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STUDY ON THE ADVANCED FIA/ ELECTROCHEMILUMINESCENCE DETECTION TECHNIQUE USING A MIRACLE REACTION CELL FEASIBLE FOR HIGHT SENSITIVE DETECTION

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Abstract

The construction of a high-sensitive electrolysis cell based on electrochemiluminescence for flow injection analysis(ECL-FIA) is described. The luminescence intensity was sensitively dependent on the orifice shape and thickness of spacer film in the cell. The thickness of spacer film should be as thin as possible in order to produce a stronger luminescence intensity. The luminescence intensities of some derivative compounds of phthalhydrazide, the commonly used labelling reagents, were compared in the flowing system. The detection limit for luminol was about 1 × 10^{-8} M, and the reproducibility of the determination at 5 × 10^{-6} M was 2.5% in terms of the relative standard deviation . N-(4-aminobutyl)-N-ethylisoluminol(ABEI) and N-(6-aminohexyl)-N-ethylisoluminol(AHEI) could be determined by using ECL-HPLC.

Introduction

Electrochemiluminescence(ECL) is designated as the light emission during the electrolysis of several compounds. As ECL has attracted the interest of researchers, much work has been on this area since 1964(1-3). These studies on ECL have led to the development of important analytical technique, providing much information about redox mechanisms of organic systems. Many attempts have been made recently to apply ECL as a detection technique for reversed-phase high performance liquid chromatography (HPLC)(4-5). In principle, there are two main experimental techniques for studying ECL as a HPLC detector. The first involves the application of an alternative potential at the working electrode, so that the radical cations and anions are produced at the same electrode in alternative cycles. The annihilation process then takes place in the vicinity of the working electrode, and the analysis of the emission intensity involves a complicated mathematical formula. The second method is based on the use of two constantly polarized electrodes. Using the thin layer cell, the diffusion of radical ions is sufficient for the concentration of both cations and anions on the surface of the electrode for procceding with the annihilation reaction.

In recent years there has been increasing interest in the development of chemiluminescence(CL) detection in high-performance liquid chromatography(HPLC) for the analysis of various biological substances. Several applications of luminol-labelled analytes in HPLC have been reported(6-8) because of their high sensitivities. On the other hand, many attempts have also been made to apply ECL as a detection technique for HPLC(9-11). Compared with CL detection, ECL detection has several advantages. First, the electrolysis current and the luminescence intensity can be obtained simultaneously by simple experiment, which can provide more information about reaction mechanism. Second, ECL detection system generally needs much simpler experimental device than CL system, because some oxidant can be produced by electrolysis. Moreover, the electro-inactive and non-luminescent molecules can be detected by ECL after the derivation of such target molecules with luminol or its analogs.

We have investigated the use of ECL as a detector for HPLC to determine oligo peptides and bovine serum albumin, using a simple electrolytic cell with a platinum electrode(12). The detection limit for luminol was about 10^{-6} M. Though it had the advantage that the base line was satisfactorily smooth even at low concentrations of luminol, the sensitivity was not so high compared with that of existing methods. Another problem was the occurrence of bubbles at the electrode surface at a higher applied potential. It causes the electrolytic current unstable. We tried to overcome such difficulties by modifing the shape of the orifice and the thickness of the spacer film of the cell . On the basis of the results obtained, we have been investigating the analytical application of ECL- FIA and ECL-HPLC in detail.

Apparatus

The experimental setups for FIA-ECL are similar to that used in the previous study(13). The schematic assembly of the cell is present in Fig.1. The injection valve (Sanuki Co.) has a 20 μl loop. All connecting tubing was 1.0 mm I.D. Teflon. The main body of the cell was composed of two pieces of Diflon and stainless steel blocks, which were tightly fixed to each other. All the thin spacer films with different orifice shapes were designed in our laboratory. The total volume of the cell was estimated by computer calculation. The cylindrical optical window made of a different kind of material, was set in front of the working electrode. The materials of the working electrode were platinum(Pt) and glassy carbon(GC), and they were well polished with fine alumina powder and then cleaned with water before use. The area of the working electrode was measured following Benschoten's method(14). The reference electrode was Ag/AgCl(-0.044V vs. SCE), and all electrode potentials are reported vs. the Ag/AgCl.

