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An overview on flow methods for the chemiluminescence determination of phosphorus

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Abstract

A review on the flow analysis of phosphorus with chemiluminescence detection is presented. A brief discussion of the chemiluminescence principles and applications is given. Particular emphasis is devoted to coupling different flow techniques (flow injection, sequential injection, multicommutation, multisyringe flow injection, multi-pumping) to chemiluminescence detection. Enzymatic and non-enzymatic methods, mostly applied to environmental samples, are summarized and compared in terms of application range, detection limits, flow configuration, repeatability and sampling rate.

Keywords: Chemiluminescence; Flow methods; Phosphorus; Phosphate

1. Introduction

Phosphorus is an essential nutrient in aquatic systems for primary productivity of the phytoplankton growth. However, increased inputs of phosphate from raw or treated wastewater, agriculture drainage, or certain industrial wastes can lead to eutrophication, a process that often is accompanied by growth of toxic algae [1]. The European Union regulatory directives set the limit of 0.1 mg L^{-1} $P–PO_4^{3-}$ (3.23 μM) as an indicator level for probable problematic algal growth. The most bioavailable form of phosphorus in aquatic ecosystems is orthophosphate, usually measured as the filterable molybdate reactive phosphorus [1], that passes through the 0.45 μm membrane. Spectrophotometric methods based on the formation of the blue or yellow form of phosphomolybdate or vanadophosphomolybdate heteropoly acids are the most frequently used to quantify phosphate concentrations between 1 and 20 mg $P–PO_4^{3-}$ L^{-1} (32.3–646 μM). For concentrations as low as 0.01 mg $P–PO_4^{3-}$ L^{-1} (0.323 μM) an additional extraction step is recommended to eliminate possible interferences. This latter sample preparation increases the complexity of the method, making its implementation more difficult as routine monitoring procedure in uncontaminated natural waters. Additionally, these samples have a highly dynamic spatial and temporal behaviour [2], resulting in the necessary real-time (in situ) monitoring. Therefore, flow methods for the automation of this determination have received increased attention in the last decades. Several flow methods were developed based on spectrophotometric detection [3]. Meanwhile, the use of chemiluminescence (CL) in flow methods is gaining wide acceptance by the analytical chemists for the determination of a wide range of analytes [4–11]. This development can be attributed to the low detection limits, high sensitivity and simple instrumentation pointed to this technique. These features are particularly important in the determination of phosphorus present at trace levels in some environmental samples.

The objective of this paper is to give an overview of the existent chemiluminescence flow methods for the determination of phosphorus. Additionally, a brief discussion of both the CL and flow techniques is presented. Special emphasis

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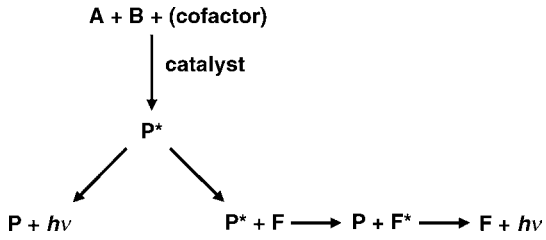


Fig. 1. Several mechanisms that lead to a chemiluminescent reaction: (A) substrate; (B) oxidant; (P) product; (P*) product in the excited state; (F) fluorophore; (F*) fluorophore in the excited state; ($h\nu$) emission of radiation.

will be given on the advantages of coupling CL detection to flow systems.

2. Chemiluminescence: principles and applications

Luminescence methods are based on the measurement of the emission spectrum obtained when previously excited atoms or molecules decay to their ground state. The emission of light is always a response to an input of energy of some kind, giving rise to different types of luminescence [8,9].

Chemiluminescence (CL) can be defined as the production of electromagnetic radiation (ultraviolet, visible or infrared) as consequence of a chemical or biochemical reaction. In this procedure, the chemical reaction produces sufficient energy to induce the transition of an electron from its ground state to an excited state. To return to its ground state the electron can either lead to the emission of a photon, a process called CL, or the excited molecule can lose energy by undergoing chemical reactions, by collisional deactivation, internal conversion or inter-system crossing. Because these radiationless processes compete with CL they are undesirable from an analytical point of view [12]. The number of chemical reactions that produce CL is small, limiting the procedure to a relatively few species.

