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Determination of the flavonoids/antioxidant levels in *Cirsium oleraceum* and *Cirsium rivulare* extracts with cerium(IV)–rhodamine 6G chemiluminescence detection

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ABSTRACT

The determination of the sum of flavonoid compounds in extracts from inflorescences (expressed as mg L^{-1} of apigenin) and leaves (expressed as mg L^{-1} of linarin) of *Cirsium oleraceum* and *Cirsium rivulare* species by flow injection system with chemiluminescence detection (FI-CL) has been carried out. The method is based on the strong enhancement by polyphenols occurring in both plants of the CL signal generated by the reaction of cerium(IV) with rhodamine 6G in a sulfuric acid medium. Under the optimized conditions, the linear working ranges of 0.1–10 and 2.5–50 μmol L^{-1} were obtained for apigenin and linarin, respectively. The developed method is simple, sensitive with the detection limits of 38 nmol L^{-1} (apigenin) and 840 nmol L^{-1} (linarin) and offers high sample throughput (up to 300 samples per hour). The relative standard deviation was 0.62% and 3.75% for 10 measurements of 5 μmol L^{-1} apigenin and linarin, respectively. The proposed method has been successfully applied to determine the flavonoids/antioxidant levels in aqueous and methanolic extracts from inflorescences and leaves of *C. oleraceum* and *C. rivulare*. A possible mechanism of the enhancement of cerium(IV)–rhodamine 6G CL system by polyphenols was briefly discussed. For comparative studies, the antioxidant activity of *C. oleraceum* and *C. rivulare* extracts was also evaluated by spectrophotometric 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging method.

1. Introduction

Polyphenolic compounds are a diverse group of natural antioxidants which are commonly found in higher plants and in food of plant origin. It is believed that these compounds posses strong *in vitro* and *in vivo* antioxidant activities and protect our body from the oxidative damage caused by radicals [1]. Epidemiological and clinical studies show that consumption of plant polyphenols reduces the risk of cardiovascular diseases, some types of cancers and diabetes. Polyphenols in plants are present in different combinations and interaction between them seems to be an important factor for their effectiveness as antioxidants [2]. The synergistic interaction of structurally different compounds may contribute to the overall antioxidant activity of plant-derived products, which could be more distinct than the activity of individual compounds.

Antioxidant activity of phenolic compounds has been studied extensively but as far, none of the previously described methods for the evaluation of the total content of phenolic compounds and antioxidant capacity was recognized as a standard method. The majority of methods are spectrophotometric [3,4]. However, they have some drawbacks, such as tedious operation, long reaction time, expensive reagents, low sensitivity and selectivity causing over- or underestimation of the results. For example Folin-Ciocalteu reagent reacts not only with phenols but also with non-phenolic compounds (*e.g.* aromatic amines, sugars, organic acids, some alkaloids and proteins) [5]. While, the antioxidant activity determined by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) (ABTS) assays based on the measurement of the scavenging ability of antioxidants against radical chromogen DPPH$^{\bullet}$ and radical cation ABTS$^{\bullet+}$ is underestimated when colored compounds are present in the sample [6].

In recent years, a significant increase of attention to chemiluminescence (particularly in combination with flow-injection analysis) has been observed as a powerful analytical tool for simple, rapid and sensitive determination of the antioxidant activity of polyphenolic compounds. The high dilution of the sample eliminates possible matrix effects and ensures good selectivity of CL methods [7]. Among different luminofores which have been exploited for the evaluation of antioxidant activity, luminol is the most commonly

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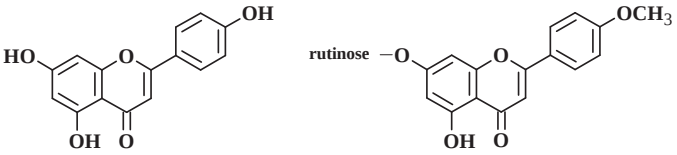

Fig. 1. The chemical structure of apigenin and linarin.

used [8]. However, as far as we know there are no examples of applying cerium(IV)-involved CL reactions for this purpose. In the literature there are only few studies which exploit the CL of cerium(IV)–rhodamine 6G [9–11] and cerium(IV)-Tween 20 [12] detection systems for the analysis of phenolic compounds. The combination of HPLC technique with post-column CL detection was applied for the determination of *p*-hydroxybenzoic acid in apple juices [12] and mixture of phenolic acids (gallic, *p*-hydroxybenzoic and 2,4-dihydroxybenzoic acid) and kaempferol in red wine samples [9]. Flow injection method with chemiluminescence detection (FI-CL) developed by Wang et al. [10] was used for the detection of ferulic acid in Taita Beauty Essence samples. A simple two-line FI-CL manifold was used by Cui et al. [11] for the investigation of a CL mechanism of cerium(IV)–rhodamine 6G-phenolic compound reaction in sulfuric acid medium.

Scientists are still looking for new sources of plant antioxidants that have not been extensively studied yet and could be potentially used in the prevention of damages caused by free radicals. *Cirsium oleraceum* (L.) Scop. (cabbage thistle) and *Cirsium rivulare* (Jacq.) All. (brook thistle), belonging to the family Asteraceae, are herbaceous perennial plants occurring in Central and Southwestern Europe [13]. They abundantly grow in Poland and have been traditionally used in Polish folk medicine: cabbage thistle as diuretic, hemostatic, anti-inflammatory and astringent and brook thistle as anxiolytic remedies. The extracts from leaves and inflorescences possess antimicrobial and antioxidant activity [14]. *C. rivulare* demonstrates anxiolytic and precognitive effects, what is mainly dependent on flavonoids apigenin and linarin [15]. Their chemical structures are shown in Fig. 1. The most important group of chemical compounds of both plants are flavonoids, mainly apigenin and metoxylated derivatives of flavones—aglycones and glycosides [16,17].

