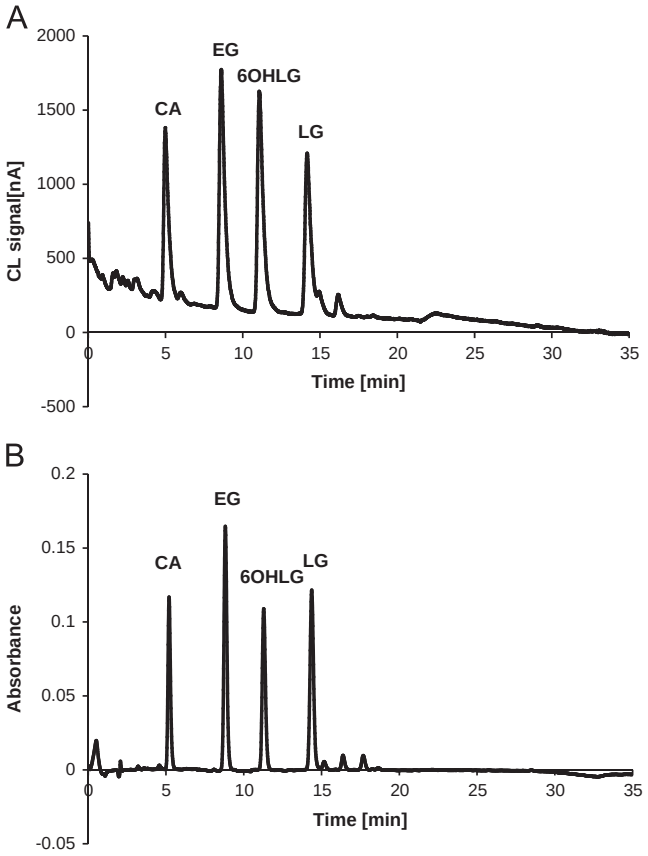

Fig. 2. Typical chromatograms of methanolic extract from leaves of *C. palustre* using: (A) manganese(IV) chemiluminescence detection; (B) UV absorbance detection. Peaks: (CA) chlorogenic acid (t_R=5.20 min), (EG) eriodictyol 7-*O*-glucoside (t_R=8.81 min), (6OHLG) 6-hydroxyluteolin 7-*O*-glucoside (t_R=11.28 min) and (LG) luteolin 7-*O*-glucoside (t_R=14.37 min). Chromatographic conditions are detailed in Section 2.3. Peaks were identified by using standard solutions.

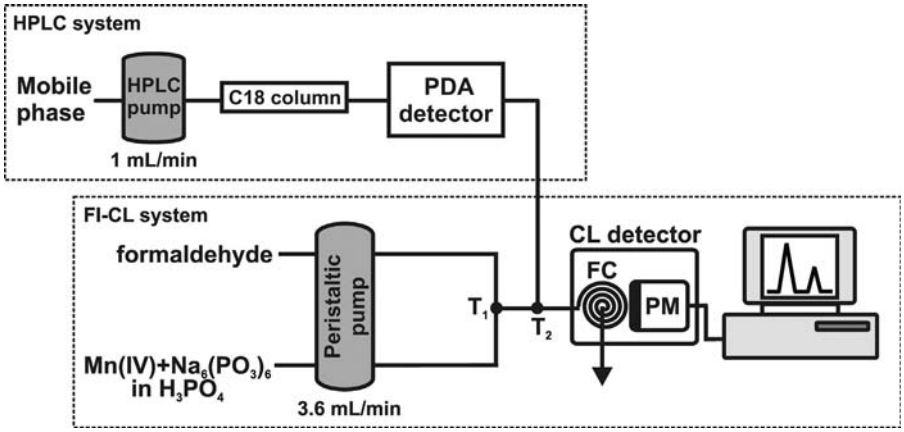

Fig. 1. Schematic diagram of the HPLC-FI-CL system for the determination of polyphenols in *C. palustre* leaves extracts. T_1, T_2: mixing points; FC: flow cell; PM: photomultiplier tube.

t-test. The calculated F and t values were compared with critical values in published *F*-distribution and *t*-distribution tables (α=0.01).

