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Flavin Mononucleotide Chemiluminescence for Determination of Traces of Copper(II) by Continuous Flow and Flow Injection Methods

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Flow injection analysis, with chemiluminescence detection, is used to determine traces of copper(II) by means of the flavin mononucleotide-hydrogen peroxide-phosphate buffer system. This permits the determination of copper(II) more selectively than any other chemiluminescence system with a detection limit of 0.03 ng(20-μl sample injection) or 0.06 ng ml^{-1} (continuous sample flow). The linear range is 3 orders of magnitude, the sampling rate is 120 h^{-1}, and the relative standard deviation is 3.1% for 1 ng Cu(II)($n=10$). Iron(II) and chromium(III and VI), the strongest enhancer after copper(II), provide signals 2—3% of that for copper(II). Effect of surfactant micelles on the signal for copper is also discussed. The method is successfully applied to real samples.

Chemiluminescence (CL) methods have been recognized as a valuable approach to sensitive analyses. In particular, the use of solution CL in analyses for inorganic and organic species at trace levels has received considerable attention because of the simplicity of instrumentation, the wide dynamic range, and the short analysis time as well as high sensitivity. An alkaline hydrogen peroxide solution of luminol, lucigenin, lophine, or gallic acid has mainly been utilized for trace metals, cobalt(II) being the most effective catalyst.[1] Copper(II) can be detected by means of its catalytic effect on the oxidation of CL reagent; its detection limit is 0.2 ppm in the lophine system,[2] 1×10^{-7} M (1 M=1 mol dm^{-3}) in the luminol system,[3] and 0.1 ppm in the lucigenin system.[4] These CL systems, however, suffer from lack of selectivity for copper determination. For instance, in the luminol system there are at least 30 different species that enhance light emission.[5]

Flow injection analysis(FIA) is very suitable for the determination of various kinds of analytes based on CL measurements. Cobalt(II) in the pg to sub-pg range has been detected by the gallic acid[6] or luminol system,[7] cadmium(II) and zinc(II) in the sub-ng range by their inhibiting effects on the luminol system,[8] hydrogen peroxide in the 10^{-8} to 10^{-9} M range by the oxalic ester[9] or luminol system,[10] iodine in the sub-ng range by the hypochlorite system,[11] sulfite in the ng range by the permanganate system,[12] fluorescent compounds in the fmol range by the oxalic ester system,[13] and albumin in the 10^{-8} M range by its inhibiting effect on the luminol system.[14]

In the preliminary work, we have made it clear that flavin mononucleotide (FMN) is usable as a CL reagent for sensitive, selective, and rapid determination of copper(II) by FI method.[15] It is based on the measurement of CL arising from the copper-catalyzed oxidation of FMN by hydrogen peroxide under nearly neutral conditions. The object of this paper is to further explore the combination of FIA and CL method and its analytical characteristics, and to verify the utility of the present FI-CL method in copper determination by applying to real samples.

Experimental

Apparatus. Prior to flow experiments, a batch system as shown in Fig. 1 was assembled in order to obtain CL decay curves, that is, to know how fast the present CL reaction proceeds. Each 0.2 ml of FMN, hydrogen peroxide, and copper(II) solutions is transferred in that order to a reaction vessel by a peristaltic pump. The light emitted is observed *via* a fiber optics by a photomultiplier tube (PMT, Hamamatsu Photonics R453) with no wavelength discrimination.

A schematic diagram of the flow system for FIA is given in Fig. 2. An aqueous solution containing FMN and phosphate

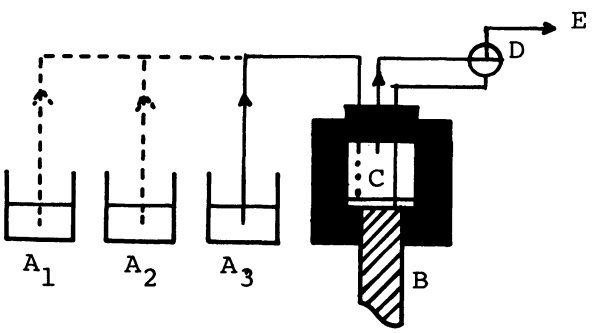

Fig. 1. Batch system for obtaining CL decay curves. A_1: 2.8×10^{-3} M FMN (phosphate buffer), A_2: 10% H_2O_2, A_3: 10^{-4} M Cu(II), B: fiber optics, C: reaction cell, D: 3-way cock, E: to peristaltic pump.

