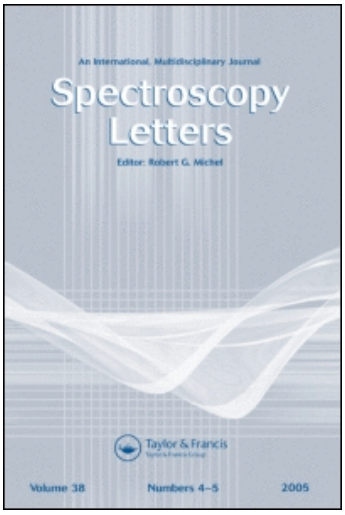


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# Sensitive Chemiluminescence Assay for Patulin in Apple Juice by Flow Injection

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# Sensitive Chemiluminescence Assay for Patulin in Apple Juice by Flow Injection

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**Abstract:** A sensitive chemiluminescence (CL) procedure for the determination of patulin by flow injection is described. It was found that patulin inhibits the CL generated from the luminol–dissolved oxygen system significantly. The decrement of chemiluminescence intensity was linear with the patulin concentration over the range from 0.04 to 10.0 ng $mL^{-1}$ with a detection limit of 0.01 ng $mL^{-1}$ ($3\sigma_{noise}$). At a flow rate of 2.0 mL $min^{-1}$, a complete analytical process could be performed within 0.5 min, including sampling and washing, with a relative standard deviation of less than 3.0% (n = 5). The proposed method was applied successfully in the determination of patulin in apple juice, and the recovery was between 96.9% and 103.9%.

**Keywords:** Apple juice, chemiluminescence, flow injection, luminol, patulin

## INTRODUCTION

Patulin {4-hydroxy-4H-furol [3, 2-c] pyran-2 (6H)-one} is a toxic metabolite produced by penicillium, aspergillus, and byssochlamys.[1,2] Patulin is a lactone (Fig. 1), that is relatively stable at low pH, and thermal processing appears to cause only a moderate reduction in patulin levels.[3] Patulin can be produced in different food products, including fruits, grains, cheese, cured meats, and especially in apples and apple products. It has become one of the most important quality criteria for apple juice.[4] In addition to being

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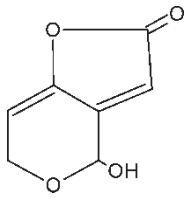


***Figure 1.*** Structure of patulin.

toxic, patulin induces mutagenesis, carcinogenicity, teratogenicity, and intestinal injuries, including epithelial cell degeneration, inflammation, ulceration, and hemorrhages.[5–8] A maximum permitted concentration has been set for 50 $\mu g\ L^{-1}$ in foodstuffs by the World Health Organization.[9]

Chromatography has been widely used for the determination of patulin, including thin-layer chromatography[10] and gas chromatography using MS detection[11–13] with the lowest detection limit of 6 $ng\ mL^{-1}$ and high-performance liquid chromatography using UV detection[14–16] with a detection limit of 1–5 $ng\ mL^{-1}$. Stable isotope dilution assay with a detection limit of 12 $pg\ mL^{-1}$,[17] and electrokinetic capillary electrophoresis with a detection limit of 3.8 $ng\ mL^{-1}$[18] have also been reported for the determination of patulin in apple products. Chemiluminescence (CL) has high sensitivity, wide linear range, simple instrumentation, high sampling efficiency, and reduced reagent consumption.[19,20] However, the authors are aware of no report on CL for the determination of patulin.

It is well-known that luminol can be oxidized by dissolved oxygen in an alkaline medium, producing CL.[21]

In the current study, it was established that patulin inhibits the CL emission of the luminol–dissolved oxygen reaction. The decrease in CL corresponded with the concentration of patulin linearly between 0.04 and 10 ng $mL^{-1}$ ($r^2 = 0.9989$) with a detection limit ($3\sigma_{noise}$) of 0.01 ng $mL^{-1}$. At a flow rate of 2.0 mL $min^{-1}$, a complete analytical process could be performed within 0.5 min, with a relative standard deviation of less than 4.0% ($n = 5$). The proposed method has been applied successfully to the determination of patulin in apple juice, and the recovery for the patulin samples was between 96.9% and 103.9%.