The HPLC was performed using a LC-6A(Shimadzu Co., Japan) liquid chromatograph equipped with a Rheodyne 7125 sample injector(Cotati, CA, USA) and a 5C_{18} AR reversed-phase column(250 × 4.6 mm id., waters). The mobile phase was 15 mM NaH_2PO_4-K_2HPO_4 buffer solution(pH 6.5), and the flow rate was 0.5 ml/min.The carrier solution was prepared by dissolving 0.3 M K_2CO_3, the flow rate was 1.0 ml/min. A VP-6537A pen recorder(Matsushita Co. Ltd., Japan) was used for recording the luminescence intensity.

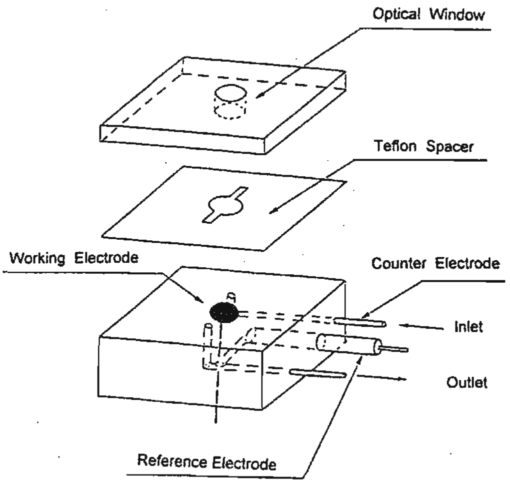

Fig. 1 Electrolytic cell for flow injection analysis

Reagents

The purchased reagents were of analytical grade. Luminol was purified following the reported proceduce(15). The molar absorptivity of the purified luminol at the maximum absorption(352 nm) in 0.05 M potassium hydroxide was $7480\ \mathrm{l \cdot mol^{-1} \cdot cm^{-1}}$, and the emission maximum was at $\lambda = 425$ nm. These values are similar to that reported by Kuwana(16). A luminol solution (5×10^{-3} M) with 0.1 M potassium carbonate was stored in a light-tight polypropylene bottle. The deionised water was twice-distilled. Based on the experimental result, 0.1 M potassium carbonate solution(pH 11.15)was used as a carrier solution at a definite flow rate throughout the experiments.

Results and discussion

Cyclic voltammetry and the relationship between ECL-intensity and the applied potential of luminol

In order to determine the optimum applied potential for the ECL study, cyclic voltammerty(CV) of luminol was performed using Pt and GC electrodes in 0.1M K_2CO_3 solution (Fig. 2A and Fig. 2B). Anodic waves with $E_p = 0.45V$ for Pt electrode and $E_p = 0.50V$ for GC electrode were obtained for the oxidation of luminol on the first potential scan in the positive direction. A second oxidation wave at +1.0V is also shown in Fig.2A for the GC electrode. It was concluded that this process involved the oxidation of the amino group in luminol molecule as mentioned in the previous study(17). The ECL intensity(I_{ECL}) at both kinds of electrodes was measured as a function of the potential as shown in Fig.3. The first I_{ECL}

peaks were +0.45V at the Pt electrode, and+0.50V at the GC electrode. A second larger IECL peak was obtained by scanning in the positive direction with Ep +1.70V to +1.82V, only in the GC electrode. The pH dependence of this peak was +180mVpH^{-1}.