3. Results and discussion

The main polyphenolic compounds identified in methanolic extracts from *C. palustre* leaves by the HPLC-PDA method were chlorogenic acid (CA, t_R=5.20 min), eriodictyol 7-*O*-glucoside (EG, t_R=8.81 min), 6-hydroxyluteolin 7-*O*-glucoside (6OHLG, t_R=11.28 min) and luteolin 7-*O*-glucoside (LG, t_R=14.37 min). The typical PDA chromatogram obtained for plant extract is shown in Fig. 2B. The peaks were identified by comparing the retention times and UV spectra with those obtained for standard solutions of polyphenols and by observation of an increase in peak area after the addition of the corresponding standard to the sample solution. The chemical structures of studied polyphenols are presented in Fig. 3.

3.1. Optimization of HPLC-FI-CL conditions

The problem in HPLC-FI-CL systems is that the mobile phase should not only be suitable for the good separation of analytes but also should be compatible with applied CL reaction. Therefore, we investigated several different compositions of mobile phases which were previously used in the literature for separation of plant polyphenols. Some of them were slightly modified in order to obtain good resolution of polyphenolic compounds present in extracts from leaves of *C. palustre*. We examined the following mobile phases: 0.25% (v/v) phosphoric acid in water (solvent A) and 100% methanol (solvent B) [30]; 1.0% (v/v) acetic acid in water (solvent A) and 100% methanol (solvent B) [31]; 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 100% methanol (solvent B) [32]; 0.02% (v/v) trifluoroacetic acid in water (solvent A) and 0.02% (v/v) trifluoroacetic acid in methanol (solvent B) [33]; water (solvent A) and 0.025% (v/v) trifluoroacetic acid in acetonitrile (solvent B) [34]; 0.05% (v/v) trifluoroacetic acid in mixture of acetonitrile:water (17:83, v/v) (solvent A) and 100% acetonitrile (solvent B) [35]. We found that the mobile phases containing acetonitrile strongly inhibited CL signals of studied polyphenols. The comparison of the CL signals obtained for methanolic and acetonitrile mobile phases is presented in Fig. 4. This is in accordance with previous observations of McDermott et al. [27] who found that acetonitrile quenched the post-column manganese(IV) chemiluminescence much more intensively than methanol. Therefore, the mobile phases containing methanol were selected for further optimization studies. The best resolution of chromatographic peaks was observed for mobile phase containing 0.25% (v/v) phosphoric acid in water (solvent A) and 100% methanol (solvent B). Therefore, this mobile phase was chosen as an optimal. The gradient elution program was also optimized. The best resolution of peaks was obtained for the following gradient elution program: 0 min: 70% A, 30% B; 25 min: 40% A, 60% B; 30 min: 10% A, 90% B; 35 min: 10% A, 90% B.

As a post-column detection after chromatographic separation of polyphenols we applied manganese(IV)-based CL reaction. Before, we had used such reaction for the determination of the total content of polyphenols in plant-derived beverages (wine, tea and fruit juices) using flow injection analysis [29]. The results were expressed as milligrams of gallic acid equivalents per litre of drink. As a sensitizer of CL reaction we applied formaldehyde. For the FI-CL determination of the total polyphenolic content (expressed as 6OHLG equivalents) in extracts from leaves of *C. palustre* we used Mn(IV) reagent, but in the presence of two sensitizers: formaldehyde and sodium hexametaphosphate [9]. Application of an additional sensitizer caused about 8-fold enhancement of signal-to-background response for the reaction of Mn(IV) with

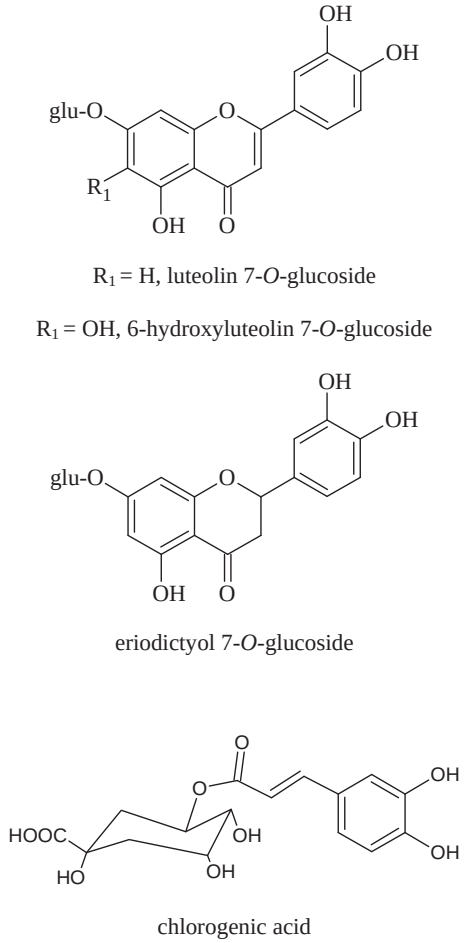

Fig. 3. The structures of the main polyphenolic compounds present in leaves of *C. palustre*.