## MATERIALS AND METHODS

### Apparatus

A schematic diagram of the CL flow injection analysis system is shown in Fig. 2. A peristaltic pump was utilized to deliver all flow streams. PTFE tubing (1.0 mm i.d.) was used as connection material in the flow system. A

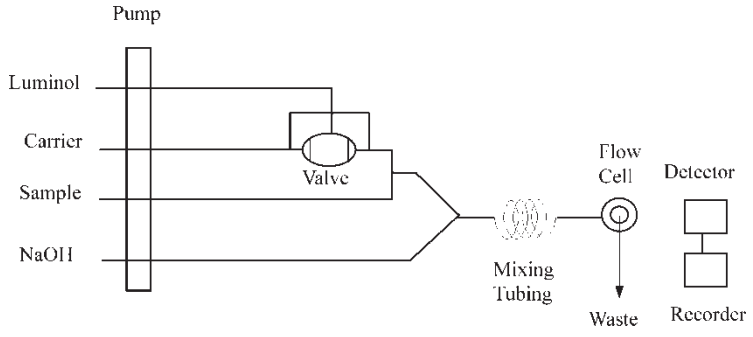


***Figure 2.*** Schematic diagram of the flow-injection system for determination of patulin.



six-way valve with a loop of 100 μL was employed for sampling. The flow cell was made by coiling 30 cm of colorless glass tube (i.d. 1.0 mm) into a spiral disk shape with a diameter of 2.0 cm and placed close to the photomultiplier tube (PMT) (model IP28, Hamamatsu, Japan). The CL signal produced in the flow cell was detected without wavelength discrimination, and the PMT output was amplified and quantified by a luminosity meter (model GD-1, Xi'an Remax Electronic Science-Tech. Co. Ltd., Xi'an, China) connected to a recorder (model XWT-206, Shanghai Dahua Instrument and Meter Plant, Shanghai, China).

## Reagents

All reagents were of analytical grade, and the water used was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Luminol (Fluka, Biochemika, Buchs, Switzerland) was obtained from Xi'an Medicine Purchasing and Supply Station (China). Standard solution of patulin (3.285 mg $L^{-1}$) was supplied by the Shaanxi Entry-Exit Inspection and Quarantine Bureau (Xi'an, China) and stored below 4°C. Working-strength solutions were prepared daily from the above stock solution as required by appropriate dilution. Luminol ($2.5 \times 10^{-2}$ mol $L^{-1}$) was prepared by dissolving 4.4 g luminol in 1 L of 0.1 mol $L^{-1}$ NaOH solution.

## Procedures

As shown in Fig. 2, flow lines were inserted into the sample, luminol, water carrier, and sodium hydroxide solutions, respectively. The pump was started at a constant speed of 2.0 mL $min^{-1}$ to wash the whole system until a stable baseline was recorded. Then 100 μL luminol solution was injected into the water carrier stream by injection valve and merged with the

solution stream of patulin. The mixed solution in an alkaline medium was delivered into the CL cell, producing CL emission, detected by the PMT and luminometer. The concentration of the sample was quantified by the decrement of CL intensity ($\Delta I_{CL} = I_o - I_s$), where $I_s$ and $I_o$ were CL signals in the presence and in the absence of patulin, respectively.

## Sample Preparation

### Treatment of Apple Juice Sample

According to the procedure described by Gökmen and Acar,[15] a 5-mL volume of apple juice was extracted twice with 10 mL of ethyl acetate by shaking for 1 min. The organic phases were combined and extracted with 2 mL of 1.5% sodium carbonate solution by shaking for 1 min. The aqueous phase was immediately extracted with 5 mL of ethyl acetate by shaking for 1 min. The combined organic phases at a total volume of 25 mL were dried with 2.5 g anhydrous sodium sulfate. Subsequently, the dried extract was filtered to remove the remaining particles of anhydrous sodium sulfate. A 2-mL excess of ethyl acetate was added to wash the filter cake layer, and the filtrate obtained was combined with the filtered extract. Then the extract was evaporated just to dryness in a water bath at 40°C under a gentle stream of nitrogen. The residue was dissolved with 1 mL acidified water (pH = 4 by addition of acetic acid). This prepared extract was kept frozen and ready for sample determination.