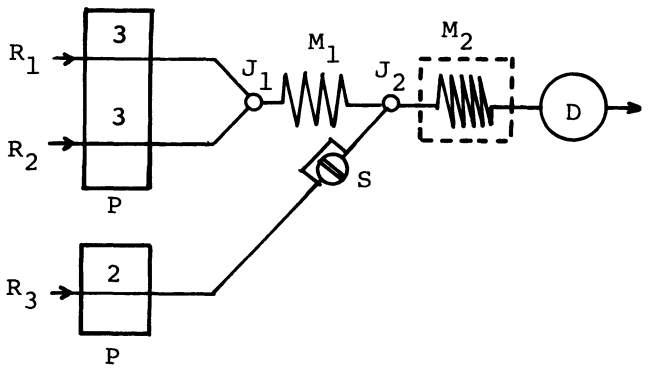

Fig. 2. Schematic diagram of the flow system for FIA (for key, see text).
Recommended conditions: 1.4×10^{-3} M FMN (pH 5.8) for R_1; 5% H_2O_2 for R_2; water for R_3. Flow rates are given as ml min^{-1}. Coil M_2 is held in a heating bath at 60 °C.

buffer is supplied through R_1 and hydrogen peroxide through R_2. A flow line R_3, which is newly placed in this experiment, represents a carrier stream for copper(II) and others. This is an aqueous copper(II) solution for the continuous flow method, or pure water when copper(II) and other species are injected by means of a 20-μl rotary valve injector S (injection method). The streams are delivered by peristaltic pumps P and mixed at Y-joints J_1 and J_2. For adequate mixing of R_1 and R_2, a 0.5-m mixing coil M_1 is placed between J_1 and J_2; a second 1-m mixing coil (reaction coil) M_2 is placed in a heating bath controlled to ±0.5 °C between J_2 and D. Teflon tubing (1-mm i.d.) is used for flow lines except the pump tubes and detection flow cell D. The distance between J_2 and D is about 5 cm (the minimum distance achieved). For D a spiral flow cell is assembled as described previously.[16] The light emitted, which has an maximum emission between 500 and 600 nm similar to the fluorescence emission of FMN (λ_{max}=*ca.* 540 nm), is observed directly by a PMT (Hamamatsu Photonics R268) with no wavelength discrimination. The signal from the PMT is fed to an electrometer and then recorded *via* a laboratory-built low-pass active filter, whose frequency cut-off is *ca.*0.1 Hz.

Fluoresecence spectra were measured by a fluorescence spectrophotometer (Hitachi MPF-4). The measurements were performed with respect to the waste solution in the flow system in Fig. 2, in which the stream of hydrogen peroxide solution was substituted by that of water.

Reagents. Chemicals of reagent grade were used as received. The water used was prepared by distillation of Millipore (milli-R) water from an all Pyrex glass apparatus. FMN (monosodium riboflavin 5′-phosphate), phosphate buffer (M/15 KH_2PO_4–M/15 Na_2HPO_4), and hydrogen peroxide solutions were prepared daily.

Analysis of Real Samples. Each 0.7 g of Pepperbush and Pond Sediment (standard reference materials) from NIES (National Institute for Environmental Studies) and of Yabukita tea leaf from National Chemical Laboratory for Industry was boiled with *ca.* 20 ml of concentrated nitric acid on a hot plate until the appearance of white fumes, and with concentrated sulfuric acid until the solution became colorless. After cooling, the solution was diluted to 100 ml. A 40-ml portion of the acid-digested sample was neutralized to about pH 6 with 2 M sodium hydroxide and diluted to 100 ml. A 10-ml portion was added to each of several 100-ml volumetric flasks for standard addition of copper before dilution to volume, and copper was determined as described above.

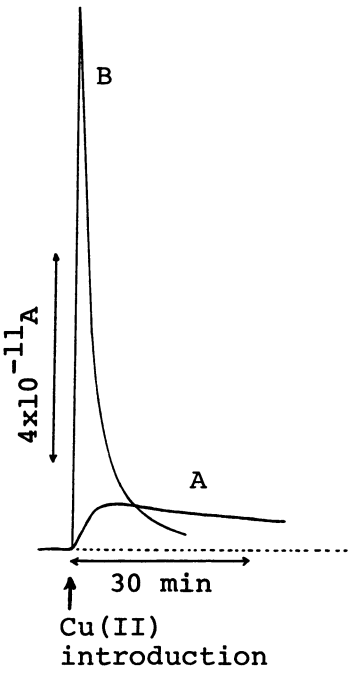

Fig. 3. CL decay curves.
A: room temperature, B: 60 °C (Each 0.2 ml of reagent solution warmed to 60 °C was introduced into the reaction cell.).