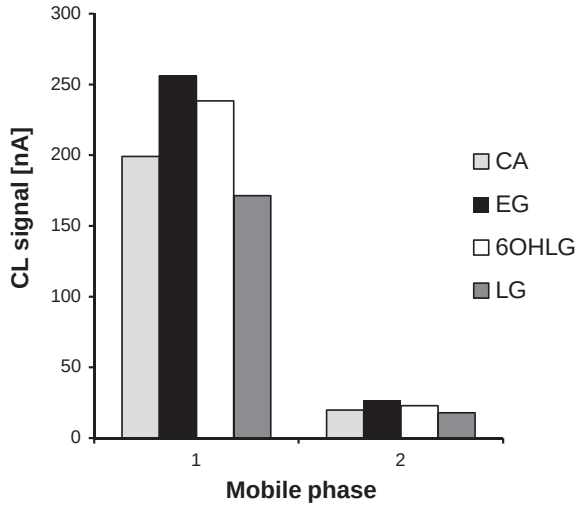

Fig. 4. The influence of organic modifier of mobile phases on the CL signals of polyphenols. 1: 0.25% (v/v) phosphoric acid in water (solvent A) and 100% methanol (solvent B), gradient profile: 0 min: 70% A, 30% B; 25 min: 40% A, 60% B; 30 min: 10% A, 90% B; 35 min: 10% A, 90% B; 2: water (solvent A) and 0.025% (v/v) trifluoroacetic acid in acetonitrile (solvent B), gradient profile: 0 min: 83% A, 17% B, 20 min: 75% A, 25% B, 25 min: 5% A, 95% B, 30 min: 5% A, 95% B.

polyphenols. Therefore, in this work as a post-column CL detection of individual polyphenols we adopted manganese(IV)–hexametaphosphate–formaldehyde system. In order to obtain the maximal CL signal intensity of investigated polyphenolic compounds, chemical and instrumental parameters affecting post-column FI-CL detection system (Fig. 1) were re-optimized. A series of univariate searches were performed on the selection of a suitable concentration of reagents, flow rate of solutions and the distance between the mixing point (T_2) and the flow cell (FC) of the CL detector (Fig. 1). As the standard compounds for the optimization studies we selected main polyphenols present in leaves of *C. palustre*: eriodictyol 7-*O*-glucoside, luteolin 7-*O*-glucoside, 6-hydroxyluteolin 7-*O*-glucoside and chlorogenic acid. The first optimized parameter was the flow rate of formaldehyde and Mn(IV) solutions. The response of the CL detection system was investigated by varying the flow rate from 1.8 mL min^{-1} to 4.6 mL min^{-1}. As it can be seen in Fig. 5A, the CL signals of polyphenols were rising with an increasing rate from 1.8 to 3.6 mL min^{-1} and remained constant beyond this value. Therefore, the flow rate of 3.6 mL min^{-1} for each line was chosen for subsequent experiments. The influence of the distance between the mixing point T_2 and the flow cell was studied in the range of 5–40 cm. The maximum signal intensities were observed when the distance to the CL detector was as short as possible. With the increase in the length, the CL signals of polyphenols decreased remarkably (by 34–42%). Therefore, the distance of 5 cm was chosen as the optimum value. Furthermore, in order to obtain the most efficient post-column CL detection, the effect of formaldehyde concentration was tested over the range of $0.2-1.0$ mol L^{-1}. The results are shown in Fig. 5B. The signals were increasing with an increase in the concentration of formaldehyde up to 0.8 mol L^{-1} and remained almost constant beyond this value. Thus, 0.8 mol L^{-1} was selected as the optimal. As a final step, the effect of concentrations of three components of the CL reagent: manganese(IV), phosphoric acid and sodium hexametaphosphate on signal intensities of polyphenols was tested in the range from 2.0×10^{-4} to 1.7×10^{-3} mol L^{-1}, from 4.0 to 6.0 mol L^{-1} and from 0 to 5% (w/v), respectively. The studies showed that the optimal concentrations of manganese(IV) (1.7×10^{-3} mol L^{-1}) and phosphoric acid (4.5 mol L^{-1}) were the same as in previously described FI-CL system [9]. The optimal concentration of sodium hexametaphosphate in the HPLC-FI-CL system was equal to 4% (Fig. 5C) and was slightly higher than in the FI-CL system [9]. This set of the optimized instrumental and chemical parameters was applied for the post-column CL determination of investigated polyphenols.