### Treatment of Human Urine and Serum Sample

The urine sample collected from a volunteer and the serum sample supplied by the Hospital of Northwest University were spiked before determination. To prepare the spiked samples, known quantities of standard solution of patulin were spiked into 1.0 mL of urine or serum. After homogenization, 0.1 mL of the spiked sample was diluted to 50 mL. The samples were determined by the proposed method directly after the dilution with factors of $1.0 \times 10^2$ for urine samples and $1.0 \times 10^3$ for serum samples.

## RESULTS AND DISCUSSION

### CL Intensity-Time Profile

From Fig. 3, the CL intensity-time profile that was tested in the static system can be seen. The CL signal of luminol–dissolved oxygen reached a maximum in 3 s after initiating the reaction and tended to vanish in the following 14 s; the CL intensity reached a maximum in 2 s and then died within 12 s in the

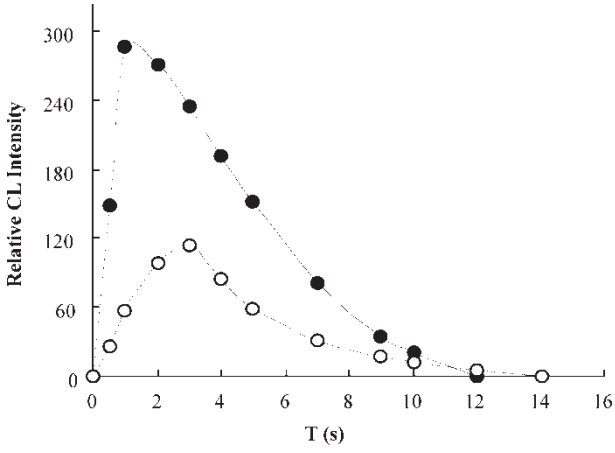


*Figure 3.* Kinetic CL intensity-time profile in static system. ●: CL intensity in the absence of patulin; ○: CL intensity in the presence of patulin (10 ng $mL^{-1}$).



presence of patulin, which demonstrated that patulin accelerates the CL reaction and inhibits CL intensity greatly.

## Effect of Flow Rate and the Length of Mixing Tube

The CL intensity was related to the flow rate. A lower flow rate caused broadening of the peak and slowed sampling rates. The ratio of signal-to-noise increased at higher flow rates. Moreover, higher flow rates could lead to an unstable baseline. A flow rate of 2.0 mL $min^{-1}$ was selected as an appropriate condition considering both the good precision and lower solution consumption. The effect of the length of mixing tube on CL intensity was also tested in pursuit of producing maximum CL intensity in the flow cell. It was observed that 5.0 cm of mixing tube afforded the best results with good sensitivity and reproducibility. Accordingly, 5.0 cm of mixing tube was considered as an optimum length.

## Effect of Luminol Concentration

The effect of luminol on the CL intensity was investigated from $1 \times 10^{-6}$ mol $L^{-1}$ to $1 \times 10^{-4}$ mol $L^{-1}$. With increasing luminol concentration, the CL signal increased steadily until luminol was $5 \times 10^{-5}$ mol $L^{-1}$, then the intensity tended to be stable. Therefore, the luminol solution of $5 \times 10^{-5}$ mol $L^{-1}$ was chosen for the optimum condition.

### Effect of Sodium Hydroxide Concentration

Owing to the nature of the luminol reaction, it is more favorable in alkaline medium. The experiments indicated there was no remarkable effect when a series of sodium hydroxide or potassium hydroxide or sodium carbonate with different concentrations (0.01, 0.025, 0.05, 0.1, and 0.5 mol $L^{-1}$, respectively) were added in the flow system to improve the sensitivity. Considering lower-cost consumption, finally sodium hydroxide was selected. It was found that the CL intensity increased when the sodium hydroxide concentration was increased up to 0.05 mol $L^{-1}$ but decreased at a higher concentration. Therefore, 0.05 mol $L^{-1}$ sodium hydroxide was used in all subsequent experiments.