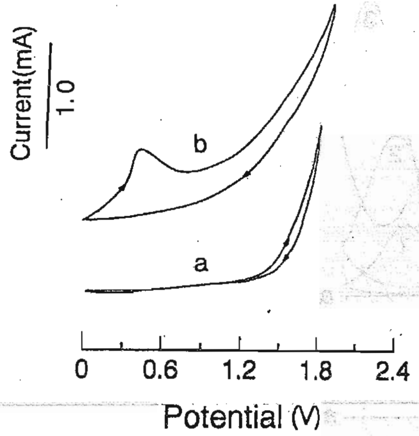

Fig. 2A Voltammetric response of luminol at Pt electrode
a: basal solution, b: 0.5 mM luminol solution
0.1 M K_2CO_3, potential scan rate: 50 mV/s

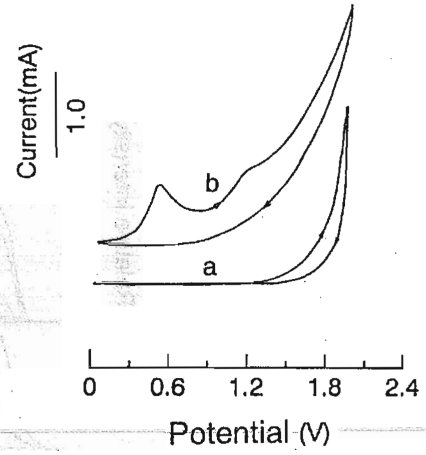

Fig. 2B Voltammetric response of luminol at GC electrode
a: basal solution, b: 0.5 mM luminol solution
0.1 M K_2CO_3, potential scan rate: 50 mV/s

Considering the results on *i*–E and IECL–E relationship for Pt and GC electrodes, there is a choice of applied potential for the ECL experiments. When the applied potential for luminol oxidation is at potential +0.50V, only luminol is oxidized to diazaquinone to give out light emission. In this instance, emission is due to the simple fluorophore. When the applied potential is more positive than +1.0V, for the GC electrode, the oxidation of the OH^- ion in alkaline solution to hydrogen peroxide takes place in addition to the oxidation of luminol. ECL at this higher potential is due to a more complicated mechanism(18).

$$2\,OH^- - 2e \longrightarrow H_2O_2$$
$$H_2O_2 - e \longrightarrow (2H^+ + O_2^{2-}) - e \longrightarrow 2H^+ + O_2 + e$$

Compared with the GC electrode, the Pt electrode seems to change its surface state, because platinum oxide layer was produced on the surface when the potential was above + 0.46V(18). In the IECL–E relationship, A peak only appeared for the Pt electrode, as the overpotential of the oxidation of hydrogen oxide decrease in the presence of a Pt(OH) film.

Although we can get more sensitivity for the GC electrode by using more positive potential, the produced oxygen adhered to the surface of the electrode, which caused a larger noise current and an instability of the luminescence base line. Considering these data, the potential +0.45V for the Pt electrode and +0.50V for the GC electrode were selected as optimal conditions.

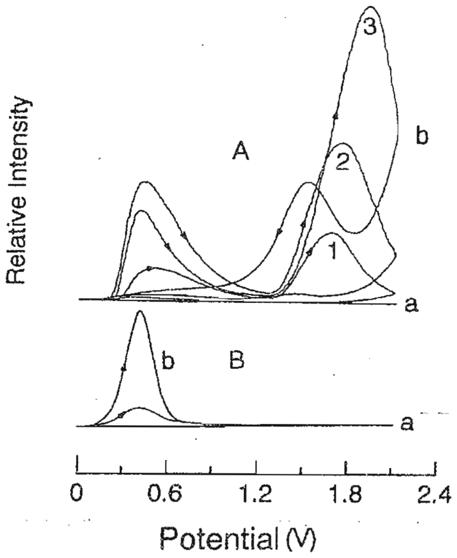

Fig. 3 ECL intensity as a function of electrode potential at Pt and GC electrode
in A, 1; pH 11.08, 2; pH 11.58, 3; pH 12.18
basal solution: 0.1 M K_2CO_3
flow rate: 1ml/min, scan rate: 50 mV/s
Pt and GC disc: 22.1 mm^2