A CL reaction generally proceeds by a variation of one of the following mechanisms pointed out in Fig. 1. Sometimes the excited particles are the products of a reaction between the analyte and a suitable reagent, usually a strong oxidant such ozone or hydrogen peroxide. In other cases, the analyte can play the role of reagent, catalyst, quencher or even an enhancer in the CL reaction. In any case a reactant undergoes a chemical reaction to form a product P* in an excited state. The excited product can lose energy by emission of radiation (direct CL), or by transferring energy to a fluorophore F that can subsequently emit radiation (indirect CL).

The CL can be affected by the characteristics of the chemiluminescence precursor and by the presence of quencher species, catalysers and metallic ions. This type of reactions is also strongly dependent on the temperature and on the properties of the medium, including the composition, pH, and ionic strength.

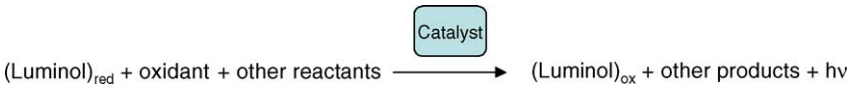
The typical signal from a chemiluminescence experiment as a function of time rise rapidly to a maximum as mixing of reagent and analyte is complete. Then a more or less exponential decay of the signal follows. Both the height of the peak or the area under the peak can be plotted as a function of concentration, and a linear relationship between signal and concentration can be observed over a concentration range of several orders of magnitude.

CL can be observed in solid, gas and liquid phase reactions and has been used for quantitative analysis in environmental, food and beverages and clinical areas [6,13]. In liquid solutions CL has been used for trace metal determinations and for quantitative analysis of biochemical and organic reactants. Many of the analysis carried out in the liquid phase make use of organic CL substances that react with oxygen, hydrogen peroxide, and many other strong oxidizing agents to produce a chemiluminescent oxidation product. Luminol provides the most common example of these compounds. In general, the luminol reaction can be schematized as follows (Scheme 1).

Several metal ions exert a pronounced effect on the chemiluminescent intensity when luminol is mixed with hydrogen peroxide or oxygen in alkaline solution. In most cases, this is due to the catalytic effect of the metal in the reaction that leads to the enhancement of the peak intensities. With a few cations, inhibition of luminescence occurs. Measurements of the increase, or decrease, in luminosity thus permit the determination of these ions at low concentration levels. Similarly, a number of organic species (like amino acids, nerve gases, certain types of insecticides, hematins, napthols, and benzene derivatives containing $-NO_2$, $-NH_2$, and $-OH$ groups) have a catalytic or inhibiting effects on the luminol reaction with hydrogen peroxide or oxygen, thus permitting their determination [14].

The instrumentation that is used for chemiluminescent quantitative analysis consists only of a suitable reaction vessel or cell, a photodetector and the associated electronics to convert and record signals (readout device). Generally, no wavelength selector is necessary since the only source of radiation is the chemical reaction between the analyte and reagent. As a detector, the photomultiplier tube is the most common instrument used. The entire apparatus except for the readout device is enclosed in a light tight box to prevent radiation from entering the box.

Chemiluminescence offers a number of attractive characteristics for chemical analysis. The method is highly sensitive



Scheme 1. General scheme of luminol CL reaction.

because low levels of light produced from the emission process are readily monitored in the absence of noise. Furthermore, the attenuation of radiation by a filter or a monochromator is avoided. In fact, detection limits are usually determined not by detector sensitivity but rather by reagent purity. Typical detection limits lie in $\mu g\,L^{-1}$ to $mg\,L^{-1}$ ranges. The simplicity, the wide dynamic ranges and the extreme sensitivity of the method account for its recent growth in chemical analysis. However, there is a drawback in this technique, the lack of selectivity due to the fact that some CL reagents can produce emission for more than one analyte.

3. Flow analysis with chemiluminescence detection

Chemiluminescence determination usually implies a previous derivatization or separation process before the CL reaction to improve selectivity or to be able to extend the range of possible analytes. These processes can result in a quite complex reaction scheme, which makes nearly impossible to perform them in a conventional batch fashion. The automation of these determinations using flow techniques is therefore crucial.

