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Determination of polyphenols by high-performance liquid chromatography with inhibited chemiluminescence detection

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Abstract

A chemiluminescence reaction detector was developed for the detection of polyphenols separated by HPLC based on the inhibition of chemiluminescence from the luminol–potassium hexacyanoferrate(III) reaction by polyphenols. The separation was carried out on a RP-C_{18} column at 37°C by using stepwise gradient elutions. The detection limits are in the range of $6.8 \cdot 10^{-7}$–$2.0 \cdot 10^{-9}$ g/ml for catechol, protocatechuic acid, chlorogenic acid, rutin, resorcinol, hydroquinone and *p-tert.*-butylpyrocatechol. The method is sensitive, selective, fast and simple. It has been successfully applied to the determination of chlorogenic acid and rutin in real tobacco samples.

Keywords: Chemiluminescence detection; Detection, LC; Polyphenols; Tobacco

1. Introduction

Polyphenols are biologically and environmentally important compounds. Some polyphenols are found in plants, fruits, wines, beers, juices, Chinese herbs, etc. They not only have important physiological functions and some pharmacological activities, but also influence the taste of some drinks. Some of the polyphenols are used in tanning, cosmetics, the pharmaceutical industry and in developing photographs and thus are important environmental pollutants. Therefore, it is very important to develop a highly sensitive and selective analytical method for the determination of polyphenols in food, biological studies, environmental monitoring, etc. High-performance liquid chromatography (HPLC) as a powerful analytical tool has been used for the determination of polyphenols with different detection systems such as UV–visible [1–4], electrochemistry [5–8].

In recent years, chemiluminescence (CL) as a detection technique used with HPLC is very attractive due to its higher sensitivity, wider linear range and simpler instrumentation. However, few CL detectors have been developed for the detection of phenolic compounds. Kwakman and co-workers [9,10] applied laryl chloride and dansyl chloride as precolumn labeling reagents for the peroxyoxalate (PO) CL detection of chlorophenols, alkylphenols and nitrophenols by HPLC.

Luminol CL reaction has been used for the indirect detection of amino acids, catecholamines, aminoglycoside antibiotics, gentamicin C, various proteins, volatile phenols, etc. in flow injection and HPLC systems based on their CL inhibition [11–15]. Recently, in our laboratory, the inhibiting behavior

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and the mechanism of luminol-induced CL by polyphenols have been studied and their analytical potential explored. The method for the determination of chlorogenic acid was developed by use of inhibited CL [16]. Meanwhile, it was found that some other polyphenols were also detectable at trace level by flow injection with inhibited CL detection under similar CL reaction conditions because of their similar CL inhibiting behaviors [17]. However, polyphenols are often present together in biological and environmental samples and similar CL reaction conditions lead to interference from one another. On the other hand, the similar CL inhibiting property of polyphenols make them well suited to the CL detection of polyphenols separated by HPLC. In this paper, a luminol CL reaction detector was developed for the determination of polyphenols including catechol, protocatechuic acid, chlorogenic acid, rutin, resorcinol, hydroquinone and *p-tert.*-butylpyrocatechol by HPLC, based on inhibition of CL from the luminol–potassium hexacyanoferrate(III) system by polyphenols. The separation of these polyphenols was studied by dividing them into two groups because catechol, protocatechuic acid, chlorogenic acid and rutin often present together in biological samples and catechol, resorcinol, hydroquinone, *p-tert.*- butylpyrocatechol in environmental samples and industrial products. The method was applied to the detection of some polyphenols in real tobacco samples.