Apigenin is one of the more common flavonoids present in plants. This compound possesses a wide spectrum of activity, among others, antioxidant, anti-inflammatory and chemopreventive [18]. Linarin and pectolinarin are detected in many species of genus *Cirsium* [16] and seem to be main constituents in leaves of *C. oleraceum* and *C. rivulare*, influencing their activity. These compounds possess analgesic and anti-inflammatory activities [19,20]. It was observed that pectolinarin and its aglycone significantly decrease the level of hepatic enzymes and exhibit hepatoprotective activity mainly through SOD antioxidant mechanism [21]. Linarin can protect osteoblasts against hydrogen peroxide-induced osteoblastic dysfunction and may exert anti-resorptive actions, at least in part, *via* the reduction of RANKL (receptor activator of nuclear factor kappa B ligand) and oxidative damage [22].

So far, the total polyphenolic and antioxidant level of *Cirsium* species has been studied spectrophotometrically [14,23,24]. The total phenolic content and antioxidant activity of aqueous extracts from leaves [14] and methanolic extracts from inflorescences and leaves [23] of *Cirsium* species was evaluated by Folin-Ciocalteu method and ABTS radical cation decolorization assay, respectively. The total content of flavonols was determined by UV spectrophotometric method based on a complex formation between flavonoid and aluminum chloride [24]. In the literature there are also described chromatographic methods which were used for determination of flavonoids and phenolic acids in *Cirsium japonicum* and *Cirsium setosum* species [25,26].

In this work, the enhancing effect of polyphenols present in *C. oleraceum* and *C. rivulare* species on the cerium(IV)–rhodamine 6G chemiluminescence reaction in a sulfuric acid medium has been studied for the first time. The aim of this study was to determine and compare the sum of flavonoid compounds (as apigenin or linarin equivalents) and indirectly the antioxidant capacity of extracts from inflorescences and leaves of *C. oleraceum* and *C. rivulare* prepared by using various types of extraction (with water and methanol under reflux and ultrasound assisted methanol extraction). For comparative studies, the antioxidant activity of these extracts was also evaluated by spectrophotometric DPPH radical scavenging method.

2. Experimental

2.1. Reagents and solutions

Linarin, apigenin, luteolin, pectolinarin, apigenin 7-*O*-glucoside, apigenin 7-*O*-glucuronide were isolated from flower heads of *C. rivulare* [17], isokaemferide and luteolin 7-*O*-glucoside were isolated from flower heads of *Cirsium palustre* [27], *p*-coumaric, caffeic, ferulic, gallic, *p*-hydoxybenzoic, chlorogenic acids were purchased from Fluka (Steinheim, Germany), syringic, vanillic acids were supplied by Roth (Karlsruhe, Germany), quercetin was from Sigma–Aldrich Chemie (Steinheim, Germany). The stock solutions of investigated polyphenols were prepared in 1.0 mol L^{-1} NaOH, kept in the dark at +4 °C and diluted with water to obtain an appropriate concentration of working solutions. Rhodamine 6G and cerium(IV) sulfate were supplied by Sigma–Aldrich Chemie (Steinheim, Germany). The 0.3 and 0.1 mmol L^{-1} rhodamine 6G solutions were prepared in water and were kept in the dark at +4 °C. The 50 and 80 mmol L^{-1} cerium(IV) sulfate solutions were prepared in 0.5 mol L^{-1} sulfuric acid. Calcein, rhodamine B, fluorescein, eosin, sodium hydroxide and sulfuric acid were purchased from POCH (Gliwice, Poland). DPPH radical was purchased from Sigma–Aldrich Chemie (Steinheim, Germany). The methanolic solution of DPPH radical (60 μmol L^{-1}) was prepared daily, before measurements.

The water used to prepare the solutions was purified in a Milli-Q Plus water purification system (Millipore S.A., Molsheim, France).

2.2. Plant material and extract preparation

Leaves and inflorescences of *C. rivulare* and *C. oleraceum* were collected in 2008, in June and July, respectively, near Białystok, situated in the north-east of Poland. Voucher specimens were deposited in the Herbarium of the Department of Pharmacognosy, Medical University of Białystok (Poland).

2.2.1. Preparation of aqueous extracts

Air-dried plant material (5 g) was extracted with 100 mL boiling water under reflux for 1 h. After filtration, the extracts were evaporated to dryness under a vacuum.

2.2.2. Preparation of methanolic extracts

Air-dried plant material (5 g) was extracted with 100 mL methanol under reflux for 1 h. The extracts were evaporated to dryness under a vacuum.

2.2.3. Ultrasound assisted methanol extraction

Air-dried plant material (5 g) was sonicated in 40 °C with 100 mL methanol for 30 min and obtained extracts were evaporated to dryness under a vacuum.

Extraction efficiency was calculated as percentage weight of the starting plant material (% w/w) and presented in Table 1.

Table 1
Yields of *C. oleraceum* and *C. rivulare* flower heads (F) and leaves (L) extracts calculated as percentage weight of the starting plant material (% w/w).

Extract	*C. oleraceum*		*C. rivulare*	
	F	L	F	L
Aqueous	38.0	42.6	16.0	35.6
Methanolic (reflux)	14.2	27.2	14.4	38.0
Methanolic (ultrasonication)	7.0	9.8	5.2	9.3

After evaporation of solvent, residues from aqueous and methanolic extracts were dissolved in 1.0 mol L^{-1} NaOH and diluted with water to adjust the concentration of polyphenols to the linear calibration range.