The limited selectivity of CL reactions can be overcome by incorporating physical separations or chemical reactions in a flow system. In fact, the majority of the determinations in real samples employ an in-line treatment to remove the matrix interferences or to modify the analyte to produce species suitable for CL detection. Furthermore, flow systems are ideally suited to monitoring liquid phase CL reactions since it provides rapid and reproducible mixing of sample and reagents. The CL reaction usually occurs in a spiral shaped flow through cell located in front of the detector. This way, it is possible to increase the intensity of radiation that reaches the detector and simultaneously monitor the fast CL reactions. Additionally, CL reactions are highly influenced by the medium conditions, like temperature, pH and ionic strength. These conditions can be easily controlled and maintained in an automated flow method. The different flow based systems allow the highly reproducible introduction of small volumes of samples and reagents, this property being particularly important for high sensitivity detection systems like CL, and for reagents or samples that are expensive or limited in quantity.

Various non-chromatographic flow techniques with potential to improve CL methods, like flow injection analysis (FIA) [15], sequential injection analysis (SIA) [16], multicommuted flow injection analysis (MCFIA) [17], and multisyringe flow analysis (MSFIA) [18], within others, are available. Separation techniques, like liquid chromatography, are out of scope of this paper.

Undoubtedly, FIA is the most widely used technique coupled to CL detection. FIA, as well as the other mentioned flow techniques, is based in reproducible sample injection or insertion in a carrier solution; controlled dispersion of the sample zone and reproducible timing of its movement from the injection point to the detection system [19].

In 1990, Ruzicka and Marshall [16] proposed a new flow technique designated as SIA, based on the same principles of FIA, and conceived as a single pump, a single valve and a single channel system. Compared to FIA, these systems allow considerable saving of reagents and a significant decrease on the chemical waste produced, since just the required amounts are aspirated and carrier is not pumped continuously. In addition, different analysis can be performed using the same manifold by simple reconfiguration of the sequence of events from the computer keyboard. Besides this, the major difference between FIA and SIA methodologies concerns the way that sample and carrier/reagent solutions are mixed inside the tubes. While in FIA the solutions are most commonly mixed in confluence points, giving rise to a concentration gradient of analyte in a constant background of reagent, in SIA efficient mixing is more difficult to achieve due to the absence of confluence points. In fact, in SIA an initial sharp boundary is formed between the adjacent sample/reagent zones stacked in the holding coil. Even after the flow reversal, only a partial overlap of analyte and reagent zones is achieved [19]. A further development of the technique led to the accommodation of solid phase chemistry, a technique designated by bead injection (BI) [20]. BI is based on the microfluidic manipulation of precise volume of suspended beads that serve as solid phase carrier for reagents or reactive groups. The injected bead suspension is trapped in an appropriate flow cell where is subsequently perfused by the analyte solution, buffers or reagents. Chemical reactions occur at the bead surface and can be analyzed on the solid phase or within the eluting liquid phase [21]. At the final step, the spent beads are discarded. The major benefit introduced by bead injection is automatic surface renewal, a critical feature when assays surfaces become contaminated or otherwise dysfunctional with repetitive use [19].

Multicommuted flow injection analysis was first described by Reis et al. [17] associated to the binary sampling approach. This technique is characterised by the use of individual commutation devices (solenoid valves) operating in a simultaneous or a sequential way, where solutions can be accessed randomly. In this approach, small plugs of sample and reagents are inserted in alternative way in the flow system and mutually dispersed while directed to the detector. Compared to other flow techniques, the main advantage introduced by the multicommuted approach is versatility based on the use of solenoid valves that can be arranged in multiple configurations. This evidence was pointed out by Zagatto et al. [22], when it mentioned that multicommutation can unify all concepts already proposed in flow analysis, considering the possibility of accommodating different flow modalities (FIA, SIA) in a system with just solenoid valves.

Multisyringe flow injection analysis was recently proposed by Cerdà et al. [18] in order to gather the advantages of the former techniques, namely the sampling rate and the ability of proper mixing of solutions of FIA, the robustness, versatility and reagent saving of SIA, and the speed of multicommutation valves of MCFIA [23]. The main limitation

Table 1
Analytical figures of chemiluminescence flow methods for the determination of phosphate ($P–PO_4^{3-}$)