2. Experimental

2.1. Chemicals and solutions

Chlorogenic acid was obtained from Rotichrom (Germany), luminol was obtained from Merck (Germany), catechol was obtained from Xi'an Chemical Company (China), rutin, protocatechuic acid, hydroquinone, resorcinol, *p-tert.*- butylpyrocatechol were obtained from the Shanghai Chemical Company (China). All other reagents were of analytical reagent grade and all aqueous solutions were prepared with redistilled water. Luminol was dissolved in 0.1 mol/l NaOH to give a $2.5 \cdot 10^{-2}$ mol/l stock standard solution. Stock solution of phenols ($1 \cdot 10^{-3}$ or $5 \cdot 10^{-4}$ mol/l) were prepared by accurate weighing and dissolving in methanol or water (for catechol, hydroquinone, resorcinol). Serial dilutions of the stock standard solution with 80% methanol yielded the required working standard solutions, which were prepared immediately prior to use. The stock standard solutions were prepared fresh weekly and stored at 4°C. The HPLC mobile phases were prepared fresh daily and filtered through a 0.2-μm membrane and then degassed prior to use.

2.2. Instrumentation

The flow system used for HPLC detection is shown in Fig. 1. Liquid chromatography (LC) ex-

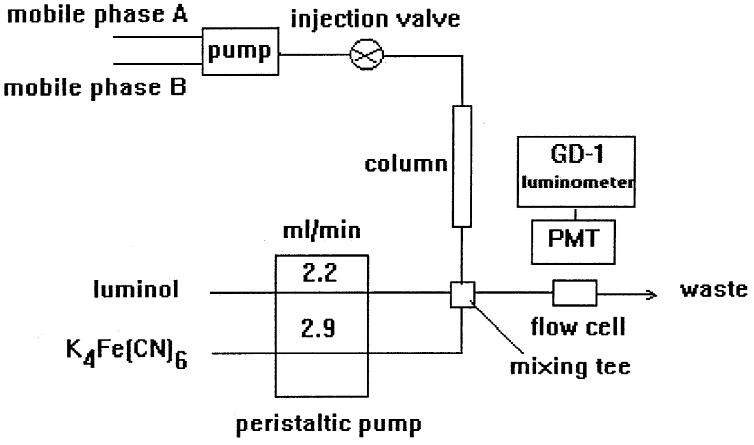

Fig. 1. Block diagram of flow system used for HPLC detection.

periments were carried out using a model LC-6A HPLC system (Shimadzu, Kyoto, Japan) consisting of two model LC-6A solvent delivery pumps, a model CTO-6A column oven, a model SCL-6A system controller, a model 7125 sampling valve (Rheodyne, Cotati, CA, USA) with a 20-μl loop. After passing through the LC system the mobile phase was mixed at a mixing-tee with luminol solution and $K_3Fe(CN)_6$ solution. Solutions of luminol and $K_3Fe(CN)_6$ were delivered with a model FIA-2400 peristaltic pump (Xintong, China) at a rate of 2.2 and 2.9 ml/min, respectively. The chemiluminescent reagents were added directly to the flow cell made by winding the desired length of tygon tubing (75×0.8 mm I.D.). The light emitted was detected by a 1P-21 photomultiplier tube (Bingshong, Beijing) biased at −700−−800 V and placed directly adjacent to the flow cell. The output current of photomultiplier tube was measured, amplified by a model GD-1 luminometer (Xi'an, China) and recorded by a flatbed recorder (Dahua, Shanghai).

2.3. HPLC separation of polyphenols

Separation of polyphenols was performed on a CapcellPak C_{18} column (5 μm, 150×4.6 mm I.D.; Japan) at 37°C using a stepwise gradient elution program with a flow-rate of 1.0 ml/min. Gradient elution conditions are given in Table 1. Gradients I, II and III were for catechol, protocatechuic acid, chlorogenic acid and rutin, for catechol, resorcinol, hydroquinone and *p-tert.*- butylpyrocatechol and for extracted solution from tobacco sample, respectively. The volume of sample injected was 20 μl in all instances (blanks, standards and samples). The peak height in chromatograms was measured for quantitative analysis.

2.4. Preparation of tobacco sample

A fresh tobacco sample was washed, dried for 2.5 h at 70°C in an oven and ground into powder. An 0.1-g amount of dried sample was defatted for 2 h with 60 ml hexane, extracted with 40 ml 80% aqueous methanol in a Soxhlet extractor and diluted to 50 ml with 80% aqueous methanol. A 20-μl volume of extracted solution was subjected to the determination of polyphenols in HPLC.