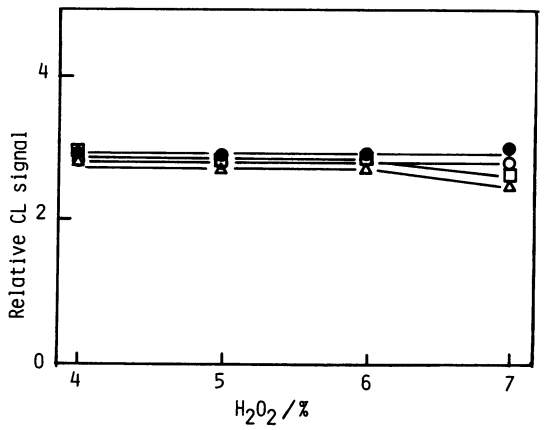

Fig. 4. Effect of the concentrations of hydrogen peroxide and FMN.
FMN concentration (10^{-3} M): △ 0.8; □ 1.0; ○ 1.2; ● 1.4, pH: 6.0, 20-μl injections of 5×10^{-7} M Cu(II), Other conditions as in Fig. 2.

Results and Discussion

Total Flow Rate and Reaction Temperature. Determinations of the total flow rate in the system and of the reaction temperature in the heating bath are very important for the FI-CL method in sensitivity and rapidity, although both are closely related to each other. At too low or too high total flow rate, most of the CL reaction, the speed of which is considerably slow at room temperature (see Fig. 3), proceeds outside the flow cell, resulting in low signal or low sampling throughput. In the previous work,[15] they were determined to be 8 ml min^{-1} and 60 °C, respectively. These are also employed in the present system, *i.e.*, 3 ml min^{-1} for R_1 and R_2, 2 ml min^{-1} for R_3, and 60 °C for M_2.

Reagent Concentrations and pH of R_1. Three reaction variables, the concentrations of FMN and hydrogen peroxide and the pH of FMN solution were optimized. Figure 4 shows that the CL signal is almost independent of the reagent concentrations. The concentrations of 1.4×10^{-3} M for FMN and of 5% for hydrogen peroxide were chosen for further experiments, which were slightly higher than those in the previous system because of the dilution of reagent solution with water of R_3.

The effect of pH is shown in Fig. 5, indicating that the optimum pH is about 5.8. The nature of the buffer

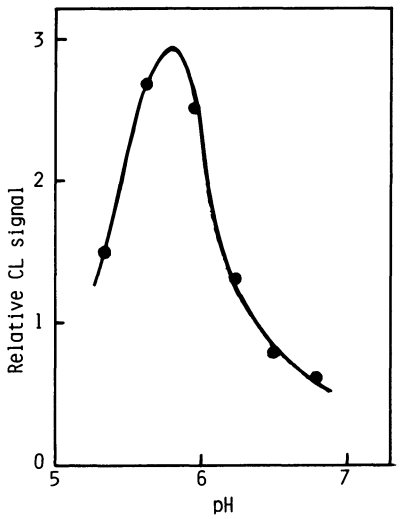

Fig. 5. Effect of pH.
1.4×10^{-3} M FMN, 6% H_2O_2, Other conditions as in Fig. 4.

components also influenced the signal, the phosphate buffer exhibiting maximum sensitivity. In sodium dihydrogencitrate-sodium tetraborate and disodium hydrogenphosphate-citric acid buffers the signals for 20-μl injections of 5×10^{-7} M copper solution were only *ca.*10 and 20% of that in the phosphate buffer, respectively; the use of sodium citrate-sodium hydroxide and sodium acetate-acetic acid buffers gave rise to no emission.

Effect of Surfactants. Over a narrow range of concentration (called the critical micelle concentration, cmc), amphiphilic surfactant molecules dynamically associate in aqueous solution to form aggregates called micelles, in which there is a sudden transition in the physical properties of aqueous surfactants. It is known that the local microenvironment in micellar media leads to significant increase in CL quantum yield.[17] We already found that the copper-catalyzed CL of 1,10-phenanthroline[18] and the FMN-sensitized CL of sulfur dioxide[19] were enhanced by some cationic and nonionic surfactant molecules, respectively.