CL reaction	CL phase	Flow mode	Sample	Working range (μM)	LOD (μM)	R.S.D. (%)	SR (h^{-1})	Reference
VMoP-HPA–luminol	Liquid	FIA—ion chromatography pos-column derivatization	River water, rice wine and seaweed	0.03–32.3	0.03	3.7		[30]
MoP-HPA–luminol		FIA—differential kinetic		3.2–322		1.8	45	[31]
VMoP-HPA–luminol	Solid	MSFIA with multicommutation approach	Mineral, ground, tap, pond waters; water from steam cycle	0.16–16.1	0.06	3.0	11	[32]
MoP-HPA–luminol	Liquid	FIA—three line with on-line sample treatment	River water	0.001–0.105; 0.53–2.11	0.001	4.7; 1.1	180	[33]
H_2O_2–luminol	Liquid	FIA—double line with immobilised enzymes	DNA	0.1–5.0	0.010	1.75		[34]
H_2O_2–TDPO	Liquid	FIA—three line with immobilised enzymes		0.1–2.0	0.039	<2.62	20	[35]
H_2O_2–luminol	Liquid	FIA—double line with immobilised enzymes	River water	4.8–160	3.2		20	[36]
H_2O_2–luminol	Solid	FIA—double line with immobilised enzymes	–	0.37–7.4	0.074		20	[37]
H_2O_2–luminol	Liquid	FIA—double line with immobilised enzymes	River water (dam)	0.16–32	0.16		13	[38]
H_2O_2–luminol	Liquid	FIA—double line with immobilised enzymes	River water (dam)	0.096–32	0.096	2.3	30	[39]
H_2O_2–luminol	Liquid	FIA—double line with immobilised enzymes	River water	0.01–30	0.01	4.3	20	[40]
H_2O_2–luminol	Liquid	FIA—double line with immobilised enzymes	River and pond waters	0.1–30	–	4.0	20	[41]

FIA, flow injection analysis; MSFIA, multisyringe flow injection analysis; CL, chemiluminescence; LOD, limit of detection; R.S.D., relative standard deviation; SR, sampling rate; TDPO, bis[2-(3,6,9-trioxadecanyloxycarbonyl)4-nitrophenyl]oxalate; VMoP-HPA, vanadomolybdophosphoric heteropoly acid; MoP-HPA, molybdophosphoric heteropoly acid.

of this approach derives from the fact that forward movement must be stopped after several operations to reload the syringes. Consequently, the sampling frequency, like in SIA systems, is lower if compared to FIA assemblies [19].

A new flow technique based on pulsed flow was exploited independently by Wang et al. [24] and by Lapa et al. [25]. This approach results in better solution mixing as it is characterised by turbulent flow with low axial dispersion. It can be carried out using a dedicated instrument [24] or by the use of several solenoid micropumps [25]. The pumps can be switched individually or in combination, in order to create a pulsed flowing stream through the analytical path. The active devices incorporate the liquid propelling units, the sample insertion port and commuting elements. The configuration and the control of the flow system can be simplified, resulting in a system with small dimensions that can be suited to field instrumentation.

The choice of the most adequate flow technique to be couple to CL detection is a difficult task, since each of the described methods presents some advantages over the others. The overwhelming use of CL detection coupled with FIA can be attributed to the easy incorporation of complex derivatization reaction schemes and separation processes. Nevertheless, all the other flow techniques have been also applied to CL detection exploiting the advantages of both [26–29].

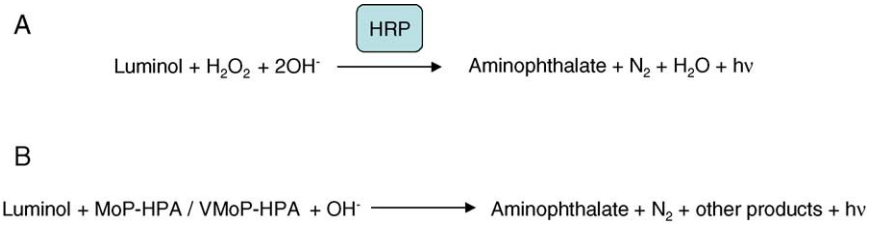
4. Phosphorus determination by flow chemiluminescence

For the CL determination of phosphorus (in the phosphate form) two types of derivatization processes were implemented in flow systems (Table 1). The first type is based on a chemical reaction of heteropoly acid formation, while the second group of applications are based on enzymatic reactions to form hydrogen peroxide. In most cases the subsequent chemiluminescence reaction is based on the oxidation of luminol while in one case [35] on the oxidation of bis[2-(3,6,9-trioxadecanyloxycarbonyl)4-nitrophenyl]oxalate (TDPO).

