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Flow and Sequential Injection—Luminescence Detection

SEQUENTIAL INJECTION CHEMILUMINESCENCE METHODOLOGY FOR OZONE EVALUATION

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A sequential injection methodology with chemiluminescence detection for the evaluation of residual ozone in waters is presented. The procedure is based on the reaction between luminol and ozone without catalysts.

Linear calibration plots were obtained for ozone concentrations between 0.05 and 2.0 mg L⁻¹, with a detection limit of 0.04 mg L⁻¹.

The developed methodology was applied to the determination of residual ozone in ozonized waters and the results complied with those furnished by the spectrophotometric reference procedure (relative deviations < 6.3%). The method exhibited good precision (RSD < 3.5%) and the sampling rate was about 140 determinations per hour.

Keywords: Chemiluminescence; Luminol; Ozone; Ozonized water; Sequential injection

INTRODUCTION

The analytical interest of chemiluminescence is in its ability to produce molecules that emit light without prior irradiation, which avoids difficulties arising from stray light, unselective excitation, and instability of the light source. Chemiluminescence (CL)-based analytical methods have played a significant role on chemical

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analysis due to its high sensitivity and simple instrumentation (Garcia-Campana and Baeyens 2000). On the other hand, CL suffers from lack of selectivity and strong temporal dependence of the CL signal. Thus, it requires strict timing control between solution mixing and detection, as measurements around the maximum emission yield enhanced sensitivity. Bearing this in mind, and considering the kinetic characteristics of both CL and flow techniques, this association seems quite adequate. Many chemiluminescence methodologies based on flow techniques have been effectively developed in recent years (Fletcher et al. 2001; Mervartova, Polasek, and Calatayud 2007). These approaches allow the immediate presentation of the reaction zone to the detector that enables an adequate measurement of the light to be emitted from the short-lived excited state intermediates formed in the chemiluminescence reaction. In fact, within flow systems, all measurements are executed under precisely defined and reproducible conditions such that all samples are treated physically and chemically in the exact same way.

The CL can be exploited in terms of signal inhibition or enhancement for the determination of a variety of substances. Luminol is one of the most commonly used chemiluminescence reagents. It has got a broad application since many different species can influence the mechanism and kinetics of its oxidation. The analyte can act as an enhancer, inhibitor, or catalyst whose concentration influences the obtained CL signal (Garcia-Campana and Baeyens 2000).

Sequential injection analysis (SIA) has been used for the implementation of a CL procedure based on luminol oxidation for the detection of substrates of oxidase enzymes that generate hydrogen peroxide (Panoutou 2005). To increase the production of luminol radicals, transition metal cations functioning as catalysts were applied, alone, namely Co^{2+} (Economou, Panoutou, and Themelis 2006; Mei et al. 2007) or in their complexed forms (e.g., ferrocene, ferricyanide) (Tucker et al. 1994; Min, Nielsen, and Villadsen 1995, 1996). Analytes have been also evaluated through the enhancement of the Mg catalyzed luminol/hydrogen peroxide reaction (Gao and Fan 2008) or quenching effect on the reaction between luminol and iodine (Min et al. 1995, 1996), superoxide anion or nitric oxide (Miyamoto et al. 2007). The biocatalysis of luminol oxidation with H_{2}O_{2} and p-iodophenol was also described in immunoassays in which horseradish peroxidase was used as analyte-label (Zhang et al. 2005).

In this paper, we report an application that relies only upon the CL reaction between luminol and ozone without the use of catalysts. The mechanism of ozone aqueous decomposition has been explored by several authors and is considered a complex radical type chain reaction (Nemes, Fabian, and Gordon 2000). However, it is known that the primary chain carrier is the ozonide ion radical (O_{3}). This radical can be involved in a serious of chain reactions that conduct to the formation of several radicals that can oxidize luminol generating chemiluminescence. This reaction has already been implemented in flow injection analysis (FIA) systems (McGowan and Pacey 1995; Jin et al. 2005) but regarding SIA, it is the first time that this determination is implemented. This technique has a great potential for solution handling and involves simple and versatile manifolds that result in reduced consumption of samples and reagents (Ruzicka and Marshall 1990).

With the use of a homemade chemiluminometric detector, it provided a simple, rapid, and economic system to assess residual ozone concentrations that could be
applied in the monitorization of aquarium and aquaculture waters with the aim of controlling the ozonization procedures.