The Effect of different orifice shape of spacer film on luminescence intensity

Considering the experimental results of CV and I_{ECL}–E relationship, I_{ECL} increased with the increase of electrolytic current until the applied potential was up to +0.50V for the Pt and GC electrodes. For the thin–layer flow cell, the following equation describes the current at the electrode for a variety of geometries of planar flow:(19)

$$i = 0.68nFCD^{2/3}v^{-1/6}(A/b)^{1/2}U^{1/2} \quad [1]$$

i: current, μA; n: number of electrons transferred per mole

F: Farady's constant, C • mol^{-1}; C: concentration, mol • L^{-1};

D: diffusion coefficient, $cm^2 \cdot s^{-1}$; v: kinematic viscosity, $cm^2 \cdot s^{-1}$;
A: electrode area, cm^2; b: channel height or thickness, cm;
U: average volume flow rate, $cm^3 \cdot s^{-1}$;

This treatment assumes that n electrones are transferred in a single step. Haapakka and Kankare(2) used $D = 3.0 \times 10^{-6}\ cm^2 \cdot s^{-1}$ (20) to analyze $i^{-1} - \omega^{-1/2}$ data with a rotating-disc electrode and they obtained n = 4 for the Pt electrode. Vitt and his co-workers(21) gave out n = 3.87 for the GC electrode and n = 3.18 for the Pt electrode. In Eq.[1], i would increase with the increase in electrode area(A), average volume flow rate(U) and with the decrease of cell thickness(b). By comparing relationships between current, the volume flow rate of solution, cell dimensions and physical constant, Weber and Purdy(22) obtained two equations for the optimum cell design at a rectangular carbon paste electrode inlaided in a channel-type flow-through cell:

$$b = (VD/0.42U)^{1/2} \qquad [2]$$

$$\text{and} \quad A = 0.52UbD^{-1} \qquad [3]$$

V: Cell volume(cm^3); the implication and units for the other symbols are the same as Eq.[1].

According to Weber's conclusion, there is no optimum shape for a given electrode and the shape of electrode is not critial for Eq.3. They suggested that some optimum thickness of cell and the area of the electrode at different average volume flow could be calculated: for example, assuming U = 1.0 ml/min, the optimum thickness of the cell is 9.7 μm, and that of the optimum thickness of the electrode area is 27.3 mm^2.

The luminescence intensity and the electrode current of luminol were compared using different orifice-shape for spacer films. It is shown in Tab.1 that the thinner the spacer film used, the larger current obtained and the brighter emission which could be observed among No.1, No.2 and No.3. The different shape of orifice for spacer films with the same thickness was compared. The shape of No.4 and No.6 are commonly used in ordinary electrolytic analysis as it is convenient for manufacturing. Compared with the electrolytic current and luminescence intensity of No.4, No.5 and No.6, their values are smaller than that of No.2. Thus, for standard amperometric analysis or luminescent measurement for thin-layer flow cells, the optimum cell design would have the orifice shape and the spacer film similar to the electrode shape, and the film would be as thin as possible. Although the optimum thickness of spacer film is reported to be 9.7 μm for an average volume flow rate of 1.0 ml/min according to Eq.2 , we used the spacer thickness of 50μm in order to achieve mechanical strength as well as the versatility of manufacturing.

Tab.1 Comparison of ECL using different films(the relative emission intensity by using 50μm film was taken as 100, Pt electrode, luminol: 5×10^{-6} M)

Spacer film(No.)	1	2	3	4	5	6
Orifice shape of spacer film						
Thickness(μm)	50	100	300	100	100	100
Volume(μl)	1.5	3.1	9.4	5.6	4.6	8.1
Max. Light intensity	100	46	27	34	37	29
Max. Oxidation Current(μA)	0.59	0.27	0.16	0.20	0.22	0.17

* The area of mark ● is taken as 22.1 mm^2 .