The CL reaction of luminol has been studied and described in various reviews [12,42]. The simplified reactions schemes involved in phosphate determination are presented in Scheme 2.

Some figures of merit, the application matrices and the applied flow techniques are summarized in Table 1.

The reactions described in the following section are all based on the initial presence of orthophosphate ion in the samples. Other organic species of phosphorus can be present in the samples however the chemiluminescence determination of these species implies additional steps to be involved in the reaction scheme to produce orthophosphate; the discussion of these possibilities was out of the scope of this review.



Scheme 2. Simplified reaction schemes involved in the phosphate determination: (A) luminol–H_2O_2–peroxidase (HRP) system; (B) luminol–molybdophosphoric heteropoly acid (MoP-HPA)/vanadomolybdophosphoric heteropoly acid (VMoP-HPA) reactions.

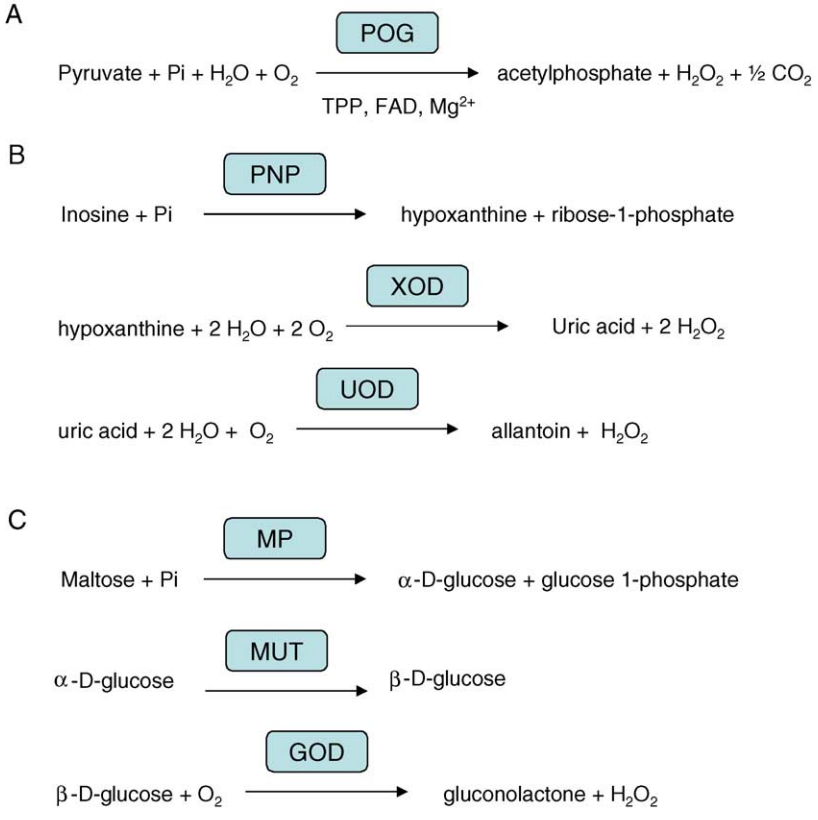
4.1. Chemical derivatization

Phosphate ion can be determined by CL based on the oxidation of luminol by a heteropolyacid complex. In acidic medium, this heteropolyacid can be formed by the reaction between phosphate and molybdate giving rise to molybdophosphoric heteropolyacid (MoP-HPA), or in the presence of vanadate resulting vanadomolybdophosphoric heteropolyacid (VMoP-HPA) (Scheme 2B).

Based on the CL reaction between luminol and MoP-HPA, a flow injection differential kinetic CL method for phosphate determination in the presence of silicate and arsenate was developed by Jiang et al. [31]. The method was based on the difference in the reaction rate of the formation of molybdophosphate, molybdosilicate and molybdoarsenate.

Recently, Yaqoob et al. [33] proposed a flow injection CL manifold for determination of phosphate in fresh waters at nanomolar concentrations. The method was based on the oxidation of luminol by MoP-HPA. A iminodiacetate chelating resin column was incorporated to remove potential cationic interferences and tartaric acid was added previous to injection to mask silicate interference. As a logical parallel study, another system was reported [43] for the determination of silicate by the same research group based on the same reaction, using a strong anion exchange resin column to eliminate the interfering effect of phosphate.