MATERIALS AND METHODS

Reagents and Solutions

All solutions were prepared using chemicals of analytical reagent grade and high purity water, with a specific conductance $<0.1 \mu S \, cm^{-1}$.

A luminol solution $4 \times 10^{-4} \, mol \, L^{-1}$ was prepared in a phosphate solution $5 \times 10^{-2} \, mol \, L^{-1}$, pH 12.4. This solution was also used as carrier in the SIA system.

A stock solution of ozone was prepared according to DIN EN ISO 7393 (Merck) by mixing, in a 100 mL flask, 20 mL of a KIO$_3$/KI solution 1:67 (m/m) with 2 mL of H$_2$SO$_4$, 0.5 mol L$^{-1}$, and NaOH 2 mol L$^{-1}$, dropwise, until the solution lost its color. The concentration of this solution was evaluated spectrophotometrically ($\lambda = 259 \, nm$) before use. Working standard solutions (0.05, 0.1, 0.25, 0.50, 0.75, 1.00, and 2.00 mg L$^{-1}$) were prepared daily by dilution of the ozone stock solution in water.

Apparatus

The SIA system used (Fig. 1) comprised a Gilson Minipuls 3 peristaltic pump equipped with PVC pump tubing (1.02 mm i.d.), a 8-port multiposition selection valve (Valco VICI C25-3180 EMH), and a chemiluminometric detector (40 $\mu L$ flow cell) similar to one already described (Miró, Estela, and Cerda 2005). The different components of the system were connected with 0.8 mm PTFE tubing. The PTFE tubing (0.8 mm i.d.) and Gilson end-fittings and connectors were used to assemble the different manifold parts.

Analytical signals were recorded on a Kipp & Zonen BD 111 (Delft, The Netherlands) strip chart recorder.

The flow system was controlled by means of a microcomputer equipped with an interface card (Advantech Corp., PCL 711B, San Jose, CA). Software was developed in QuickBasic 4.5 (Microsoft) and permitted operation of the peristaltic pump and selection valve enabling the run-time definition of all analytical parameters to be

Figure 1. SIA system for the determination of residual ozone in waters C: carrier solution (phosphate solution $5 \times 10^{-2} \, mol \, L^{-1}$, pH 12.4); PP: peristaltic pump; SV: selection valve; CL-D: chemiluminescence detector; HC: holding coil (1.5 m, eight figure); RC: reaction coil (0.1 m, eight figure).
made like flow rate, flow direction, sample volume, reagents volume, and valve positioning, as well as data acquisition and processing.

A Perkin Elmer, Lambda 45 UV/Vis spectrophotometer was used to perform the determinations by the comparison method. In this case, a quartz cell with 1 cm optical path length was used.

Ozone was generated by an ozonizer Hailea model HLO-100.

Flow Manifold and Analytical Procedure

System components were arranged as shown schematically in Figure 1. The holding coil (HC) and reaction coil were 1.5 m and 0.1 m length, respectively, both with figure eight-shaped configuration.

The analytical cycle began with the sequential aspiration of 200 μL of luminol and 200 μL of sample to the holding coil. Then, by flow reversal the reaction zone was propelled to the chemiluminometric detector and an analytical signal proportional to the concentration of ozone was registered.

Reference Procedure

In order to evaluate the accuracy of the results obtained with the developed procedure, ozone was analyzed by UV spectrophotometry at 259 nm. For the calculation of ozone concentrations a molar absorptivity of 3300 mol L⁻¹ cm⁻¹ was used (Hart, Sehested, and Holcman 1983). This procedure was also used to access the concentration of ozone in the chemical solution. For this procedure, 1 mL of the ozone solution was diluted 50 times in water and the resulting solution was analyzed immediately.