In order to investigate whether micellar media function effectively for the present CL system, hexadecyltrimethylammonium bromide (CTAB) as a cationic surfactant, sodium dodecyl sulfate(SDS) as an anionic surfactant, and polyoxyethylene (20) sorbitan trioleate (Tween 85) as a nonionic surfactant were added to the carrier stream (R_3). However, no CL enhancement was observed, regardless of the presence or absence of buffer reagent, its components, and the reaction temperature. These surfactants, except Tween 85 at lower concentrations, suppressed the signal as shown in Table 1. In the table; the effect of surfactants on the fluorescence intensity of FMN is also represented; the micellar solution of CTAB causes decreased fluorescence intensity but those of SDS and Tween 85 increased fluorescence. It can be said from the table that the suppression of CL signal, especially for SDS,

Table 1. Effect of surfactants on CL signal for copper(II)

Surfactant[a]	Concentration	Relative CL signal	Relative fluorescence intensity of FMN
none		1.0	1.0
CTAB	1×10^{-4} M	0.88	
	1×10^{-3} M	0.29	0.72
	8×10^{-3} M	ND[b]	0.47
SDS	1×10^{-3} M	0.74	
	1×10^{-2} M	0.17	1.1
	8×10^{-2} M	0.04	2.9
Tween 85	0.5 g l^{-1}	1.0	
	7 g l^{-1}	1.0	2.1
	20 g l^{-1}	0.91	3.2

a) cmc: 9×10^{-4} M for CTAB, 8×10^{-3} M for SDS, unknown for Tween 85, b) Not detected.

is mainly ascribable to the inhibition of CL reaction due to the addition of surfactant, although for CTAB it is due in part to the decreased fluorescence yield of emitter (the similarity of CL spectrum to the FMN fluorescence spectrum indicates that the emitter has the flavin residue, *i.e.*, the isoalloxazine ring). At the CTAB and SDS concentrations over their cmcs, the signals are suppressed completely or markedly. In contrast, Tween 85 does not suppress the signal or the suppression is not so rigorous as the ionic surfactants even in the addition of large amount of surfactant over its cmc. These are rationalized in terms of the micellar effect that one of the ionic reactants (Cu^{2+} and FMN^-) is repelled electrostatically from the charged micellar surface which the ionic surfactant forms, while the other is attracted, resulting in an inhibition of the CL reaction. On the contrary, the ionic reactants are not influenced by the noncharged micellar surface which the nonionic surfactant forms. This is supported by the finding that the signal for chromium(VI) with a charge of the same sign as that of FMN is enhanced by CTAB, while it is suppressed by SDS and not changed by Tween 85. Other surfactants tested like zephiramine, didodecyldimethylammonium bromide, sodium oleate, Brij-35, and Triton X-100 also did not enhance the signal for copper.

Characteristics of the System. Under the recommended conditions (as specified in Fig. 2), the analytical characteristics of the system were investigated. The background and noise currents were 1.6×10^{-11} and 2.4×10^{-13} A, respectively. The detection limit is defined by the noise level, which depends on the background signal arising presumably from copper as an impurity in the reagents and water used. Accordingly, an improvement of the detection limit would be expected with decreased background. Thus, EDTA or tetraethylenepentamine was added to the reagent stream R_1 in an attempt to mask copper as the impurity. However, no decreased background signal was observed. This indicates that the background comes from noncatalyzed CL of FMN which can not be

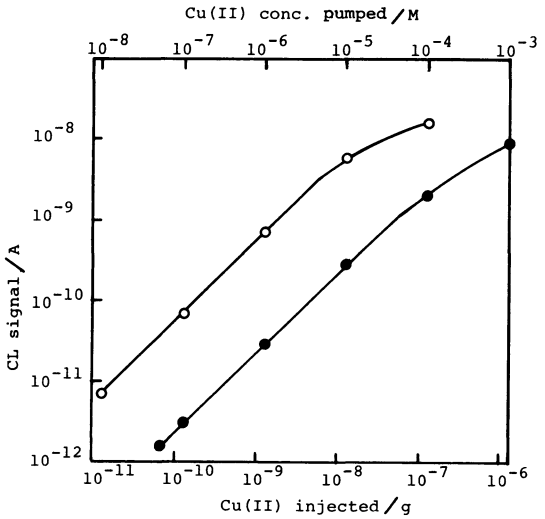

Fig. 6. Calibration graphs.
○: Continuous flow; ●: 20-μl injection.

eliminated.