Based on the reaction between luminol and VMoP-HPA, Fujiwara et al. [30] developed a flow chemiluminescence method for the determination of arsenate, germanate, phosphate and silicate, after separation by ion chromatography.



Scheme 3. Simplified reaction schemes involved in the enzymatic phosphate (Pi) determination: (A) POG—pyruvate oxidase G; TPP—thiamine pyrophosphate; FAD—flavin adenine dinucleotide; (B) PNP—purine nucleoside phosphorylase; XOD—xanthine oxidase; UOD—urate oxidase; (C) MP—maltose phosphorylase; MUT—mutarotase; GOD—glucose oxidase.

A flow-through solid-phase based optical sensor was described by Morais et al. [32] for the phosphate determination in different waters exploiting the MSFIA concept with multicommutation. The proposed system relied upon the VMoP-HPA formation and the transient immobilisation of the VMoP-HPA in a *N*-vinylpyrrolidone/divinylbenzene copolymer packed in a spiral shaped flow through cell. The method was able to determine trace levels of phosphate in high silicate content samples.

4.2. Enzymatic derivatization

When chemiluminescence detection is considered, oxidases are used as they give rise to hydrogen peroxide, which can be detected through its reaction with luminol.

Phosphate ion is used by many enzymes as a substrate [44]. Various enzymes and enzyme combinations have been applied with the objective of the generation of hydrogen peroxide for the determination of phosphate.

One of the possible enzymes is pyruvate oxidase G (POG, EC 1.2.3.3) that catalysis the formation of acetylphosphate from pyruvate in the presence of phosphate [36–39]. The reaction scheme is presented in Scheme 3A. This system has the advantage of using a single enzyme for the formation of hydrogen peroxide. However, two cofactors, thiamine pyrophosphate (TPP) and flavin adenine dinucleotide (FAD), are indispensable for the reaction. Additionally, the TPP cofactor needs further purification [36,37] because of the phosphate impurities that adversely affected the analysis.

Other possibility is the use of a two-enzyme system containing purine nucleoside phosphorylase (PNP, EC 2.4.2.1) and xanthine oxidase (XOD, EC 1.1.3.22) to generate hydrogen peroxide (Scheme 3B) [34,35]. One of the advantages of this system is the generation of two moles of peroxide for each mole of phosphate, which consequently should increase the sensitivity. However, as inosine is not a stable substrate [37] it is difficult to use for monitoring purposes. Additionally, inosine seems to inhibit the second (XOD) reaction or the CL reaction at higher concentrations [35]. The equilibrium of the second reaction could be altered using a third enzyme, urate oxidase (UOD, EC 1.7.3.3) [34]. This approach further increases the sensitivity since one of the products of this reaction is also hydrogen peroxide.

Later a three-enzyme system was proposed [40,41] for the generation of hydrogen peroxide. The system consisted of maltose phosporylase (MP, EC 2.4.1.8), mutarotase (MUT, EC 5.1.3.3) and glucose oxidase (GOD, EC 1.1.3.4), the reaction scheme is presented on Scheme 3C. In this case, no co-factors are involved in the reactions and the substrate maltose is stable and inexpensive. However, the determination do relies upon three consecutive enzymatic reactions, making the optimization process difficult [41]. As a result of this work, a prototype of the phosphate sensor was presented, capable of continuously monitoring phosphate concentrations of 1.0 μM during at least 2 weeks.

5. Concluding remarks

Chemiluminescence detection can provide a rather important alternative for the determination of phosphorus at trace levels. When compared to existing spectrophotometric methods most of the developed CL flow techniques show the advantage of lower detection limits. For the spectrophotometric detection of phosphorus with batch, segmented flow and flow injection analysis techniques detection limits of 0.3, 0.03 and 0.02 μM were reported, respectively [1]. The coupling of flow methods to chemiluminescence detection enhance the possibilities of this technique because it allows: (i) to improve selectivity by performing in-line sample treatment such as physical separations and chemical reactions; (ii) reproducible conditions of reaction time, pH, temperature, ionic strength; (iii) to minimise reagent consumption and effluent production. Additionally, the use of a flow through cell located in front of the detector allows monitoring the generally fast CL reactions.

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