3.2. Analytical figures of merit

Under the optimized conditions described above, analytical parameters of the proposed HPLC-FI-CL method such as linearity, sensitivity, correlation coefficients, limits of detection and precisions were evaluated. Calibration graphs were constructed by plotting the CL peak height versus the concentration of analytes. For all tested polyphenols linearity was observed in the concentration range of 0.5–40.0 μg mL^{-1}. The other parameters are summarized in Table 1. The results demonstrate that the method is sensitive for the determination of all studied compounds. The best sensitivity was obtained for chlorogenic acid and 6-hydroxyluteolin 7-*O*-glucoside. The detection limits calculated as signal-to-noise ratios of 3 ($S/N=3$) were in the range of 0.06–0.11 μg mL^{-1} indicating full capacity of the method for the determination of each polyphenolic compound in samples of plant extracts. The intra-day precision of the proposed method was tested with 6 repeated injections of solution containing all polyphenolic standards at the concentration level of 5.0 μg mL^{-1}. The relative standard deviations (RSD) were $\leq$4.0%. The obtained RSD values indicate a satisfactory precision of the proposed method. The inter-day precision, expressed as RSD of the slopes of the

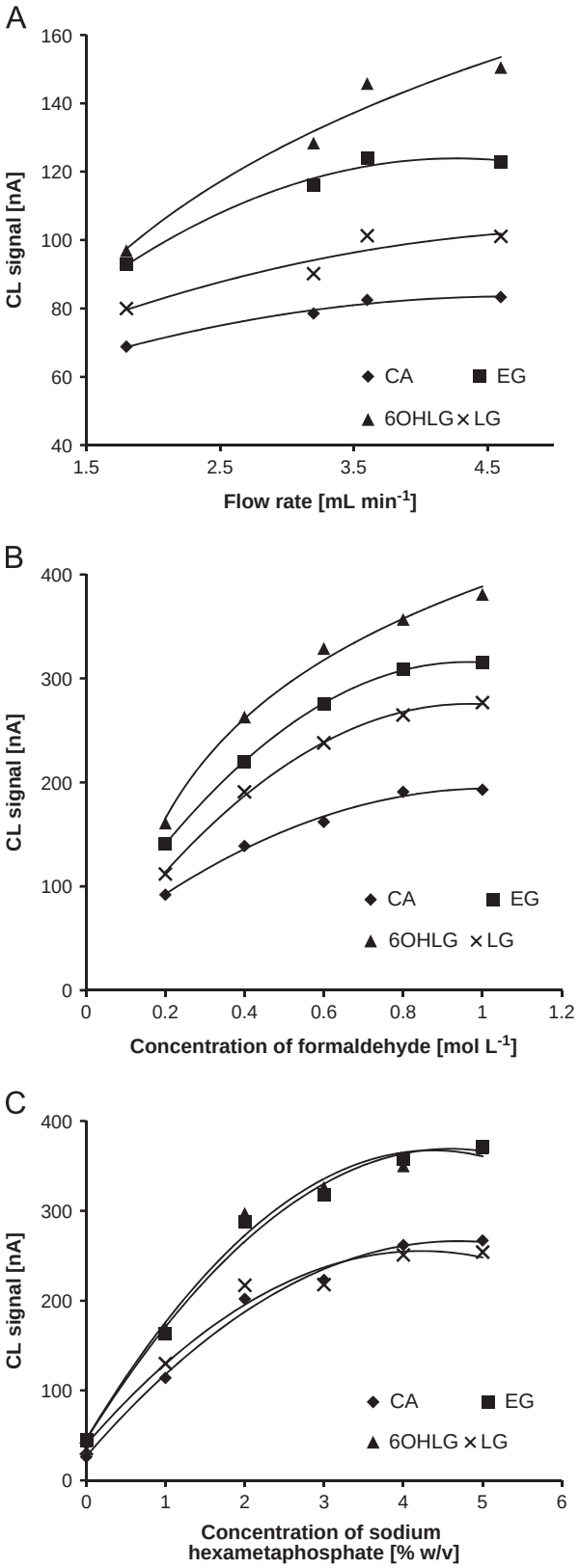

Fig. 5. Optimization of the post-column FI-CL system for chlorogenic acid (CA), 6-hydroxyluteolin 7-*O*-glucoside (6OHLG), luteolin 7-*O*-glucoside (LG) and eriodictyol 7-*O*-glucoside (EG) determination: (A) flow rate of reagents; (B) concentration of formaldehyde; (C) concentration of sodium hexametaphosphate. Concentration of CA, 6OHLG, LG and EG was 2 μg mL^{-1}.

Table 1
Analytical figures of merit for polyphenolic standards using HPLC-FI-CL method.

Analyte	Slope of the calibration graph ± SD ($n=3$)[a]	Correlation coefficient r	Limit of detection [μg mL^{-1}]	Intra-day precision RSD [%] ($n=6$)[b]	Inter-day precision RSD [%] ($n=3$)[a]
Chlorogenic acid	114.7 ± 7.0	0.9975	0.06	4.0	6.1
Eriodictyol 7-*O*-glucoside	83.26 ± 4.66	0.9996	0.11	2.3	5.6
6-Hydroxyluteolin 7-*O*-glucoside	112.8 ± 6.9	0.9989	0.09	3.5	6.1
Luteolin 7-*O*-glucoside	76.00 ± 2.30	0.9991	0.11	3.1	3.0

[a] Three independent calibration graphs obtained in three different days.
[b] Concentration of analyte was 5 μg mL^{-1}.

Table 2
The content of polyphenolic compounds in methanolic extracts from leaves of *C. palustre* determined by HPLC-FI-CL and HPLC-PDA methods.

Analyte	Mean ± SD [mg g^{-1} dw of extract]		The relative error [%]	Mean ± SD [mg g^{-1} dw of extract]		The relative error [%]