Ozonization of Water Samples

There are several factors affecting the decomposition of ozone in water and so some studies involving the use of an ozonizer were performed in order to define the ozonization conditions. Considering that ozone exhibits maximum stability in water at pH 2 (McGowan and Pacey 1995), an initial comparative study was performed involving the ozonization of acidified and non-acidified water samples. The ozone content of both samples was monitored spectrophotometrically, and it was observed that acidification of the sample resulted in an increase of ozone concentration of about 85% confirming the influence of pH on the stability of ozone. An acidified water sample was then ozonized for distinct periods of time up to 2 hours, and it was noted that the ozone concentration increased very slightly with the increase of ozonization time up to one hour. Longer ozonization periods did not led to a significant change in the ozone concentration due to its fast decomposition. Indeed, it is known that ozone is poorly soluble in water and decomposes very rapidly in aqueous environments so that, in the analytical point of view, only the residual ozone can be considered. In the described conditions the maximum ozone concentration was about 0.5 mg L⁻¹ confirming that in aqueous media this specie is unstable and that higher concentrations can only be reached in specific conditions namely with ozonization under pressure.

Thus, the samples for analysis were obtained by instrumental ozonization (30 minutes) of Milli-Q water samples acidified to pH 2, with phosphoric acid. In order
to obtain samples with ozone concentration higher than 0.5 mg L\(^{-1}\) a batch of samples was spiked with ozone stock solution. The samples obtained by both procedures were analyzed immediately in the SIA system and spectrophotometrically.

RESULTS AND DISCUSSION

In order to obtain the maximum relative CL intensity, the conditions of the SIA system were optimized. We investigated the effect of flow rate, order of aspiration, volume, and concentrations of sample and reagents as well as composition of the carrier solution on the CL intensity. This section describes and discusses the application of the developed methodology to the analysis of ozonized water samples, as well as the validation of the obtained results.

Optimization of the Flow System

One of the most important advantages of the SIA technique is that it is not necessary to adjust sample dilution prior to its introduction in the system in order to bring the signal height within the linear range of the detector. It is possible to insert accurately variable sample volumes by combining time and flow rate without physical reconfiguration of the system. The influence of sample volume on the chemiluminometric signal was studied in the range between 12.5 and 250 \(\mu\)L. The studies proceeded with 200 \(\mu\)L of sample since the analytical signals increased up to this volume and no significant variation was observed for higher volumes (Fig. 2).

![Figure 2. Effect of sample and luminol volume and luminol concentration in the analytical signals. The presented results correspond to the analysis of an ozone standard solution 0.5 mg L\(^{-1}\).](image-url)
The oxidation of luminol was the key step of the developed CL methodology such that the optimization of its volume and concentration was of the most importance for the sensitivity of the procedure. The plot of luminol volume in the range of 12.5–250 μL vs. CL intensity displayed an initial increase up to 200 μL and then leveled-off. Initially the CL intensity increased as the overlapping between luminol and sample zones increased. Maximum overlap was achieved for a luminol volume of 200 μL and by further increasing this slug, the leading luminol portion was not subjected to overlap. Therefore, the volume of luminol zone was set at 200 μL. In reference to luminol concentration, the optimization studies were conducted between $5 \times 10^{-5}$ and $2.5 \times 10^{-4}$ mol L$^{-1}$. The results showed that the utilization of a luminol solution with concentration superior to $2 \times 10^{-4}$ mol L$^{-1}$ did not lead to a significant improvement in sensitivity. Thus, the determination was performed with luminol $2 \times 10^{-4}$ mol L$^{-1}$ (Fig. 2).

Since the sequence of addition of reagents greatly affects the sensitivity of the determinations in SIA systems, the order of aspiration was studied by changing the sequence of insertion of sample and luminol aliquots. The optimized procedure involved the sequential aspiration of luminol and sample since this situation proved to be more favorable in terms of sensitivity, probably due to the reduced dispersion of the sample zone associated with its aspiration before the change of flow direction.

The short reaction coil (volume $5 \times 10^{-3}$ mL, length 0.10 m) inserted just before the detector was used to give a final intimate mixing between sample and reagent. Initially, an increase in the CL intensity was recorded as the mixing coil was increased, and this was due to a more effective mixing of the zones in the longer coil. For coil lengths greater than 10 cm, a decrease was observed, and this was attributed to the more pronounced loss of emitted light long before the sample reached the detection cell.

Increasing the delivery flow rate in the range 2–5.5 mL min$^{-1}$ resulted in increased CL intensity as the mixed zones were transported faster to the detector and less radiation was lost during their travel in the mixing coil. A delivery flow rate of 5 mL min$^{-1}$ was used to minimize wear of the pump.