2.3. Apparatus and procedure

The configuration of the FI-CL system employed in this work is shown in Fig. 2. A peristaltic pump Minipuls 3 (Gilson, Australia) delivered the solutions of cerium(IV) sulfate, rhodamine 6G and the carrier stream at a proper flow rate to the CL detector (KSP, Poland). An appropriate volume of sample solution was injected into sodium hydroxide stream using a four-way rotary injection valve (Model 5041, Rheodyne, USA) and then merged with the mixture of rhodamine 6G and acidic Ce(IV) solutions in a Perspex T-piece. A mixing coil of 0.8 mm i.d. of an appropriate length was used to improve the efficacy of CL reactions. The emitted light was collected with a photomultiplier tube (operated at 1100 V) placed in a light-tight box. The flow cell was a flat spiral PTFE coil of 1.0 mm i.d. (length of 25 cm in six windings) placed in front of a photomultiplier. The data acquisition and the control of the system were performed through special software purchased from the manufacturer of the luminometer.

Absorption spectra were monitored using a model 8452A diode array spectrophotometer (Hewlett–Packard, Germany). Chemiluminescence spectra were carried out on a Hitachi F-7000 fluorescence spectrophotometer (Hitachi Ltd., Japan) with the light source switched off.

2.4. DPPH free radical scavenging method

Radical scavenging activity of extracts from leaves and inflorescences of *C. rivulare* and *C. oleraceum* against a DPPH radical was measured spectrophotometrically by the method of Brand-Williams et al. [28] which was modified by Miliauskas et al. [29]. Radical scavenging activity was calculated according to the formula:

$$\%\,\text{Inhibition} = \left[\frac{A_B - A_A}{A_B}\right] \times 100,$$

where A_B—absorption of blank sample ($t = 0$); A_A—absorption of tested plant extract solution ($t = 15$ min).

3. Results and discussion

The oxidation reaction between Ce(IV) and rhodamine 6G in sulfuric acid medium is accompanied by weak chemiluminescence [9,11,30,31]. Some phenolic compounds which are known as strong antioxidants could enhance chemiluminescence of the cerium(IV)–rhodamine 6G system in acidic aqueous solution [11].

The most recognized group of compounds in *C. oleraceum* and *C. rivulare* are flavonoids, but one fact is worthy noticing, that in individual species and parts of plant diversified qualitative composition is observed. On the basis of the review of literature and own observation we stated that in flower heads of both plants apigenin and its derivatives occur in the vast majority, whereas in the leaves dominate linarin and pectolinarin. Therefore, apigenin and linarin were selected as the standard compounds for the determination of the sum of flavonoid substances, respectively, in inflorescences and leaves of both plants. Preliminary studies have shown that apigenin, linarin and its derivatives strongly enhanced the CL of Ce(IV)–rhodamine 6G system. In order to obtain a reliable method for the determination of both compounds a number of chemical and instrumental parameters affecting an FI-CL system (Fig. 2) were optimized. A series of experiments were performed on the selection of a suitable fluorophore, concentration of rhodamine 6G, cerium(IV), sulfuric acid and sodium hydroxide, volume of injected sample, flow rate, length of mixing coil and the distance between the mixing point and the detector. All these parameters were optimized for 10 μmol L^{-1} apigenin and linarin with respect to the sensitivity on the basis of the peak height (ΔI). The net CL intensity was calculated according to the formula: $\Delta I = I_S - I_0$, where I_0—a background emission from the Ce(IV)- rhodamine 6G reaction, I_S—CL signal obtained after injection of phenolic compounds.

3.1. Effect of chemical and instrumental variables

Firstly, the effects of other sensitizers such as rhodamine B, fluorescein, calcein and eosin were investigated. When they substituted for rhodamine 6G solution one by one, the CL signals were so weak that could not be used for assay. Only when the rhodamine B solution was mixed with cerium(IV) in sulfuric acid medium a CL emission was observed apparently but it was still few times weaker than that using rhodamine 6G. Therefore, rhodamine 6G was selected as fluorophore for further investigations.

The influence of the concentration of rhodamine 6G on the CL signals was examined in the range of 0.01–0.7 mmol L^{-1}. With the increase of the concentration of rhodamine 6G, ΔI increased and reached maximum when 0.1 mmol L^{-1} rhodamine 6G was used for the determination of apigenin and 0.3 mmol L^{-1} for the determination of linarin, therewith it decreased slightly (Fig. 3(A)). Thus, these values were chosen for further studies. Reduction of CL emission, which has been observed when higher concentrations of rhodamine 6G were used is due to the formation of nonfluorescent dimers of rhodamine 6G which effectively quench the CL of the monomer [32].

The effects of cerium(IV) concentration was tested within the range of 0.5–90 mmol L^{-1}, as shown in Fig. 3(B). At the beginning with increasing concentrations of cerium(IV) the value of ΔI increased, then it reached a plateau. The maximum value of ΔI was obtained when 50 mmol L^{-1} of cerium(IV) concentration was used for the determination of apigenin and 80 mmol L^{-1} for linarin.

Chemiluminescence of cerium(IV)–rhodamine 6G system greatly depends on the concentration of sulfuric acid in cerium(IV) sulphate. Therefore, the influence of sulfuric acid concentration on the CL reaction was examined over the range of 0.5–2.0 mol L^{-1} which is shown in Fig. 3(C). In both cases, the optimum value of ΔI was observed for the 0.5 mol L^{-1} concentration of sulfuric acid. The use of lower concentrations of H_2SO_4 than 0.5 mol L^{-1} resulted in further increase of ΔI but we noted that after some time in the solution of cerium(IV) sulfate precipitate was formed. Therefore, 0.5 mol L^{-1} was chosen for further studies.

The stock solutions of the examined polyphenols were diluted in sodium hydroxide and injected into the sodium hydroxide carrier stream. It was observed that the peak height strongly depends on the concentration of NaOH. Therefore, the effect of NaOH concentration was studied in the range of 0.1–2.0 mol L^{-1}. The results are shown in Fig. 3(D). ΔI increased significantly with the increasing concentration of NaOH until 0.5 mol L^{-1} was reached. When higher concentrations were used, the peak height decreased sharply. Thus, 0.5 mol L^{-1} was chosen as the optimal for both compounds.