3. Results and discussion

3.1. Optimization of CL reaction conditions

It was noted that polyphenols inhibited the chemiluminescence of several reaction systems induced by luminol. Thus, several CL reaction systems such as luminol–H_2O_2–Co^{2+}, luminol–H_2O_2–Cr^{3+}, luminol–$KMnO_4$, luminol–$K_3Fe(CN)_6$ were studied for our purpose. It was found that the luminol–$K_3Fe(CN)_6$ system was suitable for CL detection of polyphenols in RP-HPLC because of the simple flow system and the strong inhibiting signal. Therefore, the luminol–$K_3Fe(CN)_6$ system was chosen for the CL detection of polyphenols. The optimization of the CL system was carried out in our previous studies [16,18].

The effect of luminol pH on CL intensity in RP-HPLC is shown in Fig. 2. The optimal pH is slightly different for different polyphenols. Luminol

Table 1
Gradient elution conditions for the separation of polyphenols

Gradient no.	Mobile phase A	Mobile phase B	Gradient conditions
I	MeOH–0.025% H_3PO_4	0.025% H_3PO_4	78%B 3 min, 78%B–50%B 1 min 50%B 16 min
II	MeOH	0.05%H_3PO_4	95%B min, 95%B–50%B 1 min 50%B 14 min
III	MeOH	0.1 mol/l KH_2PO_4	92%B 4 min, 92%B–65%B 1 min 65%B 9 min, 65%B–50%B 1 min 50%B 5 min, 50%B–30%B 1 min

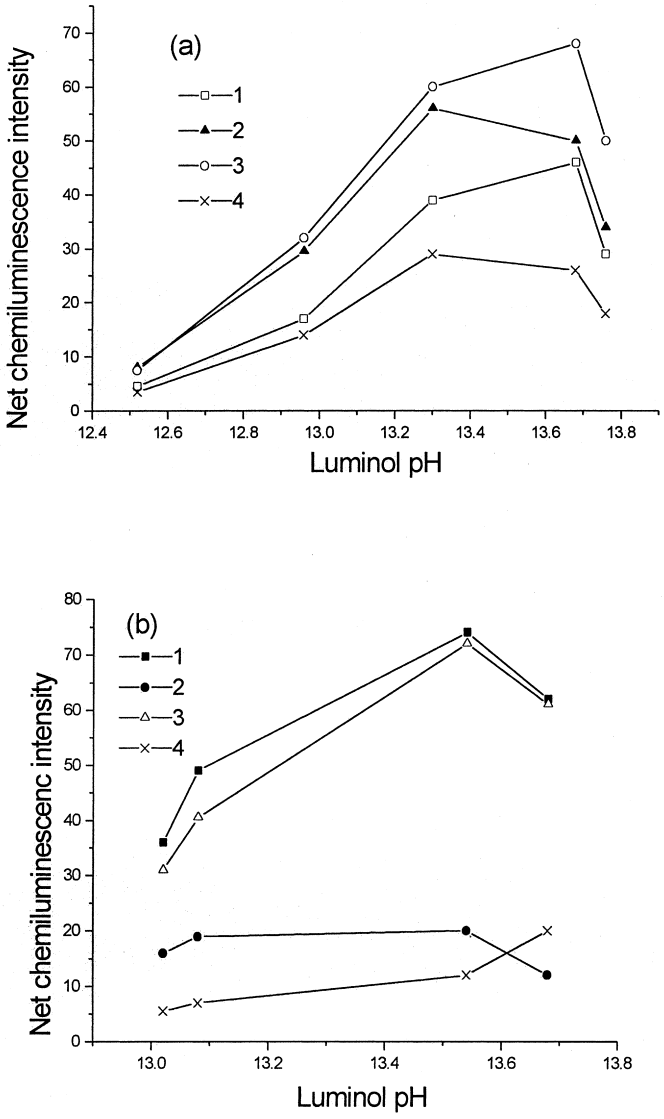

Fig. 2. Effect of pH on CL intensity. (a) Separation condition: gradient I. 1=protocatechuic acid, 2=catechol, 3=chlorogenic acid, 4=rutin (b) Separation condition: gradient II. 1=hydroquinone, 2=resorcinol, 3=catechol, 4=*p-tert.*- butylpyrocatechol.

pH was adapted to 13.3 for protocatechuic acid, catechol, chlorogenic acid and rutin and 13.5 for hydroquinone, resorcinol, catechol, *p-tert.*-butylpyrocatechol.