### Performance of Proposed Method for Patulin Determination

Under these conditions, the linearity of the results was examined by measuring a series of standard solutions. The decreased CL intensity was found to be proportional to patulin concentration, and the response to the concentration was linear over the range from 0.04 to 10 ng $mL^{-1}$; the detection limit was 0.01 ng $mL^{-1}$ ($3\sigma_{\text{noise}}$, n = 5). The regression equation was, $\Delta I_{CL} = 3.0667\ C_{patulin} + 168.22$ ($r^2 = 0.9989$) with detection limit of 0.01 ng $mL^{-1}$ ($3\sigma_{\text{noise}}$), and the relative standard deviation was less than 4.0%. ($\Delta I_{CL} = I_o - I_s$, where $I_s$ and $I_o$ were CL signals in the presence and in the absence of patulin, respectively). At a flow rate of 2.0 mL $min^{-1}$, the determination could be performed in 0.5 min, including sampling and washing.

### Interference Studies

The influence of foreign species was examined by analyzing patulin solution (0.2 ng $mL^{-1}$) to which increasing amounts of interfering species were added. The tolerable limit of foreign substances was taken as a relative error less than 10%. The tolerated concentrations were more than 10 ng $mL^{-1}$ for $Cl^-$, $NO_3^-$, $Ac^-$, $I^-$, $SO_4^{2-}$, $PO_4^{3-}$, $Cr_2O_7^{2-}$, borate, and urea; more than 0.5 ng $mL^{-1}$ for $Cu^{2+}$, $Fe^{3+}$, uric acid, oxalic acid; 20 ng $mL^{-1}$ for malic acid; and 200 μg $mL^{-1}$ for fructose.

### Test of Precision in the CL Flow System

One hundred microliters luminol was flow-injected through the system in the presence of 80, 320, 480, and 720 pg $mL^{-1}$ patulin, and the CL intensity was recorded to test the precision of the CL flow system. The experiment lasted for 5 days, and the flow system was regularly used more than 5 h per day. The relative standard deviation (RSD) data in Table 1 shows the precision of the CL flow system.

*Table 1.* The precision of the CL flow system

| | RSD% with different patulin concentration (n = 5) | | |
|---|---|---|---|
| Day | 80 pg $mL^{-1}$ | 320 pg $mL^{-1}$ | 720 pg $mL^{-1}$ |
| 1 | 2.59 | 2.00 | 1.18 |
| 2 | 2.09 | 1.70 | 1.15 |
| 3 | 2.23 | 1.92 | 1.04 |
| 4 | 2.17 | 2.23 | 1.93 |
| 5 | 1.45 | 1.36 | 0.82 |

## Applications

### Determination of Patulin in Apple Juice

Following the procedure described, the samples of apple juice were analyzed via a standard addition method where a known quantity of patulin was added. The results are summarized in Table 2, with recovery varying from 96.9% to 103.9% and RSDs of less than 3.0%, which were in good agreement with results obtained by HPLC (Agilent 1100, $C_{18}$).[22]

### Determination of Patulin in Spiked Human Serum and Urine

Following the procedure described, the samples of human serum and urine were analyzed. The recovery studies were performed on each of the

*Table 2.* Results of determination for patulin in apple juice[a]

| | | | | | Patulin content (ng $mL^{-1}$) | |
|---|---|---|---|---|---|---|
| Sample no. | Added (pg $mL^{-1}$) | Found (pg $mL^{-1}$) | RSD (%) | Recovery (%) | By proposed method | By HPLC |
| 1 | 0 | 73 | 2.66 | 102.5 | 137.8 | 138.4 |
| | 80 | 155 | 1.06 | | | |
| 2 | 0 | 135 | 1.53 | 96.3 | 139.1 | 139.0 |
| | 80 | 212 | 1.41 | | | |
| 3 | 0 | 129 | 2.95 | 97.5 | 138.0 | 138.1 |
| | 160 | 285 | 2.78 | | | |
| 4 | 0 | 300 | 2.63 | 100.6 | 139.6 | 139.4 |
| | 160 | 461 | 1.18 | | | |
| 5 | 0 | 319 | 2.02 | 99.4 | 139.3 | 139.1 |
| | 320 | 637 | 1.13 | | | |

[a]The average of five determinations.