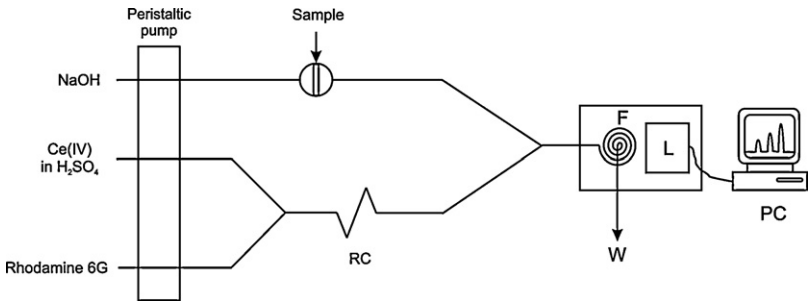

Fig. 2. Schematic diagram of the FI-CL system for determination of apigenin and linarin. RC: mixing coil; L: luminometer; F: flow cell; PC: computer; W: waste.

Furthermore, the volume of sample injected was studied over the range from 100 to 1000 μL by varying the length of the sample loop placed in the injection valve. It was observed that, the peak height became remarkably higher with an increase in the sample volume up to 600 μL (apigenin) and 400 μL (linarin), then ΔI decreased slightly. Therefore, these volumes were selected as the optimal.

The CL reactions of cerium(IV) with rhodamine 6G and polyphenols are very fast. Therefore, the flow rate of the reagent streams and carrier stream was varied from 1.5 to 9.0 mL min^{-1}. For apigenin, ΔI increased significantly with the increasing flow rate until 9.0 mL min^{-1}, higher values of flow rate resulted in instability of the baseline and high consumption of reagents. Thus, 9.0 mL min^{-1} was chosen for further studies. For linarin, ΔI increased up to 5 mL min^{-1} where it reached a plateau. The highest CL response was observed for 5 mL min^{-1}; thus, this flow rate was selected as the optimal.

To improve the efficiency of the CL reaction between cerium(IV) and rhodamine 6G and to maximize the enhancing effect of polyphenols a mixing coil (RC) (Fig. 2) of 0.8 mm i.d. was used. The influence of the length of mixing coil on CL enhancement was studied from 6 to 102 cm. It was found that, as the length of RC coil increased, the peaks corresponding to apigenin and linarin become gradually higher until the length of 35 cm. Above this value decrease of ΔI was observed. Therefore, in both cases 35 cm was chosen as an optimum because ΔI reached the highest value.

As a final step, the distance between the mixing point and the CL detector was also investigated. With the increase in the length, the peak height decreased remarkably. The maximum signal intensity was observed when the distance between the mixing point and the

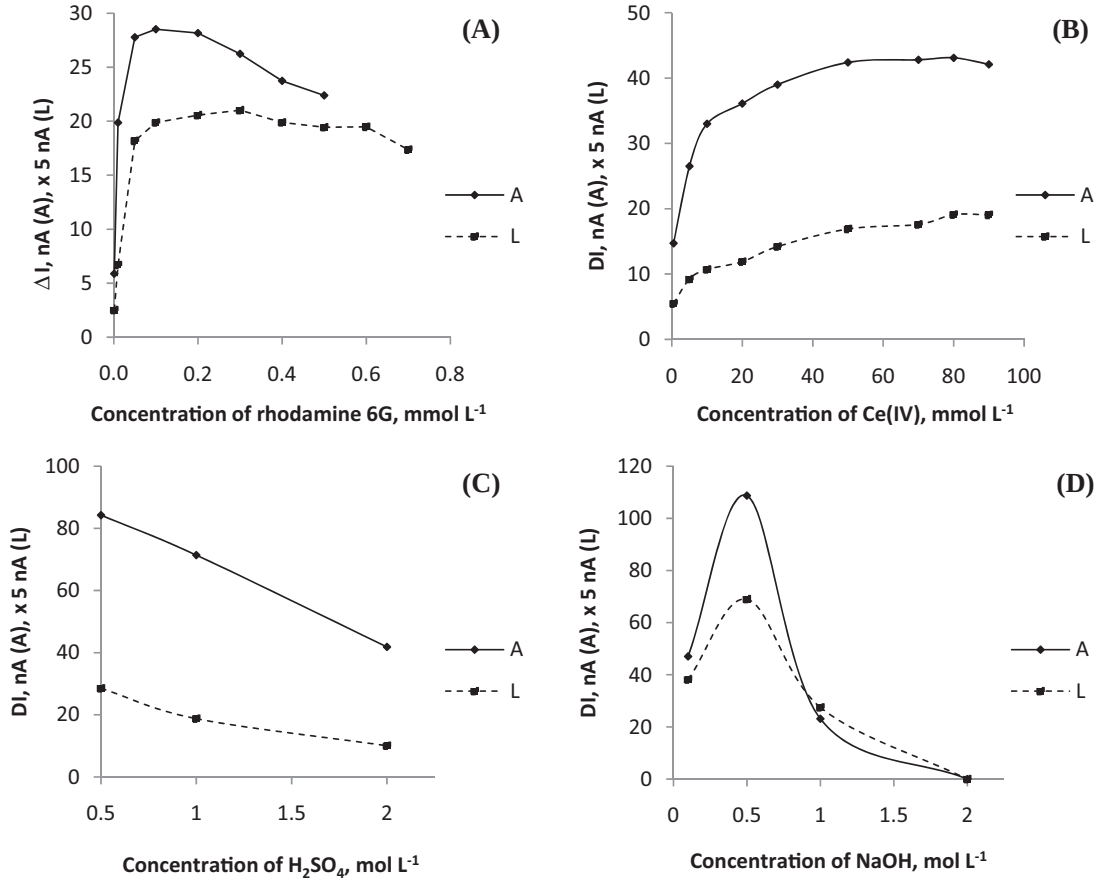

Fig. 3. Optimization of the FI-CL system for apigenin and linarin determination: (A) concentration of rhodamine 6G; (B) concentration of Ce(IV) (in H_2SO_4); (C) concentration of sulfuric acid; (D) concentration of sodium hydroxide. A—apigenin, L—linarin. Concentration of A and L was 10 μmol L^{-1} in 0.5 mol L^{-1} NaOH.