The effect of the luminol concentration on CL intensity in RP-HPLC is shown in Fig. 3. When the concentration of luminol is $1.0 \cdot 10^{-2}$ mol/l, the inhibiting signal ΔI reached maximum for all the tested polyphenols. Therefore, the optimal concentration of luminol was $1.0 \cdot 10^{-2}$ mol/l.

The effect of potassium hexacyanoferrate(III) concentration on CL intensity was studied in the range $5 \cdot 10^{-5}$–$1 \cdot 10^{-7}$ mol/l. It was found that when the concentration of potassium hexacyanoferrate(III) is lower and comparable with the concentration of polyphenol, the inhibiting signal ΔI is higher. Previ-

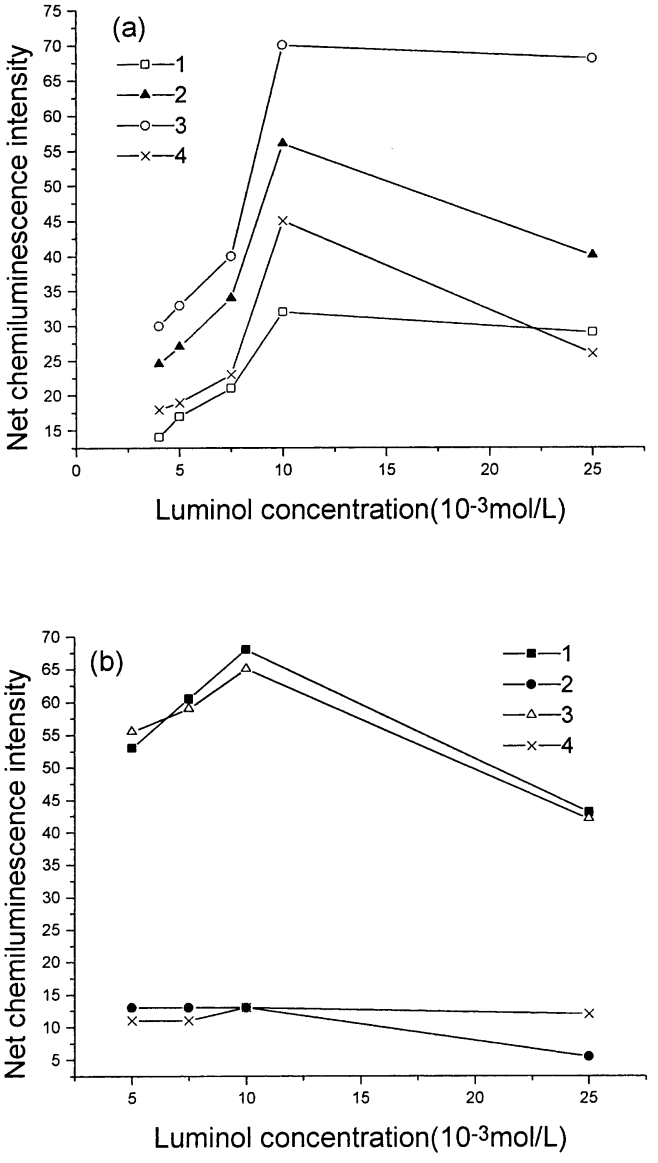

Fig. 3. Effect of luminol concentration on CL intensity. (a) Separation condition: gradient I. 1=protocatechuic acid, 2=catechol, 3=chlorogenic acid, 4=rutin (b) Separation condition: gradient II. 1=hydroquinone, 2=resorcinol, 3=catechol, 4=*p-tert*.- butylpyrocatechol.

ous studies showed that CL intensity decreased due to the competition of polyphenol with luminol for potassium hexacyanoferrate(III) and the inhibiting signal appeared when the concentration of polyphenol was comparable with the concentration of potassium hexacyanoferrate(III) [16]. However, when the concentration of potassium hexacyanoferrate(III) was too low, it was almost consumed by polyphenols and the inhibiting signal was too strong, leading to poorer detection limits. A concentration of $5 \cdot 10^{-7}$ mol/l potassium hexacyanoferrate(III) was found to be suitable for the detection of polyphenols.