Logarithmic calibration graphs were linear over three decades for 20-μl sample injections and for continuous sample flow (2 ml min^{-1}) (Fig. 6). The detection limit for a signal-to-noise ratio of 3 is 0.06 ng ml^{-1} for continuous sample flow and 0.03 ng for 20-μl sample injections. This is much lower than the 30 ng (30 ng ml^{-1}) achieved by the flash-photolytically initiated riboflavin CL[20] and the 6 ng ml^{-1} by the luminol electrochemiluminescence.[3] The relative standard deviation is 3.1% when 20-μl of the 1×10^{-6} M solution (*ca.* 1 ng) is successively injected ten times. The sampling rate is 120 h^{-1}.

Effect of Other Substances. Most solution CL methods suffer from poor selectivity. The luminol, lucigenin, and lophine systems permit 30, 20, and 11 species, respectively, to enhance the luminescent reaction.[5] In contrast, the FMN system is fairly selective and only iron(II) and chromium(III,VI) provide CL signals 2—3% of that for copper(II); other species enhance the emission very weakly, or give rise to no emission (Table 2). The signals which S^{2-}, SCN^-, and $S_2O_3^{2-}$ provide seem to derive from the FMN-sensitized CL produced by the energy transfer from excited sulfur molecules (max. emission wavelength= *ca.* 440 nm) to FMN molecules in such a way that the CL based on the hydrogen peroxide-oxidation of sulfide is sensitized by fluorescein (max. fluorescence wavelength λ_{fl}=535 nm) or rhodamine B (λ_{fl}=575 nm).[21] This is supported by the fact that the emission from these species are also sensitized by brilliant sulfoflavine (CI=56205, λ_{fl}=*ca.* 520 nm), another efficient sensitizer used in a previous work.[12] For ascorbic acid, cyanide, and EDTA, the emission may arise from the CL process involving singlet oxygen molecules as in the gallic acid-,[22] pyrogaroll-,[23] tannic acid-,[24] or formaldehyde-hydrogen peroxide CL system[25] and in the urea-, gelatin-, or guanidine-hypochlorite CL system.[25] The background suppression due to iodide is ascribable to the decreased fluorescence yield of emitter because the presence of 10^{-3} M iodide decreases the FMN fluorescence intensity by *ca.* 20%.

To check the effect of concomitant species on the signal for copper, 20 μl of a 10^{-4} to 10^{-6} M solution of each common species was injected into the stream R_3 of 2×10^{-8} M copper(II) solution. The results are

TABLE 2. SELECTIVITY OF THE FMN CL SYSTEM

Species[a]	Relative molar signal	Species[a]	Relative molar signal
Cu(II)	1000		
Cr(VI)	34	S^{2-}	8.7
Fe(II)	28	SCN^-	3.6
Cr(III)	18	Ascorbic acid	2.1
Pb(II)	1.7	$S_2O_3^{2-}$	0.9
Co(II)	1.6	CN^-	0.03
Fe(III)	1.2	EDTA	0.01
Mn(II)	0.1	I^-	−0.02

a) 20-μl injection of 10^{-3} M solution of chloride or nitrate, or sodium or potassium salt except ammonium sulfate for Fe(II,III), and of 10^{-4} M solution of nitrate for Cu(II). Mg(II), Ca(II), Ba(II), Al(III), Sn(II), Ni(II), Zn(II), Cd(II), Ag(I), Hg(II), As(V), NH_4^+, F^-, Cl^-, Br^-, NO_2^-, NO_3^-, CO_3^{2-}, SO_3^{2-}, SO_4^{2-}, $S_2O_8^{2-}$, ClO_4^-, IO_3^-, $B_4O_7^{3-}$, $[Fe(CN)_6]^{3-}$, acetate, citrate, oxalate, and urea give rise to no emission.