	HPLC-FI-CL[a] Reflux extraction	HPLC-PDA[b]		HPLC-FI-CL[a] Ultrasound assisted extraction	HPLC-PDA[b]	
Chlorogenic acid	54.99 ± 1.39	53.14 ± 1.05	3.5	50.71 ± 1.40	52.95 ± 0.67	−4.2
Eriodictyol 7-*O*-glucoside	123.3 ± 3.6	130.7 ± 1.3	−5.7	143.0 ± 0.4	148.2 ± 2.4	−3.5
6-Hydroxyluteolin 7-*O*-glucoside	77.29 ± 1.52	76.13 ± 1.26	1.5	88.41 ± 2.14	84.64 ± 1.30	4.5
Luteolin 7-*O*-glucoside	94.66 ± 0.65	99.53 ± 1.16	−4.9	100.5 ± 2.7	94.75 ± 1.83	6.1

[a] Mean values ± SD of two independent determinations.
[b] Mean values ± SD of three independent determinations.

calibration graphs obtained in three different days, was ≤6.1%. Due to the fact that the RSD is higher than 5% the calibration graphs should be registered the same day as the real sample is analysed.

3.3. Method application for the analysis of plant extracts

The HPLC-FI-CL method was applied to the determination of four polyphenols in extracts from leaves of *C. palustre* and the results were compared to those obtained by using the HPLC-PDA method. The methanolic extracts were prepared by using two types of extraction methods: reflux and ultrasound assisted extraction according to the procedures detailed under Section 2.2. The typical CL and PDA chromatograms obtained in the optimized conditions are shown in Fig. 2. The average values of CL determinations are summarized in Table 2. Both methanolic extracts (obtained by UAE and RE methods) displayed quite similar compositions of polyphenols. Slightly higher concentrations of flavonoids were observed in extracts prepared in an ultrasonic bath. The additional advantages of this type of extraction are shorter extraction time, simpler manipulation, lower temperature and energy consumption compared to reflux extraction [36]. The major compound in methanolic extracts was eriodictyol 7-*O*-glucoside (143.0 ± 0.4 mg g^{-1} dw of UAE extract, 123.3 ± 3.6 mg g^{-1} dw of RE extract). The concentrations of the remaining polyphenols decreased in the following order: luteolin 7-*O*-glucoside, 6-hydroxyluteolin 7-*O*-glucoside and chlorogenic acid. The total contents of polyphenols calculated by the summation of the values obtained for four separated compounds dominating in methanolic extracts (350.24 mg g^{-1} dw of RE extract, 382.62 mg g^{-1} dw of UAE extract) were slightly higher than the total polyphenolic levels determined by FI-CL in our previous work [9]. These differences can be explained by the fact that during determination of the total content of polyphenols in the mixture, the CL signal is the combined response of all constituents and interactions between them affect the signal intensity.