3.2. Chromatographic separation

The mobile phase composition was simply optimized. As with all postcolumn reaction schemes, the selection of mobile phase which is compatible with the postcolumn reaction is a critical step in the development of the method. Several kinds of mobile phase such as methanol–water [7], acetic acid–water [5] and methanol–phosphoric acid–water [19] have been utilized for separation of polyphenols on RP-C_{18} columns and were tested for the separation of the studied polyphenols. Of these mobile phases, methanol–phosphoric acid–water was found to be the best. It was also found that isocratic elution was not suitable for the separation of the tested polyphenols because the great difference in molecular polarity among polyphenols led to longer retention time and serious peak tailing. Good separation was obtained by use of a gradient elution. Fig. 4 shows a chromatogram for the separation and detection of a mixture of protocatechuic acid, catechol, chlorogenic acid and rutin. A rapid and complete separation of these four polyphenols was achieved by the use of gradient I. Good separation of another mixture containing hydroquinone, resorcinol, catechol, *p-tert.*- butylpyrocatechol was carried out by the use of gradient II and the chromatogram is shown in Fig. 5. In both chromatograms, baseline drift was observed. For the detection of polyphenols other than *p-tert.*-butylpyrocatechol, baseline drift was not serious. However, for the detection of *p-tert.*-butylpyrocatechol, baseline drift resulted in poorer precision. Baseline drift is a problem often encountered in HPLC–CL detection when gradient elution is used because CL intensity is sensitive to a variety of environmental factors such as solvent, pH, ionic strength, etc. [20]. In this case, the baseline drift is

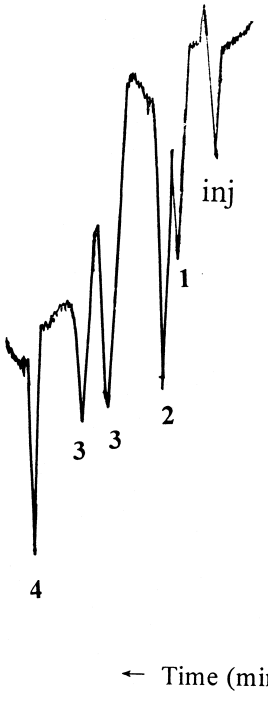

Fig. 4. Chromatogram of a mixture of some polyphenols detected by inhibited chemiluminescence. Separation condition: gradient I, luminol: $1\cdot10^{-2}$ mol/l, luminol pH: 13.3, $K_3Fe(CN)_6$: $5.0\cdot10^{-7}$ mol/l; peaks: 1=protocatechuic acid (3.62 min, $7.5\cdot10^{-7}$ g/ml), 2=catechol (4.68 min, $5.0\cdot10^{-7}$ g/ml), 3=chlorogenic acid (6.90 min, 8.55 min, $1.0\cdot10^{-6}$ g/ml), 4=rutin (10.80 min, $2.5\cdot10^{-6}$ g/ml).