***Table 3.*** Results of determination for patulin in spiked human serum[a]

| Sample no. | Added (pg mL$^{-1}$) | Found (pg mL$^{-1}$) | RSD (%) | Recovery (%) | $t$ ($t_{0.05,5} = 2.57$) | Patulin content (ng mL$^{-1}$) In sample | Spiked |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 95 | 2.16 | 97.5 | 0.92 | 9.8 | 10.0 |
| | 80 | 173 | 1.14 | | | | |
| 2 | 0 | 110 | 2.76 | 106.3 | 0.74 | 11.8 | 10.0 |
| | 80 | 195 | 2.44 | | | | |
| 3 | 0 | 350 | 2.19 | 96.9 | 0.88 | 52.8 | 50.0 |
| | 320 | 660 | 2.09 | | | | |
| 4 | 0 | 316 | 2.90 | 101.9 | 0.65 | 49.1 | 50.0 |
| | 320 | 642 | 2.26 | | | | |
| 5 | 0 | 1030 | 3.35 | 98.3 | 0.60 | 102.1 | 100.0 |
| | 720 | 1738 | 2.97 | | | | |
| 6 | 0 | 1023 | 3.14 | 102.0 | 0.64 | 99.0 | 100.0 |
| | 1040 | 2084 | 3.09 | | | | |



analyzed samples by adding a known amount of patulin to the sample before the recommended treatment, and the experimental results were also verified by *t*-test. The results are shown in Table 3 with recovery from 95.5% to 107.2% for serum samples and in Table 4 with recovery from 94.9% to 106.4% for urine samples.

***Table 4.*** Results of determination for patulin in spiked human urine[a]

| Sample no | Added (pg mL$^{-1}$) | Found (pg mL$^{-1}$) | RSD (%) | Recovery (%) | $t$ ($t_{0.05,5} = 2.57$) | Patulin content (ng mL$^{-1}$) In sample | Spiked |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 91 | 2.86 | 111.3 | 0.70 | 9.7 | 10.0 |
| | 80 | 180 | 2.61 | | | | |
| 2 | 0 | 140 | 2.37 | 92.5 | 0.88 | 12.3 | 10.0 |
| | 80 | 214 | 2.19 | | | | |
| 3 | 0 | 160 | 3.06 | 106.3 | 0.50 | 50.0 | 50.0 |
| | 160 | 330 | 2.88 | | | | |
| 4 | 0 | 312 | 2.18 | 99.4 | 0.86 | 49.8 | 50.0 |
| | 320 | 630 | 1.96 | | | | |
| 5 | 0 | 470 | 2.75 | 106.9 | 0.64 | 96.9 | 100.0 |
| | 720 | 1240 | 2.62 | | | | |
| 6 | 0 | 742 | 2.14 | 99.6 | 0.90 | 102.6 | 100.0 |
| | 720 | 1459 | 1.76 | | | | |

## CONCLUSIONS

The presented CL method combined with flow injection technique offered prominent advantages including instrumental simplicity, high sampling efficiency, reducing reagent consumption, and analytical sensitivity compared with the existed methods.