Since the oxidation of luminol to excited 3-aminophtalate with light emission only occurs in alkaline medium, a phosphate solution with pH 12.4 was tested as carrier solution in the SIA system. This possibility was considered as an alternative to the utilization of water that resulted in a total loss of analytical signal because the pH of the reaction media was not adequate for luminol oxidation. In reference to pH, it was noted that the sensitivity of the determination decreased about 7 times when it was changed from 12.4 to 11 units. Thus, a phosphate solution $5 \times 10^{-2}$ mol L$^{-1}$ with pH 12.4 was used as carrier in the SIA system.

**Interferences**

Considering that the developed methodology was to be applied in the analysis of ozonized water samples it was important to assess the potential interfering effect of several oxidant species that can be present in these samples.

Standard solutions with a fixed amount of ozone (2 mg L$^{-1}$) and increasing concentrations of the potential interfering species were analyzed by the automatic SIA methodology. A species was considered as non-interfering when the analytical signal variation regarding the one obtained in its absence was lower than 3%.
The results showed that $\text{H}_2\text{O}_2$ is not an interferent even in concentrations around $5\text{mg L}^{-1}$ and that Mn(VII) exhibited a tolerance limit of $0.001\text{mg L}^{-1}$.

The main components of the ozone chemical solution, KI and KIO$_3$, were also studied, and it was noted that none of the species affected the analytical signals in the concentrations in which they were used to prepare the stock solution.

**Figures of Merit**

After the optimization of all the parameters affecting the analytical signal, the developed methodology was evaluated for ozone concentrations between 0.05 and $2.00\text{mg L}^{-1}$ and linear calibration plots were obtained. The typical calibration plot was:

$$\text{CL (mV)} = 0.1696(\pm0.0034) \text{C (mg L}^{-1}) - 0.0051(\pm0.0031), \quad R^2 = 0.9998$$

(CL: chemiluminescence signal; C: ozone concentration).

The detection and quantification limits of the determination, calculated as $3\sigma$ and $10\sigma$ of $S (y/x)$ (Miller 1991), were 0.04 and 0.13 $\text{mg L}^{-1}$, respectively. These values are similar to those achieved by most FIA methodologies for the same purpose (Straka, Pacey, and Gordon 1984; Straka, Gordon, and Pacey 1985; Darby et al. 1995; Baeza, Alonso, and Bartroli 2005) being applicable in continuous monitoring systems since residual ozone concentration is usually higher than $0.05\text{mg L}^{-1}$.

The optimized procedure allowed performing about 140 determinations per hour.

**Analysis of Water Samples**

The developed methodology was then applied to the analysis of ozonized water samples. Fifteen samples were prepared either by spiking with the ozone chemical solution or by instrumental ozonization with the aim of mimic aquarium and aquaculture waters in which the monitorization of residual ozone can be important for the control and efficiency of the ozonization procedure.

The analysis of tap water samples showed, as expected, that the ozone content of this kind of sample is below the detection limit of the methodology as it is usually disinfected by chlorination.

In natural waters, where putative interferent compounds can be present producing background chemiluminescence, the concentration of ozone must be quantified by increased CL intensity against total blank, according to the expression $\text{CL} = \text{CL}_s - \text{Bt}$, where $\text{CL}_s$ is the signal of sample after ozonization and $\text{Bt}$ is the total blank corresponding to the sum of reagents blank and sample blank (before ozonization).

In order to evaluate the accuracy of the automatic method, the results of the analysis of the samples were compared with those furnished by the reference spectrophotometric procedure (Table 1). No significant disparity was obtained between both methods with relative deviations, expressed in percentage, lower than 6.7%. This resemblance was confirmed by a paired $t$-student test, which, for a 95% confidence level, showed no significant statistical differences between the results furnished.
by both methods ($t$ calculated = 0.23; $t$ tabulated = 2.16) (Miller 1991). Along with the evaluation of the correlation significance using the $t$-test a linear relationship between the two methods was established: SIA (mg L$^{-1}$) = 1.0005(±0.0448) Ref (mg L$^{-1}$) − 0.0017(±0.0262), confirming the null hypothesis.

No significant differences (rsd < 3.5%) were obtained in the repetitive analysis ($n = 15$) of samples with different concentrations of ozone (0.42 and 1.38 mg L$^{-1}$) confirming the repeatability of the developed procedure.