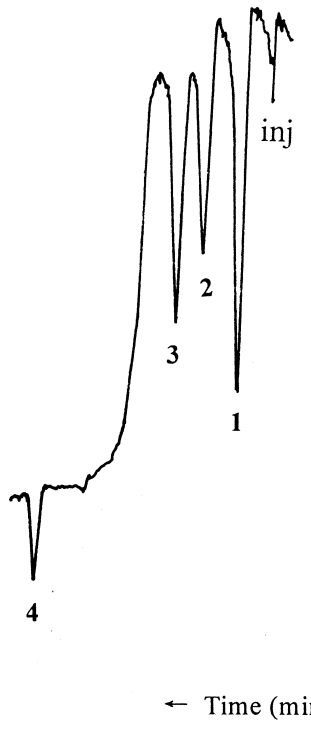

Fig. 5. Chromatogram of a mixture of some polyphenols detected by inhibited chemiluminescence. Separation conditions: gradient II, luminol: $1\cdot10^{-2}$ mol/l, luminol pH: 13.5, $K_3Fe(CN)_6$: $5\cdot10^{-7}$ mol/l; peaks: 1=hydroquinone (4.02 min, $1.0\cdot10^{-7}$ g/ml), 2=resorcinol (6.40 min, $2.5\cdot10^{-5}$ g/ml), 3=catechol (8.04 min, $5.0\cdot10^{-7}$ g/ml), 4=*p-tert.*- butylpyrocatechol (16.90 min, $1.0\cdot10^{-6}$ g/ml).

Table 2
Parameters of regression equations for polyphenols

Elution conditions	Polyphenols	Linear range (g/ml)	Regression equation[a] log $y = a \log x + b$	Correlation coefficient
Gradient I	Protocatechuic acid	$8.0\cdot10^{-8}$–$1.0\cdot10^{-5}$	$\log y = 0.85 \log x + 0.07$	$r = 0.9938$
	Catechol	$5.0\cdot10^{-8}$–$5.0\cdot10^{-6}$	$\log y = 0.82 \log x + 0.59$	$r = 0.9940$
	Chlorogenic acid	$5.0\cdot10^{-7}$–$1.0\cdot10^{-5}$	$y = 47.88 \log x + 12.96$	$r = 0.9942$
	Rutin	$1.0\cdot10^{-7}$–$1.0\cdot10^{-5}$	$\log y = 0.89 \log x + 0.74$	$r = 0.9969$
Gradient II	Hydroquinone	$5.0\cdot10^{-9}$–$1.3\cdot10^{-6}$	$\log y = 0.74 \log x + 0.49$	$r = 0.9900$
	Resorcinol	$1.0\cdot10^{-6}$–$5.0\cdot10^{-5}$	$\log y = 0.67 \log x + 0.16$	$r = 0.9994$
	Catechol	$5.0\cdot10^{-9}$–$5.0\cdot10^{-6}$	$\log y = 0.62 \log x + 0.20$	$r = 0.9923$
	p-tert.-Butylpyrocatechol	$1.0\cdot10^{-7}$–$5.0\cdot10^{-5}$	$\log y = 0.62 \log x + 0.21$	$r = 0.9907$

y, Peak height; *x*, concentration of polyphenol.

due to the change in the concentration of methanol in the mobile phase when gradient elution is used. The effect of some modifiers such as methanol, acetonitrile, ethanol, isopropanol on the CL intensity has been studied. They decreased CL intensity of the luminol reaction system and the net inhibiting signal was −25, −18, −15 and −7 for 80% aqueous methanol, acetonitrile, ethanol and isopropanol, respectively. Acetonitrile is a little better than methanol, but methanol is cheaper. Further research work is under way for deducting baseline by use of wavelet analysis.

3.3. Working curve and detection limits

The working curves of polyphenols were established under optimum separation and detection conditions. The parameters of the regression equations are shown in Table 2. Linear ranges are about 2 to 4 orders of magnitude for the detection of tested polyphenols. The detection limits at a signal-to-noise ratio of 3 were in the range $2.0\cdot10^{-9}$–$6.8\cdot10^{-7}$ g/ml and the standard deviations ($n=5$) were in the range 1.0–7.0%. The data for each polyphenol is given in Table 3. The results show that the CL detection method is sensitive for the determination of polyphenols.

A comparison with UV–visible detection systems for polyphenols shows that the detection limits for hydroquinone, catechol and resorcinol are an improvement of 2–4 and 1 orders of magnitude, respectively, over the detection limits with UV detection [3] and the postcolumn reaction colored complex detection [4] and the detection limit for chlorogenic acid is an improvement of 1 order of magnitude over the detection limit with UV detection [2].