ECL as a function of electrode material

With the increase in flow rate, the luminescence intensity increased for the Pt and GC electrodes until flow rate was up to 0.9 ml/min(Fig.4). The anodic current for luminol was also measured as a function of the flow rate for two kinds of electrodes (Fig. 5). The experiments were performed by keeping the potential at +0.45V for the Pt electrode and +0.50V the for GC electrode according to the CV experiment. The slopes of the linear plot of i_p vs $U^{-1/2}$ are 4.86μA · $s^{1/2}$ · $cm^{-3/2}$ and 4.03μA · $s^{1/2}$ · $cm^{-3/2}$ at the GC and Pt electrodes(A: 22.1mm^2), respectively. Values calculated for these slopes according to Eq.1 are 4.85μA · $s^{1/2}$ · $cm^{-3/2}$ and 3.99μA · $s^{1/2}$ · $cm^{-3/2}$ for the GC and Pt electrodes. Larger values of slope at the GC electrode would mean that the GC is more active for the oxidation of luminol than the Pt electrode.

Comparison of the plots of the IECL for luminol on the GC and Pt electrodes shows the electrode materials is in the order of Pt>GC for IECL. The IECL is not a reliable indication of the local current density when $E_{\mathrm{applied}} \gg E_{1/2}$(23,24). Several assumptions of I and IECL for the different area of Pt and GC electrodes are listed in Tab.2.

Eq.1 indicated that electrolytic current increased when the larger working electrode was used. Eq.3 showed that a suitable area of working electrode for an average volume flow rate of 1.0 ml/min would be 27.3 mm^2: the larger electrode does not necessary give out the brighter light emission. Table 2 shows that the luminescence sensitivity for the electrode of 58.1 mm^2 is low despite its larger electrolytic current.

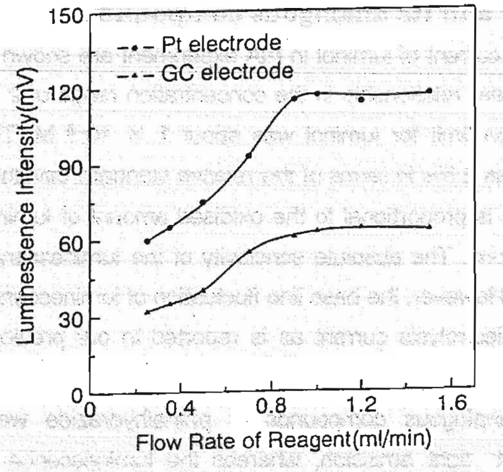

Fig. 4 Effect of flow rate on the luminescence of luminol
carrier solution: 0.1 M K_2CO_3
Electrode: Pt and GC disc, area 22.1 mm^2
Applied potential: + 0.45V(vs.Ag/AgCl) for Pt electrode and + 0.50 V(vs.Ag/AgCl) for GC electrode

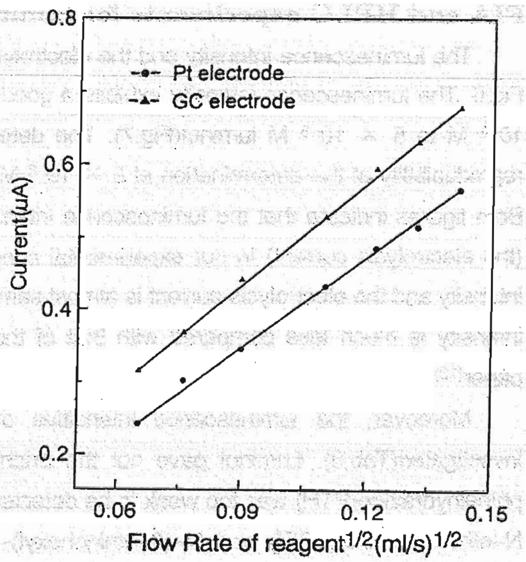

Fig. 5 Current density as a function of flow rate
conditions: same as for Fig.4

Tab. 2 Peak current and luminescence intensity of luminol for different areas of the Pt and GC electrodes(Applied potential + 0.50 V for the GC electrode, + 0.45 V for the Pt electrode, luminol: 5 X 10^{-6} M)