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## REFERENCES

1. Fliege, R.; Metzler, M. Electrophilic properties of patulin. *N*-Acetylcysteine and glutathione adducts. *Chem. Res. Toxicol.* **2000**, *13*, 363–372.
2. Boonzaaijer, G.; Bobeldijk, I.; van Osenbruggen, W. A. Analysis of patulin in Dutch food, an evaluation of a SPE based method. *Food Control* **2005**, *16*, 587–591.
3. Leggot, N. L.; Shephard, G. S. Patulin in South African commercial apple products. *Food Control* **2001**, *12*, 73–76.
4. Selmanoglu, G.; Kockaya, E. A. Investigation of the effects of patulin on thyroid and testis, and hormone levels in growing male rats. *Food Chem. Toxicol.* **2004**, *42*, 721–727.
5. Ito, R.; Yamazaki, H.; Inoue, K. Development of liquid chromatography-electrospray mass spectrometry for the determination of patulin in apple juice: investigation of its contamination levels in Japan. *J. Agric. Food Chem.* **2004**, *52*, 7464–7468.
6. Wu, T. S.; Yu, F. Y.; Su, C. C. Activation of ERK mitogen-activated protein kinase in human cells by the mycotoxin patulin. *Toxicol. Appl. Pharmacol.* **2005**, *207*, 103–111.
7. Kadakal, C.; Nas, S.; Ekinci, R. Ergosterol as a new quality parameter together with patulin in raw apple juice produced from decayed apples. *Food Chem.* **2005**, *90*, 95–100.
8. Ritieni, A. Patulin in Italian commercial apple products. *J. Agric. Food Chem.* **2003**, *51*, 6086–6090.
9. Russell, R.; Paterson, M. Primers from the isoepoxydon dehydrogenase gene of the patulin biosynthetic pathway to indicate critical control points. *Food Control* **2006**, *17*, 741–744.
10. Sheu, F.; Shyu, Y. T. Analysis of patulin in apple juice by diphasic dialysis extraction with in situ acylation and mass spectrometric determination. *J. Agric. Food Chem.* **1999**, *47*, 2711–2714.
11. Llovera, M.; Balcells, M.; Torres, M. Parallel synthesis: a new approach for developing analytical internal standards. Application to the analysis of patulin by gas chromatography-mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 6643–6648.

12. Rupp, H. S.; Turnipseed, S. B. Confirmation of patulin and 5-hydroxymethylfurfural in apple juice by gas chromatography/mass spectrometry. *J. AOAC Int.* **2000**, *83*, 612–620.
13. Melchert, H. U.; Pabel, E. Reliable identification and quantification of trichothecenes and other mycotoxins by electron impact and chemical ionization-gas chromatography–mass spectrometry, using an ion-trap system in the multiple mass spectrometry mode candidate reference method for complex matrices. *J. Chromatogr. A* **2004**, *1056*, 195–199.
14. Gökmen, V.; Acar, J.; Sarioğlu, K. Liquid chromatographic method for the determination of patulin in apple juice using solid-phase extraction. *Anal. Chim. Acta* **2005**, *543*, 64–69.
15. Gökmen, V.; Acar, J. Simultaneous determination of 5-hydroxymethylfurfural and patulin in apple juice by reversed-phase liquid chromatography. *J. Chromatogr. A* **1999**, *84*, 769–774.
16. Llovera, M.; Viladrich, R.; Torres, M.; Canela, R. Analysis of underivatizated patulin by a GC/MS technique. *J. Food Protection* **1999**, *62*, 202–205.
17. Rychlik, M.; Schieberle, P. Quantification of the mycotoxin patulin by a stable isotope dilution assay. *J. Agric. Food Chem.* **1999**, *47*, 3749–3755.
18. Tsao, R.; Zhou, T. Micellar electrokinetic capillary electrophoresis for rapid analysis of patulin in apple cider. *J. Agric. Food Chem.* **2000**, *48*, 5231–5235.
19. Powe, A. M. Molecular fluorescence phosphorescence, and chemiluminescence spectrometry. *Anal. Chem.* **2004**, *76*, 4614–4634.
20. Fletcher, K. A. Molecular fluorescence, phosphorescence, and chemiluminescence spectrometry. *Anal. Chem.* **2006**, *78*, 4047–4068.
21. Song, Z. H.; Hou, S. Sub-picogram determination of vitamin $B_{12}$ in pharmaceuticals and human serum using flow injection with chemiluminescence detection. *Anal. Chim. Acta* **2003**, *488*, 71–79.
22. Zhang, Y. B.; Li, Y. B.; Sun, L. P.; Zhang, P. Method for the Determination of Patulin in Beverage for Export, by People's Republic of China Import and Export Commodity Inspection Bureau. SN 0598, 1996.