Hydroquinone, catechol, resorcinol can be detected at ng level using amperometric detection [8], so the detection limits for hydroquinone and catechol using CL detection are 2 orders of magnitude greater than that using amperometric detection and the

Table 3
Detection limits and precision for polyphenols

Elution condition	Polyphenol	Detection limit (g/ml)	Precision ($n=5$) (g/ml)	RSD (%)
Gradient I	Protocatechuic acid	$3.2\cdot10^{-8}$	$5.0\cdot10^{-6}$	2.1
	Catechol	$3.7\cdot10^{-8}$	$5.0\cdot10^{-6}$	3.0
	Chlorogenic acid	$4.3\cdot10^{-8}$	$5.0\cdot10^{-6}$	2.1
	Rutin	$8.0\cdot10^{-8}$	$5.0\cdot10^{-6}$	2.6
Gradient II	Hydroquinone	$2\ 0\cdot10^{-9}$	$2.5\cdot10^{-7}$	1.9
	Resorcinol	$6.8\cdot10^{-7}$	$5.0\cdot10^{-5}$	4.0
	Catechol	$3.2\cdot10^{-9}$	$7.5\cdot10^{-7}$	1.0
	p-tert.-Butylpyrocatechol	$4.4\cdot10^{-8}$	$2.5\cdot10^{-6}$	7.0

detection limit for resorcinol is comparable with amperometric detection.

3.4. Application

The method was used for the detection of chlorogenic acid and rutin in a real tobacco sample. It was found that the mobile phase used above was not suitable for a real tobacco sample because other polyphenols were present in the sample and interfered with the detection of chlorogenic acid and rutin; a gradient of methanol and 0.1 mol/l KH_2PO_4 was used. The optimum concentration and pH of luminol were $2 \cdot 10^{-3}$ mol/l and 13.0 for the CL detection reaction under the separation condition. The chromatogram is shown in Fig. 6. The number of peaks with the CL detection is much less than with UV detection and thus the method is more selective than UV detection. The recovery of chlorogenic acid and rutin in tobacco sample was 90% and 95% ($n=4$), respectively. These results indicate that the method is applicable to the detection of chlorogenic acid and rutin in real tobacco samples.

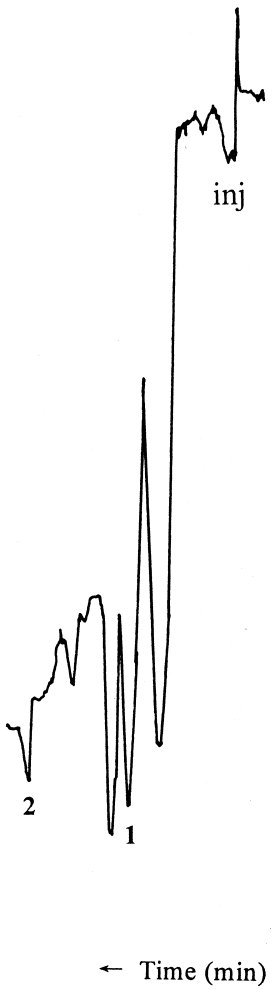

Fig. 6. Chromatogram of the extracted solution from tobacco sample detected by inhibited chemiluminescence. Separation condition: gradient III, luminol: $2 \cdot 10^{-3}$ mol/l, luminol pH: 13.0, $K_3Fe(CN)_6$: $5 \cdot 10^{-7}$ mol/l, peaks: 1=chlorogenic acid (9.20 min), 2=rutin (14.6 min).

Several unknown peaks were also observed in Fig. 6 and believed to be other polyphenols. Therefore, it is possible to detect other polyphenols in real tobacco samples by the presented method. Further research work is in progress.

4. Conclusion

A HPLC procedure coupled with inhibited chemiluminescent detection was developed for simultaneous determination of polyphenols. The method has the merits of high sensitivity, good selectivity, short analytical time and simple instrumentation. Another chromatographic mode coupled with inhibited chemiluminescent detection is under investigation in order to overcome baseline drift. It is also possible to utilize the inhibited chemiluminescent detector for the detection of other polyphenols separated by HPLC. Extensive and systematic studies are in progress.

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