Table 2
The calibration equations of phenolic antioxidants present in flower heads (F) and leaves (L) of *C. oleraceum* and *C. rivulare*.

Part of plant	Investigated compound	Calibration equation	*r*	Part of plant	Investigated compound	Calibration equation	*r*
F	Apigenin	$y=(71.1\pm2.3)x+(25.6\pm4.1)$	0.994	L	Isokaempferide	$y=(18.1\pm0.2)x+(40.6\pm5.2)$	0.995
	Isokaempferide	$y=(65.2\pm0.1)x+(28.3\pm0.5)$	0.996		Chlorogenic acid	$y=(9.47\pm0.07)x+(14.6\pm0.98)$	0.999
	Quercetin	$y=(46.9\pm0.1)x+(2.58\pm0.88)$	1.000		*p*-Hydroxybenzoic acid	$y=(9.08\pm0.09)x+(22.8\pm1.51)$	0.995
	Luteolin 7-*O*-glucoside	$y=(43.2\pm0.1)x+(3.13\pm0.37)$	1.000		Caffeic acid	$y=(9.04\pm0.13)x+(14.1\pm1.5)$	0.998
	Caffeic acid	$y=(41.9\pm0.1)x+(1.78\pm0.42)$	1.000		Apigenin	$y=(6.67\pm0.05)x+(15.3\pm1.68)$	0.997
	Chlorogenic acid	$y=(35.6\pm0.1)x+(3.48\pm0.57)$	1.000		*p*-Coumaric acid	$y=(4.28\pm0.06)x+(14.0\pm0.7)$	0.995
	p-Hydroxybenzoic acid	$y=(23.3\pm0.2)x+(21.8\pm0.8)$	0.991		Gallic acid	$y=(2.82\pm0.02)x+(0.30\pm0.26)$	1.000
	Luteolin	$y=(20.6\pm0.2)x+(0.46\pm0.25)$	1.000		Vanillic acid	$y=(1.05\pm0.06)x+(2.01\pm1.22)$	0.997
	p-Coumaric acid	$y=(13.8\pm0.1)x+(4.3\pm0.17)$	0.994		Linarin	$y=(1.57\pm0.04)x+(4.10\pm0.55)$	0.994
	Gallic acid	$y=(12.7\pm0.1)x+(1.31\pm0.16)$	0.999		Pectolinarin	$y=(1.23\pm0.06)x+(5.08\pm0.91)$	0.999
	Apigenin7-methylglucuronide	$y=(13.3\pm0.2)x+(6.19\pm0.34)$	0.998		Syringic acid	$y=(0.31\pm0.01)x+(1.29\pm0.46)$	0.999
	Vanillic acid	$y=(6.93\pm0.16)x+(1.95\pm0.30)$	1.000		Ferulic acid	$y=(0.29\pm0.03)x+(2.01\pm0.86)$	1.000
	Apigenin7-*O*-glucoside	$y=(3.96\pm0.07)x+(1.73\pm0.10)$	0.999				
	Linarin	$y=(2.63\pm0.05)x+(0.94\pm0.24)$	0.999				
	Pectolinarin	$y=(1.33\pm0.07)x+(0.07\pm0.14)$	1.000				
	Ferulic acid	$y=(0.967\pm0.059)x+(0.662\pm0.076)$	0.996				
	Syringic acid	$y=(0.225\pm0.028)x+(0.309\pm0.083)$	0.996				

detector was as small as possible. Therefore, 6 cm was selected as the optimal for both compounds.

3.2. Analytical performance characteristics

Under the optimized experimental conditions given above, the peak height (ΔI, nA) was linearly proportional to the apigenin and linarin concentrations (C, $\mu mol\,L^{-1}$) ($n=6$) in the range of 0.1–10 and 2.5–50 $\mu mol\,L^{-1}$ respectively. The regression equation was obtained as $\Delta I=(71.1\pm2.3)C+(25.6\pm4.1)$ ($r=0.994$) for apigenin and $\Delta I=(1.57\pm0.04)C+(4.10\pm0.55)$ ($r=0.994$) for linarin. The relative standard deviation for 10 determinations was 0.62% for 5 $\mu mol\,L^{-1}$ of apigenin and 3.75% for 5 $\mu mol\,L^{-1}$ of linarin. The theoretical limit of detection defined as signal-to-noise ratio of 3, was 38 $nmol\,L^{-1}$ (apigenin) and 840 $nmol\,L^{-1}$ (linarin). The proposed FI-CL method is fast and enables 300 samples h^{-1} (apigenin) and 128 samples h^{-1} (linarin) to be determined.

Chemiluminescent enhancement of the cerium(IV)–rhodamine 6G system by other polyphenols identified in leaves and flower heads of both plants was studied in the optimal experimental conditions for apigenin (over the range of 0.1–10 $\mu mol\,L^{-1}$, $n=6$) and linarin (over the range of 2.5–50 $\mu mol\,L^{-1}$, $n=6$). The regression equations are summarized in Table 2. The results demonstrate that the FI-CL method is very sensitive for the detection of apigenin, which is known as an antioxidant, and is present in extracts of inflorescences of *C. oleraceum* and *C. rivulare* in a large quantity. Therefore its contribution to the antioxidant activity of these extracts will be significant. The slope values of majority of investigated phenolics present in the extracts of leaves were higher than the slope value of linarin. However, their contribution to the antioxidant activity of extracts from leaves will be negligible because they are present there in traces.