To the best of our knowledge, the present study is the first example of application of HPLC-FI-CL method for the determination of individual polyphenolic compounds in extracts from *C. palustre*. In the literature there are only papers on the qualitative analysis of aqueous extracts from leaves [37] and diethyl ether fractions of methanolic extracts from leaves and inflorescences of this plant [38]. These studies were carried out by HPLC-PDA methods.

The accuracy of the developed HPLC-FI-CL method was confirmed by the comparison of the results with those obtained by the HPLC-PDA method (Table 2). The relative error of determination did not exceed 6.1%. Statistical analysis (*F*-test and *t*-test) showed that the differences between the results are not significant at the 99% confidence level (F calculated $< F$ critical and t calculated $< t$ critical). However, the HPLC-FI-CL method has proved to be much more sensitive (40–65 times higher slopes of the calibration graphs) than the HPLC-PDA method. Despite a significant increase in sensitivity, the LOD values of CL detection for all analytes were only about 3 times lower than those of PDA detection. This may be attributed to the fact that in CL detection we observed higher noise of the baseline. However, literature survey shows that improvement in detection limits is not always observed. For example, the LOD of the HPLC-FI-CL method of determination of rutin and quercetin in Chinese medicine plants obtained by Wu et al. [22] was not superior than that of the HPLC-UV method.

As far as we know, our method is the first HPLC-FI-CL method for the determination of EG, 6OHLG and LG. In the literature we have found two HPLC-FI-CL methods for the quantification of CA [24,39]. Both of them are based on the on-line inhibition of luminol chemiluminescence by polyphenols. These methods are characterized by 1.4-times better [24] and 2-times better [39] detection limits compared to our method. However, Cui et al. [24] obtained shorter linearity range (0.5–10 μg mL^{-1}), while Dapkevicius et al. [39] did not specify analytical parameters of the developed method, except of LOD. Moreover, in both methods, due to application of alkaline solution of luminol, strong baseline drift was observed.

Literature survey shows that LODs of CA, EG, 6OHLG and LG obtained in this work by HPLC-FI-CL method are better or comparable with LODs of HPLC methods with spectrophotometric

detection used for the determination of these compounds in different herbs [40–43]. Only Ren et al. [40] reported three times lower LOD for LG. However, in our newly developed method linearity range for all polyphenolic compounds begins with 0.5 μg mL^{-1}, while in HPLC methods with spectrophotometric detection it starts from higher concentrations [40–43], for example 14.79 μg mL^{-1} for LG and 51.75 μg mL^{-1} for EG [40]. An additional advantage of our method is the ability to estimate antioxidant activity of polyphenolic compounds. In our previous work [9] we observed a linear correlation between the concentration of polyphenols in *C. palustre* extracts determined by Mn(IV)–hexametaphosphate–formaldehyde CL detection system and their ability to scavenge DPPH and ABTS radicals.

Analyzed flavonoids have been reported to exhibit strong antioxidant activity. Eriodictyol 7-*O*-glucoside demonstrated significant scavenging effects on hydroxyl radical and superoxide anion radical (IC_{50} 280 and 300 μM, respectively) [44]. This compound was more active than epigallocatechin gallate (IC_{50} 580 and 530 μM, respectively), the main active constituent of green tea extract. Also 6-hydroxyluteolin 7-*O*-glucoside is a good free radical scavenger, and was reported to have higher superoxide radical anion scavenging activity than luteolin-7-*O*-glucoside due to an additional OH group on the molecule [45,46].