	7.1 mm^2		22.1 mm^2		58.1 mm^2	
Material	i (µA)	I ECL(mV)	i (µA)	I ECL(mV)	i (µA)	I ECL(mV)
Glassy carbon	0.12	40.8	0.58	63.6	2.55	20.9
Bright platinum	0.09	88.0	0.50	116.8	2.10	45.6

The Effect of the material of the optical window on ECL

Besides the above-mentioned study, we are still seeking for factors influencing on sensitivity, and have been encouraged by the result that an optical window made of different kinds of material also influences light intensity. Compared with glass and polymer plastic, a 1.1 times and 1.2 times larger luminescence intensity was obtained by using quartz, as it absorbs less light at the wavelength λ_{425} nm.

FIA and HPLC experiments for luminol and its analogous compounds

The luminescence intensity and the electrolysis current of luminol in FIA experiment are shown in Fig.6. The luminescence intensity exhibits a good linear relationship in the concentration range of 2×10^{-8} M to 5×10^{-5} M luminol(Fig.7). The detection limit for luminol was about 1×10^{-8} M. The reproducibility of the determination at 5×10^{-6} M was 2.5% in terms of the relative standard deviation. Both figures indicate that the luminescence intensity is proportional to the oxidised amount of luminol (the electrolysis current) in our experimental conditions. The absolute sensitivity of the luminescence intensity and the electrolysis current is almost same. However, the base line fluctuation of luminescence intensity is much less compared with that of the electrolysis current as is reported in our previous paper(13).

Moreover, the luminescence intensities of analogous compounds of phthalhydrazide were investigated(Tab.3). Luminol gave out the brightest light emission, whereas the luminescence of phthalhydrazide(PTH) was too weak to be detected. The popular labelling reagents, N-(4-aminobutyl)-N-ethylisoluminol(ABEI) and N-(6-aminohexyl)-N-ethylisoluminol(AHEI) are less luminescent than luminol. The HPLC separation of both compounds is shown in Fig.8. This figure shows that the difference in a functional group of ABEI and AHEI causes a rather distinct difference in the retention time of both compounds in HPLC using reversed-phase column. As shown in Tab.3, the luminescence intensity of ABEI is much strong compared with that of AHEI at the same concentration, though the electrolysis current of both compounds is almost same. We are investigating the determinations of biomolecules using such kind of new selectivity for the derivatized compounds. The detection limit of ABEI and AHEI in this system is 4×10^{-8} M and 8×10^{-8} M, respectively. Both compounds will be applied to the ECL determination of some biochemical substances, such as amino acids,or fatty acids, after derivatized them together.

Tab.3 The comparison of relative luminescence intensity(luminol as 100) for derivative compounds of phthalhydrazide(concentration 5.0×10^{-6} M, Pt working electrode, 22.1 mm^2)

Compound	PTH	Isoluminol	Luminol	ABEI	AHEI
Luminescence Intensity	0.5	7.5	100	37.5	14.6

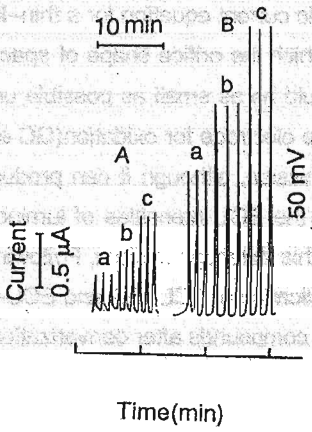

Fig.6 The typical recording of luminol in FIA

A: Current B: Luminescence

a, 3.5×10^{-6}M; b, 5.0×10^{-6}M; c, 7.0×10^{-6}M

(0.1 M K_2CO_3, Pt working electrode, flow rate: 1.0ml/min)