3.3. Method application

The proposed method was applied for the determination of the sum of flavonoids expressed as apigenin or linarin equivalent in aqueous and methanolic extracts from flower heads and leaves of *C. oleraceum* and *C. rivulare* according to the procedure detailed under Section 2.2. Table 3 shows the results, calculated and expressed as milligram apigenin/linarin equivalent per liter of extract. The antioxidant activity of the extracts was also evaluated spectrophotometrically by the DPPH radical scavenging method described in Section 2.4 (Table 3). It was found that the results obtained by the proposed FI-CL method and the DPPH method revealed a good correlation ($r=0.965$). This indicates that there is the relation between the total phenolic content in the samples and their antioxidant activity. These results also show that the highest content of phenolic compounds/antioxidant activity was found in methanolic extracts, the lowest in aqueous extracts of the plants tested. Therefore, alcoholic extracts of inflorescences and leaves of *C. oleraceum* and *C. rivulare* could be used in medicine as a source of bioactive compounds with antioxidant properties. The total phenolic content and antioxidant activity of extracts from inflorescences and leaves of *Cirsium* species has been already determined spectrophotometrically [14,23]. However, a comparison between the content of polyphenols in different extracts prepared by using various types of extraction (with water and methanol under reflux and ultrasound assisted methanol extraction) has been carried out for the first time.

3.4. Discussion of the enhancement mechanism

The mechanism of CL of Ce(IV)–rhodamine 6G-phenolic compound system has been extensively examined by Cui et al. [11]. The reaction of cerium(IV) with some phenolic compounds in acidic aqueous solution generates weak chemiluminescence. The emitter of this system is the excited Ce(III)* and the maximum emission occurs at 360 nm [11,30,31]. In the presence of rhodamine 6G, the maximum of CL emission occurs at about 560 nm and corresponds to the maximum fluorescence peak of rhodamine 6G. It indicates that luminophore of Ce(IV)–rhodamine 6G system is rhodamine 6G in an electronically excited state. In this work, the determination of plant polyphenols present in *C. rivulare* and *C. oleraceum* is based on the strong enhancement by these compounds of the CL arising during the reaction of cerium(IV) with rhodamine 6G in sulfuric acid medium. In order to elucidate the possible mechanism of CL reaction and the emitter of this system, the emission spectra of Ce(IV)–rhodamine 6G system in the presence and in the absence of polyphenolic compounds (apigenin was chosen as an example) were examined (Fig. 4). The results showed that all CL spectra are identical with the maximum of CL emission at about 560 nm (the same as the characteristic maximum emission spectrum of rhodamine 6G). However, the relative CL intensity was much higher in the presence of polyphenols. It indicated that the maximum emission is independent of analytes and the emitter of Ce(IV)–rhodamine 6G-polyphenol system could be ascribed to rhodamine 6G.

The possible enhancement mechanism of apigenin and linarin on Ce(IV)–rhodamine 6G system has been also investigated based

Table 3
The content of phenolic compounds expressed as apigenin and linarin equivalents in extracts from flower heads (F) and leaves (L) of *C. oleraceum* and *C. rivulare* determined by FI-CL method and antioxidant activity determined by the DPPH method.

Extract	*C. oleraceum*				*C. rivulare*			
	CL method		DPPH inhibition[c] (%)		CL method		DPPH inhibition[c] (%)	
	F[a]	L[b]	F	L	F[a]	L[b]	F	L
Aqueous	1.13 ± 0.02	1.85 ± 0.33	13.2 ± 0.25	2.15 ± 0.43	1.04 ± 0.02	3.80 ± 0.32	15.3 ± 0.22	10.3 ± 0.09
Methanolic (reflux)	2.89 ± 0.01	6.18 ± 0.69	30.1 ± 0.38	15.5 ± 0.26	1.93 ± 0.01	7.87 ± 0.67	17.6 ± 0.19	16.1 ± 0.31
Methanolic (ultrasonication)	3.24 ± 0.02	5.82 ± 0.37	28.1 ± 0.31	16.7 ± 0.28	1.79 ± 0.02	8.73 ± 0.46	17.1 ± 0.20	18.3 ± 0.26

[a] Mean of three determinations expressed as mg apigenin equivalent per L ± S.D.
[b] Mean of three determinations expressed as mg linarin equivalent per L ± S.D.
[c] Mean of three determinations ± S.D.

on the analysis of UV–vis absorption spectra (Fig. 5) of polyphenols (in this case apigenin) (a), Ce(IV) (b) and Ce(IV)-apigenin mixture (c). It was found that light absorption of the mixed solutions of Ce(IV) and apigenin (c) differed from the algebraic sum of individual light absorption spectra of Ce(IV) and apigenin (d). Moreover, the characteristic absorption of Ce(IV) at 316 nm and apigenin at 262 and 334 nm disappeared and a new small peak appeared with the maximum at 298 nm. That indicates that apigenin was oxidized by Ce(IV) and probably a new compound was formed. The UV–vis absorption spectra of Ce(IV) and rhodamine 6G confirmed the results presented in the literature [11,30,31]. The algebraic sum of absorption spectra of the individual compounds was different from the light absorption spectrum of the mixed solutions of Ce(IV) and rhodamine 6G. Furthermore, the absorptive peak of rhodamine 6G with the maximum at 526 nm disappeared and a new peak with the maximum at 470 nm appeared, which indicated that rhodamine 6G was oxidized by Ce(IV). Additionally, it was observed that the absorption spectrum of the mixed solutions of rhodamine 6G and each flavonoid (apigenin or linarin) was exactly the same like the addition of the two individual spectra of rhodamine 6G and apigenin (or linarin). It suggests that there is no chemical reaction between these compounds.