4. Conclusion

In this study, the first analytical method for simultaneous determination of four polyphenolic antioxidants in *C. palustre* leaves extracts was developed by using high performance liquid chromatography with flow injection chemiluminescence detection. The CL determination of polyphenols was based on the enhancing effect of analytes on the chemiluminescence generated upon mixing acidic colloidal manganese(IV), sodium hexametaphosphate and formaldehyde. As far as we know it is the first use of manganese(IV)-based CL reaction as a post-column detection of plant polyphenols.

Mn(IV)–hexametaphosphate–formaldehyde detection system offers better compatibility with the water–methanol mobile phase of HPLC and stability of the baseline than luminol-based systems. However, for manganese(IV) chemiluminescence detection we do not recommend using of mobile phases which contain acetonitrile due to its quenching effect on the CL signals.

The HPLC-FI-CL method has been successfully applied to quantify four major polyphenols in methanolic extracts from leaves of *C. palustre*. Considering the results of the experiments, we evaluate this plant material as a rich source of flavonoid compounds. The highest contents of polyphenols were determined in methanolic extracts obtained by ultrasound assisted extraction. Therefore, we recommend this type of extraction for isolation of polyphenolic compounds from *C. palustre* leaves. Moreover, the UAE method fulfills the criteria of 'green chemistry', due to its simple procedure, short time of extraction and low energy input.

The proposed HPLC-FI-CL method was shown to be a viable alternative to traditional HPLC-PDA method. The CL detection offers greater sensitivity (40–65 times higher slopes of the calibration graphs) and lower detection limits (about 3 times) with the use of simple and inexpensive instrumentation (no external light source is required).