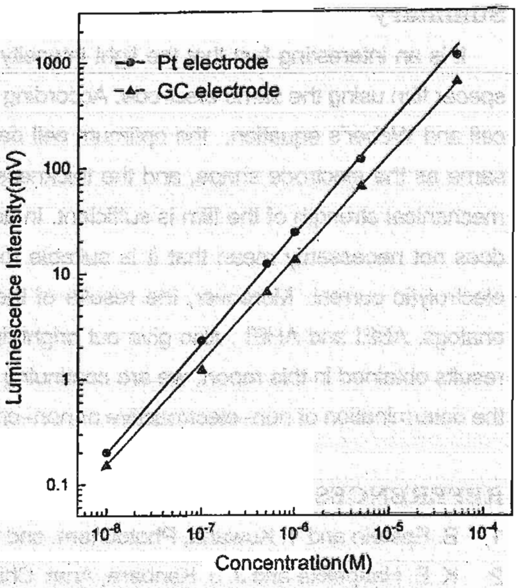

Fig. 7 The calibration curves for luminol

Caption and experimental conditions are same as for Fig.4

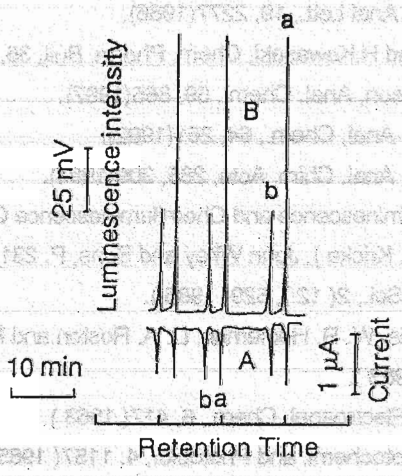

Fig. 8. Chromatograms for ABEI and AHEI

A: Current B: Luminescence

a: 1.0 ×10^{-5} M ABEI; b: 1.0 ×10^{-5} M AHEI

Eluent: 0.015MNaH_2PO_4–K_2HPO_4 pH 6.5, Flow rate: 0.5ml/min

Carrier solution: 0.3 M K_2CO_3, Flow rate: 1.0 ml/min,

Applied potential: +0.45V(vs.Ag/AgCl), Pt working electrode

Separation column 5C_{18} AR(250 × 4.6 mm id),

Sample volume 20 μl, Temperature 25℃.

Summary

It is an interesting fact that the light intensity can be increased by modifing the orifice-shape of the spacer film using the same electrode. According to the electrolytic current equation for a thin-layer flow cell and Weber's equation, the optimum cell design is one in which the orifice shape of spacer film is same as the electrode shape, and the thickness of the film should be as small as possible unless the mechanical strength of the film is sufficient. In addition, the active electrode for oxidation(GC electrode) does not necessarily mean that it is suitable for strong light emission, although it can produce larger electrolytic current. Moreover, the results of the comparison of the ECL intensities of luminol and its analogs, ABEI and AHEI , also give out bright light emission in this detection system. Following on the results obtained in this report, we are continuing the study of application of ECL-FIA and ECL-HPLC to the determination of non-electroactive or non-chemiluminescent compounds after derivatization.

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新作反応セルを用いた高感度及び高性能フローインジェクション/電解化学発光検出法の研究

フロインジェークション分析または、ＨＰＬＣへの応用のための電解化学発光（ＥＣＬ）測定用高感度セルの試作を行った。薄層電解セルの電極材質及びスペーサの形状と厚さの発光強度への影響等について研究し、適切条件を見出した。ルミノールについての検出下限は２×１０$^{-8}$Ｍである。この新規な検出系を応用したＦＩＡ及びＨＰＬＣ・ＥＣＬ分析法を用い、発光標識として通常よく使われているＡＢＥＩとＡＨＥＩの発光強度、測定条件等について検討した。

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