On the basis of literature data [11,30,31] and the analysis of CL and UV–vis absorption spectra it was found that the possible mechanism of the enhancement of cerium(IV)–rhodamine 6G CL system by polyphenols can be expressed as following:

$$\mathrm{Ce(IV)} + \mathrm{Rh6G} \rightarrow \mathrm{Ce(III)^*} + \mathrm{Rh6G_{ox}}$$

$$\mathrm{Ce(IV)} + \mathrm{polyphenol} \rightarrow \mathrm{Ce(III)^*} + \mathrm{polyphenol_{ox}}$$

$$\mathrm{Ce(III)^*} + \mathrm{Rh6G} \rightarrow \mathrm{Ce(III)} + \mathrm{Rh6G^*}$$

$$\mathrm{Rh6G^*} \rightarrow \mathrm{Rh6G} + h\nu$$

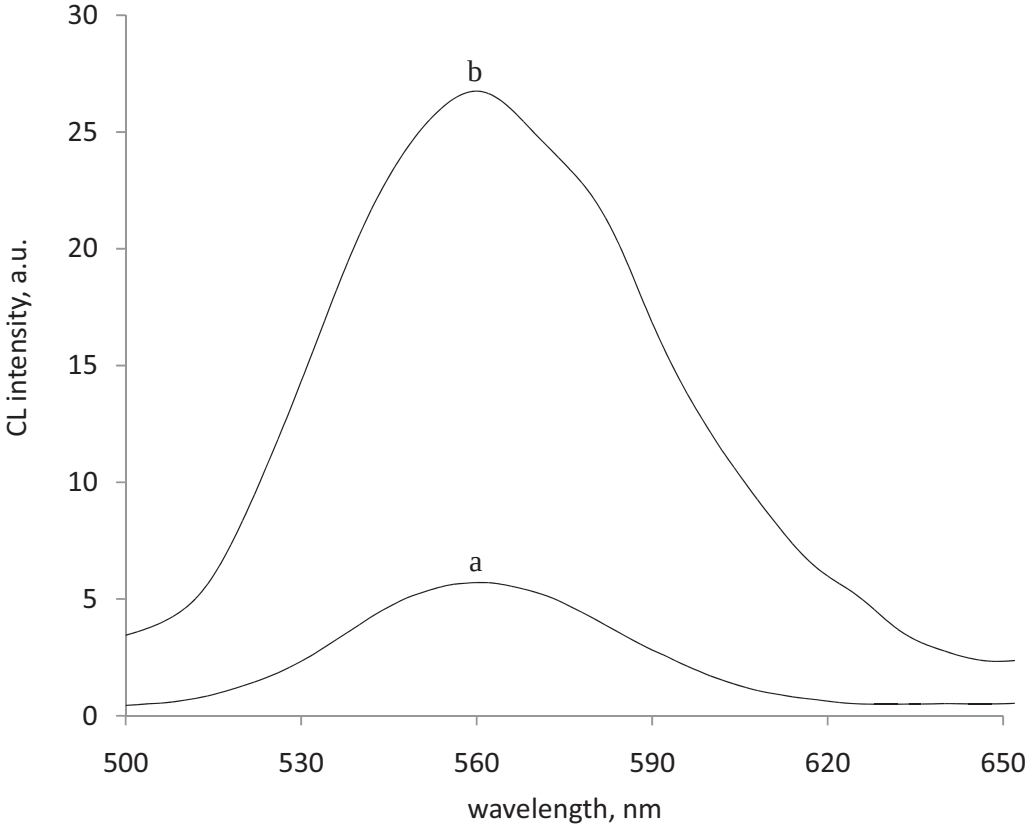

Fig. 4. Chemiluminescence spectra: (a) Ce(IV) + Rh6G; (b) Ce(IV) + Rh6G + apigenin. Ce(IV): 80 mmol L^{-1} in 0.5 mol L^{-1} H_2SO_4; rhodamine 6G (Rh6G): 0.3 mmol L^{-1}; apigenin: 50 μmol L^{-1} in 0.5 mol L^{-1} NaOH.

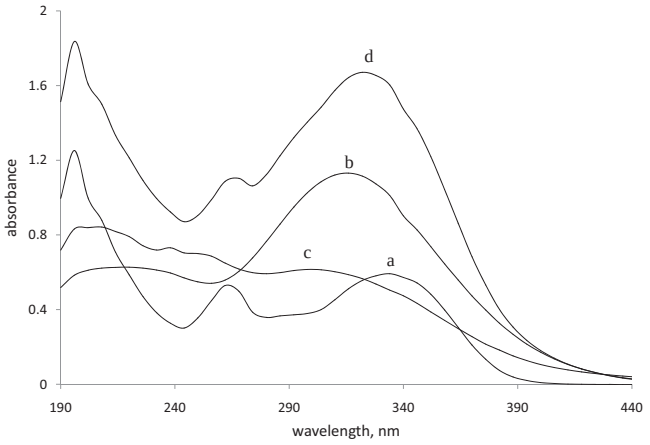

Fig. 5. UV–vis absorption spectra: (a) apigenin; (b) Ce(IV); (c) Ce(IV) + apigenin; (d) algebraic sum of individual light absorption spectra of Ce(IV) and apigenin. Apigenin: 50 μmol L^{-1} in 0.5 mol L^{-1} NaOH; Ce(IV): 3.8 mmol L^{-1} in 0.5 mol L^{-1} H_2SO_4; blank: 0.5 mol L^{-1} NaOH + 0.5 mol L^{-1} H_2SO_4.

where Rh6G is rhodamine 6G, $Rh6G_{ox}$ is rhodamine 6G in its oxidized form, $polyphenol_{ox}$ is polyphenol in its oxidized form. The product of the reaction of Ce(IV) with rhodamine 6G and polyphenols could be the excited-state Ce(III). Ce(III)* transfers the excess of energy to rhodamine 6G to form rhodamine 6G*, which emits its characteristic radiation at about 560 nm.