References

[1] T.G. Tutin, V.H. Heywood, N.A. Burges, D.M. Moore, D.H. Valentine, S.M. Walters, D.A. Webb, Flora Europaea, Plantaginaceae to Compositae (and Rubiaceae), vol. 4, Cambridge University Press, London, 1976.
[2] H. Ishida, T. Umino, K. Tsuji, T. Kosuge, Chem. Pharm. Bull. 35 (1987) 861–864.
[3] S. Liu, X. Luo, D. Li, J. Zhang, D.L. Qiu, W. Liu, L. She, Z. Yang, Int. Immunopharmacol. 6 (2006) 1387–1393.
[4] H.A. Jung, S.E. Jin, B.-S. Min, B.-W. Kim, J.S. Choi, Food Chem. Toxicol. 50 (2012) 2171–2179.
[5] A. Walesiuk, J. Nazaruk, J.J. Braszko, J. Ethnopharmacol. 129 (2010) 261–266.
[6] J. Nazaruk, Biochem. Syst. Ecol. 37 (2009) 525–527.
[7] J. Nazaruk, A. Galicka, Phytother. Res. (2014), http://dx.doi.org/10.1002/ptr.5143.
[8] J. Nazaruk, Fitoterapia 79 (2008) 194–196.
[9] J. Malejko, E. Nalewajko-Sieliwoniuk, J. Nazaruk, J. Siniło, A. Kojło, Food Chem. 152 (2014) 155–161.
[10] R.L. Prior, X. Wu, K. Schaich, J. Agric. Food Chem. 53 (2005) 4290–4302.
[11] L.M. Magalhães, M. Santos, M.A. Segundo, S. Reis, J.L.F.C. Lima, Talanta 77 (2009) 1559–1566.
[12] H.A.G. Niederlander, T.A. van Beek, A. Bartasiute, I.I. Koleva, J. Chromatogr. A 1210 (2008) 121–134.
[13] B. Kusznierewicz, A. Piasek, A. Bartoszek, J. Namiesnik, J. Food Compos. Anal. 24 (2011) 1073–1080.
[14] S.-Y. Shi, H.-H. Zhou, Y.-P. Zhang, X.-Y. Jiang, X.-Q. Chen, K.-L. Huang, Trends Anal. Chem 28 (2009) 865–877.
[15] X.-P. Ding, X.-T. Wang, L.-L. Chen, J. Qi, T. Xu, B.-Y. Yu, Food Chem. 120 (2010) 929–933.
[16] X.-P. Ding, J. Qi, Y.-X. Chang, L.-L Mu, D.-N Zhu, B.-Y. Yu, J. Chromatogr. A 1216 (2009) 2204–2210.
[17] P.S. Francis, J.W. Costin, X.A. Conlan, S.A. Bellomarino, J.A. Barnett, N. W. Barnett, Food Chem. 122 (2010) 926–929.
[18] G.P. McDermott, X.A. Conlan, L.K. Noonan, J.W. Costin, M. Mnatsakanyan, R. A. Shalliker, N.W. Barnett, P.S. Francis, Anal. Chim. Acta 684 (2011) 134–141.
[19] Q. Zhang, H. Cui, A. Myint, M. Lian, L. Liu, J. Chromatogr. A 1095 (2005) 94–101.
[20] H. Cui, J. Zhou, F. Xu, C.-Z. Lai, G.-H. Wan, Anal. Chim. Acta 511 (2004) 273–279.
[21] H. Kodamatani, H. Shimizu, K. Saito, S. Yamazaki, Y. Tanaka, J. Chromatogr. A 1102 (2006) 200–205.
[22] H. Wu, M. Chen, Y. Fan, F. Elsebaei, Y. Zhu, Talanta 88 (2012) 222–229.
[23] Q.L. Zhang, L. Wu, C. Lv, X.Y. Zhang, J. Chromatogr. A 1242 (2012) 84–91.
[24] H. Cui, C. He, G. Zhao, J. Chromatogr. A 855 (1999) 171–179.
[25] A.J. Brown, C.E. Lenehan, P.S. Francis, D.E. Dunstan, N.W. Barnett, Talanta 71 (2007) 1951–1957.
[26] Z.M. Smith, J.M. Terry, C.M. Hindson, N.W. Barnett, P.S. Francis, Microchem. J. 111 (2013) 67–73.
[27] G.P. McDermott, J.M. Terry, X.A. Conlan, N.W. Barnett, P.S. Francis, Anal. Chem. 83 (2011) 6034–6039.
[28] M. Jáky, M. Zrinyi, Polyhedron 12 (1993) 1271–1275.
[29] E. Nalewajko-Sieliwoniuk, I. Tarasewicz, A. Kojło, Anal. Chim. Acta 668 (2010) 19–25.
[30] D.M. Jeong, H.A. Jung, J.S. Choi, Arch. Pharm. Res. 31 (2008) 28–33.
[31] D. Cristea, I. Bareau, G. Vilarem, Dyes Pigm 57 (2003) 267–272.
[32] N.T.P. Thao, T.D. Cuong, T.M. Hung, Arch. Pharm. Res. 34 (2011) 455–461.
[33] D. Wen, C. Li, H. Di, Y. Liao, H. Liu, J. Agric. Food Chem. 53 (2005) 6624–6629.
[34] M. Ganzera, A. Pocher, H. Stuppner, Phytochem. Anal 16 (2005) 205–209.
[35] V. Svehlikova, R.N. Bennett, F.A. Mellon, Phytochemistry 65 (2004) 2323–2332.
[36] M.K. Khan, M. Abert-Vian, A.S. Fabiano-Tixier, O. Dangles, F. Chemat, Food Chem. 119 (2010) 851–858.
[37] J. Nazaruk, S.K. Czechowska, R. Markiewicz, M.H. Borawska, Nat. Prod. Res. 22 (2008) 1583–1588.
[38] J. Nazaruk, Herba Polonica 55 (2009) 32–36.
[39] A. Dapkevicius, T.A. van Beek, H.A.G. Niederländer, J. Chromatogr. A 912 (2001) 73–82.
[40] D.-M. Ren, Z. Qu, X.-N. Wang, J. Shi, H.-X. Lou, J. Pharm. Biomed. Anal. 48 (2008) 1441–1445.
[41] Z.-M. Qian, H.-J. Li, P. Li, J. Chen, D. Tang, Biomed. Chrom 21 (2007) 649–654.
[42] A. Nugroho, M.-H Kim, J. Choi, N.-I. Baek, H.-J. Park, Arch. Pharm. Res. 35 (2012) 1403–1411.
[43] J. Wen, L. Kang, H. Liu, Y. Xiao, X. Zhang, Y. Chen, Polypodiaceae, Pharmacogn. Res 4 (2012) 148–153.
[44] H. Yao, Z.X. Liao, Q. Wu, G.Q. Lei, Z.J. Liu, D.F. Chen, J.K. Chen, T.S. Zhou, Chem. Pharm. Bull. 54 (2006) 133–135.
[45] D. de Beera, E. Jouberta, C.J. Malherbea, D.J. Brand, J. Chromatogr. A 1218 (2011) 6179–6186.
[46] Y. Lu, L.Y. Foo, Food Chem. 75 (2001) 197–202.