**CONCLUSIONS**

The implementation of the chemiluminescence reaction of luminol without the use of catalysts in a SIA system confirmed, once again, the possibility of associating profitably SIA with chemiluminescence detection.

Furthermore, the proposed method resulted in a drastic reduction on reagents consumption and effluent generation when compared to the existing FIA methods in which reagents (Straka et al. 1984; Straka et al. 1985; Chung, Bellamy, and Dasgupta 1992; Darby et al. 1995; McGowan and Pacey 1995; Baeza et al. 2005; Jin et al. 2005; Takayanagi and Dasgupta 2005), in one case sample, (Baeza et al. 2005) flow continuously. In each analytical cycle 0.014 mg of luminol were consumed and only 1.2 mL of effluent were produced being the methodology in good agreement with the actual concerns of green chemistry. Generally, the SIA procedure proved to be faster (140 determination per hour) than the majority of the existing flow methodologies for residual ozone monitoring that perform between 60 and 120 determinations per hour (Straka et al. 1984; Straka et al. 1985; McGowan and Pacey 1995; Baeza et al. 2005).

Furthermore, the resulting SIA manifold was simple and robust allowing the real-time analysis of residual ozone in a fully-automated way, with minimal operator

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**Table 1.** Results of the analysis of water samples by the SIA-CL and spectrophotometric methodologies

<table>
<thead>
<tr>
<th>Sample</th>
<th>UV ± SD$^a$ (mg L$^{-1}$)</th>
<th>SIA ± SD$^b$ (mg L$^{-1}$)</th>
<th>Relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.188 ± 0.010</td>
<td>0.191 ± 0.004</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>0.188 ± 0.008</td>
<td>0.200 ± 0.008</td>
<td>6.3</td>
</tr>
<tr>
<td>3</td>
<td>0.129 ± 0.002</td>
<td>0.123 ± 0.003</td>
<td>−5.0</td>
</tr>
<tr>
<td>4</td>
<td>0.276 ± 0.014</td>
<td>0.290 ± 0.008</td>
<td>5.0</td>
</tr>
<tr>
<td>5</td>
<td>0.240 ± 0.010</td>
<td>0.235 ± 0.006</td>
<td>−1.9</td>
</tr>
<tr>
<td>6</td>
<td>0.371 ± 0.010</td>
<td>0.383 ± 0.003</td>
<td>3.4</td>
</tr>
<tr>
<td>7</td>
<td>0.208 ± 0.008</td>
<td>0.215 ± 0.008</td>
<td>2.9</td>
</tr>
<tr>
<td>8</td>
<td>0.451 ± 0.001</td>
<td>0.460 ± 0.006</td>
<td>2.1</td>
</tr>
<tr>
<td>9</td>
<td>0.647 ± 0.010</td>
<td>0.638 ± 0.006</td>
<td>−1.4</td>
</tr>
<tr>
<td>10</td>
<td>0.807 ± 0.010</td>
<td>0.764 ± 0.006</td>
<td>−5.3</td>
</tr>
<tr>
<td>11</td>
<td>0.400 ± 0.010</td>
<td>0.379 ± 0.003</td>
<td>−5.1</td>
</tr>
<tr>
<td>12</td>
<td>0.749 ± 0.010</td>
<td>0.715 ± 0.025</td>
<td>−4.6</td>
</tr>
<tr>
<td>13</td>
<td>1.304 ± 0.008</td>
<td>1.361 ± 0.003</td>
<td>4.4</td>
</tr>
<tr>
<td>14</td>
<td>0.412 ± 0.008</td>
<td>0.428 ± 0.009</td>
<td>3.9</td>
</tr>
<tr>
<td>15</td>
<td>0.926 ± 0.022</td>
<td>0.892 ± 0.009</td>
<td>−3.7</td>
</tr>
</tbody>
</table>

$^a$SD – standard deviation of four replicates.

$^b$SD – standard deviation of three replicates.
intervention. It is, however, a good option for the analysis of aquarium and aquaculture waters that are usually disinfected by ozonization as an alternative to chlorination that is associated to the formation of byproducts (such as halogenated trihalomethanes and haloacetic acids) harmful for living organisms. In these cases, the methodology seemed to be suitable for on-line monitorization of residual ozone with the aim of control the ozonization conditions.

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