4. Conclusion

A novel FI-CL detection method has been developed for the determination of plant phenolic compounds (apigenin and linarin) based on the phenolic compound-enhanced cerium(IV)–rhodamine 6G CL in a sulfuric acid medium. The method has been successfully applied to estimate the flavonoids/antioxidant levels in extracts from inflorescences and leaves of *C. oleraceum* and *C. rivulare*. All tested extracts show antioxidant activities. However, the highest content of flavonoids/antioxidant activity was found in methanolic extracts. Therefore, alcoholic extracts of *C. oleraceum* and *C. rivulare* could be potentially used in medicine as effective natural remedies against aging and diseases caused by free radicals. A correlation between the results obtained by FI-CL with those obtained by DPPH• spectrophotometric method was positive and highly significant. However, the proposed method is far more simple, reproducible and sensitive. Moreover, it is faster and enables up to 300 samples per hour to be determined. To the best of our knowledge, it is the highest sample throughput among previously reported methods used for the determination of the polyphenolic content or antioxidant activity. Therefore, it could be concerned as a routine method for the evaluation of the total phenolic level in plant extracts (especially those containing large amounts of apigenin or linarin) and their antioxidant activity.

References

[1] E. Cadenas, L. Packer (Eds.), Handbook of Antioxidants, second ed., Marcel Dekker, Inc., New York, 2002.
[2] X. Yao, X. Xu, G. Fan, Y. Qiao, S. Cao, S. Pan, Eur. Food Res. Technol. 229 (2009) 743–750.
[3] K. Robarts, J. Chromatogr. A 1000 (2003) 657–691.
[4] M. Naczk, F. Shahidi, J. Pharm. Biomed. Anal. 41 (2006) 1523–1542.
[5] M.J. Bermúdez-Soto, F.A. Tomás-Barberán, Eur. Food Res. Technol. 219 (2004) 133–141.
[6] M.B. Arnao, Trends Food Sci. Tech. 11 (2000) 419–421.
[7] E. Nalewajko-Sieliwoniuk, I. Tarasewicz, A. Kojło, Anal. Chim. Acta 668 (2010) 19–25.
[8] E. Nalewajko-Sieliwoniuk, J. Nazaruk, E. Antypiuk, A. Kojło, J. Pharm. Biomed. Anal. 48 (2008) 579–586.
[9] Q. Zhang, H. Cui, A. Myint, M. Lian, L. Liu, J. Chromatogr. A 1095 (2005) 94–101.
[10] J.P. Wang, N.B. Li, H.Q. Luo, Spectrochim. Acta A 71 (2008) 204–208.
[11] H. Cui, Q. Zhang, A. Myint, X. Ge, L. Liu, J. Photochem. Photobiol. A 181 (2006) 238–245.
[12] H. Cui, J. Zhou, F. Xu, C.-Z. Lai, G.-H. Wan, Anal. Chim. Acta 511 (2004) 273–279.
[13] T.G. Tutin (Ed.), Flora Europaea, vol. 4, Cambridge University Press, Cambridge, 1976.
[14] J. Nazaruk, S.K. Czechowska, R. Markiewicz, M.H. Borawska, Nat. Prod. Res. 22 (2008) 1583–1588.
[15] A. Walesiuk, J. Nazaruk, J.J. Braszko, J. Ethnopharmacol. 129 (2010) 261–266.
[16] I.E. Jordon-Thaden, S.M. Louda, Biochem. Syst. Ecol. 31 (2003) 1353–1396.
[17] J. Nazaruk, P. Jakoniuk, J. Ethnopharmacol. 102 (2005) 208–212.
[18] D. Patel, S. Shukla, S. Gupta, Int. J. Oncol. 30 (2007) 233–245.
[19] M. Martinez-Vazquez, T.O. Ramírez Apan, A.L. Lastra, R. Bye, Planta Med. 64 (1998) 134–137.
[20] H. Lim, K.H. Son, H.W. Chang, K.H. Bae, S.S. Kang, H.P. Kim, Biol. Pharm. Bull. 31 (2008) 2063–2067.
[21] Y.-M. Yoo, J.-H. Nam, M.-Y. Kim, J. Choi, H.-J. Park, Biol. Pharm. Bull. 31 (2008) 760–764.
[22] Y.H. Kim, Y.S. Lee, E.M. Choi, Cell. Immunol. 268 (2011) 112–116.
[23] J. Nazaruk, Fitoterapia 79 (2008) 194–196.
[24] J. Nazaruk, Ł. Szoka, Herba Pol. 55 (2009) 32–37.
[25] M. Ganzera, A. Pocher, H. Stuppner, Phytochem. Anal. 16 (2005) 205–209.
[26] Y. Lu, W. Song, X. Liang, D. Wei, X. Zhou, Chromatographia 70 (2009) 125–131.
[27] J. Nazaruk, Biochem. Sys. Ecol. 27 (2009) 525–527.
[28] W. Brand-Williams, M.E. Cuvelier, C. Berset, Lebensm. Wiss. Technol. 28 (1995) 25–30.
[29] G. Miliauskas, P.R. Venskutonis, T.A. van Beek, Food Chem. 85 (2004) 231–237.
[30] Y. Huang, Z. Chen, Talanta 57 (2002) 953–959.
[31] W. Liu, Y. Huang, Anal. Chim. Acta 506 (2004) 183–187.
[32] T. Taguchi, S. Hirayama, M. Okamoto, Chem. Phys. Lett. 231 (1994) 561–568.