TABLE 3. INTERFERENCE STUDY FOR CATIONIC SPECIES

Species	Relative error[a] /%		
	10^{-4}M	10^{-5}M	10^{-6}M
Co(II)	370(80)	40(12)	ND
Cr(VI)	320(500)	50	ND
Cr(III)	260(300)	35	ND
Fe(II)	190(240)	50	ND
Zn(II)	110(ND)	58(ND)	12(ND)
Mn(II)	100(40)	15(ND)	ND
Fe(III)	71(12)	27(ND)	ND
Hg(II)	60(9)	15(ND)	ND
Pb(II)	55(ND)	10(ND)	ND
Cd(II)	30(ND)	ND	
Ni(II)	15(ND)	ND	
Sn(II)	−15(ND)	ND	

a) Values in parentheses: For the carrier solution containing 10^{-4} M Zn(II), ND: Not detected. 10^{-4} M Mg(II), Ca(II), Ba(II), Al(III), Ag(I), and As(V) do not interfere.

TABLE 4. INTERFERENCE STUDY FOR ANIONIC SPECIES

Species	Relative error/%	
	10^{-4}M	10^{-5}M
S^{2-}	85	ND
Ascorbic acid	64	
CN^-	35	ND
$C_2O_4^{2-}$	23	ND
SCN^-	15	ND
$S_2O_3^{2-}$	9	ND
I^-	−10	ND
$[Fe(CN)_6]^{3-}$	−20	ND
EDTA	−100	−100

10^{-4} M F^-, Cl^-, Br^-, ClO_4^-, IO_3^-, NO_2^-, NO_3^-, SO_3^{2-}, SO_4^{2-}, $S_2O_8^{2-}$, CO_3^{2-}, NH_4^+, $B_4O_7^{3-}$, acetate, citrate, and urea do not interfere.

shown in Tables 3 and 4.

Interesting features which appear in Table 3 are that species (Zn, Hg, Cd, Ni, Sn) eliciting no emission *per se* enhance the copper(II)-catalyzed emission (Sn suppresses it) and that species which elicit emission *per se* provide larger signal than those which they provide in the absence of copper. Such interferences are reduced or eliminated by the addition of zinc(II) to the carrier stream as shown in parentheses in the table. On the other hand, the addition of zinc(II) slightly increases the signals for chromium(III,VI) and iron(II) (and also for copper). Although these phenomena can not be readily interpreted, it may be correlated with the stability of FMN–metal complex which is likely to be formed during the CL reaction;[26] the stability of the complex decreasing in the order Cu>Zn>Co>Mn>Ni[27] may account for the reduction and elimination of interference owing to the addition of zinc(II). For anionic species, oxalate which does not chemiluminesce by itself provides signal, in addition to the emissive species (Table 4). Although the interference due to oxalate is not fully explainable, it is closely related to the fact that the oxalate–hydrogen peroxide system emits weak light which is easily sensitized by an appropriate fluorescent compound.[28] It is conceivable that the presence of copper enhances the sensitized-emission which can not be detected in the absence of copper.

Application to Real Samples. The applicability of the proposed method was evaluated by assaying NIES standard reference materials (Pepperbush[29] and Pond Sediment[30]) and Yabukita tea leaf.[31] Standard additions of copper to the acid-digested samples indicated that the results obtained for Pepperbush (12, 13, 13 $\mu g\,g^{-1}$), Pond Sediment (220, 213, 222 $\mu g\,g^{-1}$), and Yabukita tea leaf (9.1, 9.0 $\mu g\,g^{-1}$) by the continuous sample flow method were within the certified values (12±1, 210±12, and 9.09±0.97 $\mu g\,g^{-1}$, respectively).

Conclusion

The above results indicate that ultratrace amounts of copper(II) can be selectively and rapidly determined by FIA based on the FMN CL system. This provides a lower detection limit (0.03 ng or 0.06 $ng\,ml^{-1}$) for copper(II) than that by any other method, the detection limit being lower than those for flame atomic-absorption (18 $ng\,ml^{-1}$) and -fluorescence methods (1.5 $ng\,ml^{-1}$)[32] and neutron activation analysis (0.1 ng),[33] and comparable to those for flameless atomic absorption and inductively coupled plasma emission methods (0.04 $ng\,ml^{-1}$).[32]

Although no micellar enhanced CL is observed here, CL in oriented systems like micelles is very attractive in the analytical point of view because there is much possibility of enhancing quantum efficiency or energy transfer efficiency and because they permits the use of CL reagents and sensitizers insoluble in water. Furthermore, electrostatic interaction between charged analytes and the ionic micellar surface serves to improve selectivity. In the present CL system, for instance, cationic surfactant micelles may enable selective detection of chromium (anionic analytes) because the micellar effect works to suppress emission for cationic analytes like copper and iron, the emission for copper being completely suppressed by the presence of CTAB micelles as shown in Table 1.

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