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11 Monolithic Column Chromatography Coupled With Chemiluminescence Detection

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11.1 Introduction

Chemiluminescence is the emission of light from an electronically excited intermediate in a chemical reaction [1, 2]. As the light can be measured against a dark background, and the number of emitted photons is dependent on the concentration of reacting species, this phenomenon can be exploited for highly sensitive chemical detection. However, the production of the excited intermediate depends on the physical processes of solution mixing and the kinetics of a chemical reaction, which complicates the sample and reagent handling required for accurate detection.

With inherent temporal and spatial control of solutions, flow-based analytical techniques, such as FIA and HPLC, provide an ideal means to merge samples (or separated sample components) reproducibly with chemiluminescence reagents and present the reacting mixture to a light detector. A typical manifold for HPLC with chemiluminescence detection is shown in Figure 11.1a. Sample components eluted from the column are merged with a reagent and propelled through a transparent spiral-shaped flow cell (Figure 11.1b) mounted against a photomultiplier tube within a light-tight box. Although this flow manifold is more complex than that required for detection based on the ultraviolet (UV) or visible absorbance or on the native fluorescence of the analytes, the detector components are simpler, because an excitation light source is not required.

Monolithic Chromatography and its Modern Applications, edited by Perry G. Wang. © 2010 ILM Publications, a trading division of International Labmate Limited.

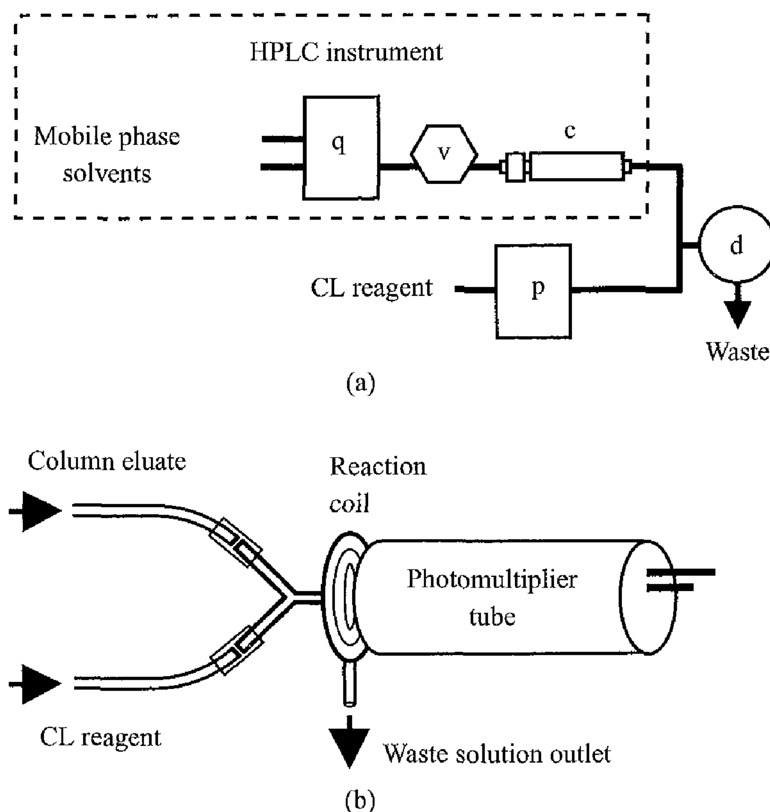

Figure 11.1 Examples of (a) a manifold for HPLC with chemiluminescence detection; (b) a chemiluminescence flow cell (q = quaternary pump; v = injection valve; c = column; p = peristaltic pump; d = detector; CL = chemiluminescence).

11.2 Commonly Used Chemiluminescence Reagents

Although many analytical applications that involve flow analysis with chemiluminescence detection have been reported, the majority are based on a small number of commonly used reagents, such as luminol (5-amino-2,3-dihydrophthalazine-1,4-dione), diaryl oxalates and oxamides, tris(2,2′-bipyridine)ruthenium(III), and potassium permanganate [3].

Under suitable conditions, the oxidation of luminol evokes a brilliant blue luminescence with maximum intensity at 420–425 nm [3–5]. Although some discrepancies regarding the light-producing pathway persist in the scientific literature, it is generally accepted that the emission emanates from an electronically excited 3-aminophthalate dianion (Figure 11.2) [3].

The most common oxidant used to initiate this light-producing reaction is hydrogen peroxide, in conjunction with a range of co-reactants or catalysts such as peroxidase enzymes and certain transition metal ions and complexes [3, 4]. This class of chemiluminescence reaction has been used in a wide range of applications, such as to visualise blood at crime scenes [5], in immunoassays that incorporate peroxidase labels and to determine biomolecules using FIA or HPLC with either immobilised peroxide-producing enzymes or luminol-based labelling reagents [4]. A range of organic molecules have been found to enhance or inhibit the chemiluminescence from

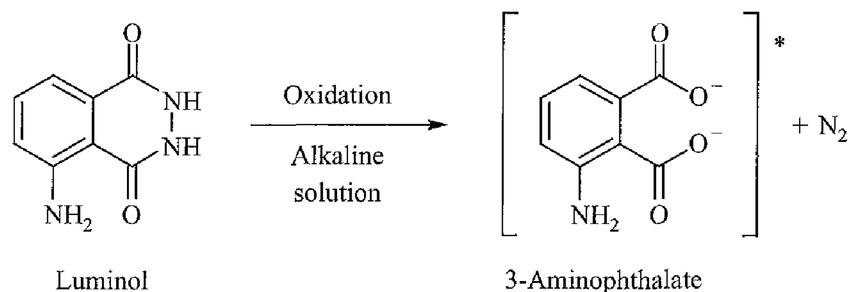

Figure 11.2 The chemiluminescent oxidation of luminol.

various luminol systems, which has also been utilised for sensitive chemical measurement [4].

The light-producing reactions of substituted diaryloxalates or diaryloxamides with hydrogen peroxide are collectively known as 'peroxyoxalate chemiluminescence' (Figure 11.3) [6, 7]. The high-energy intermediates formed in these reactions are capable of exciting a variety of fluorescent compounds through what is believed to be an electron-exchange process [6], which leads to an emission that matches the characteristic photoluminescence of the added fluorophores. This class of reaction has been used successfully with FIA and HPLC methodology to determine hydrogen peroxide, substrates of enzymes that generate hydrogen peroxide, compounds that exhibit native fluorescence and compounds that have been derivatised with efficient fluorophores.

Tris(2,2′-bipyridine)ruthenium(III) (Figure 11.4) is an effective chemiluminescence reagent for the detection of certain amines (particularly aliphatic tertiary amines), proline, nicotinamide adenine dinucleotide, organic acids, alkaloids and

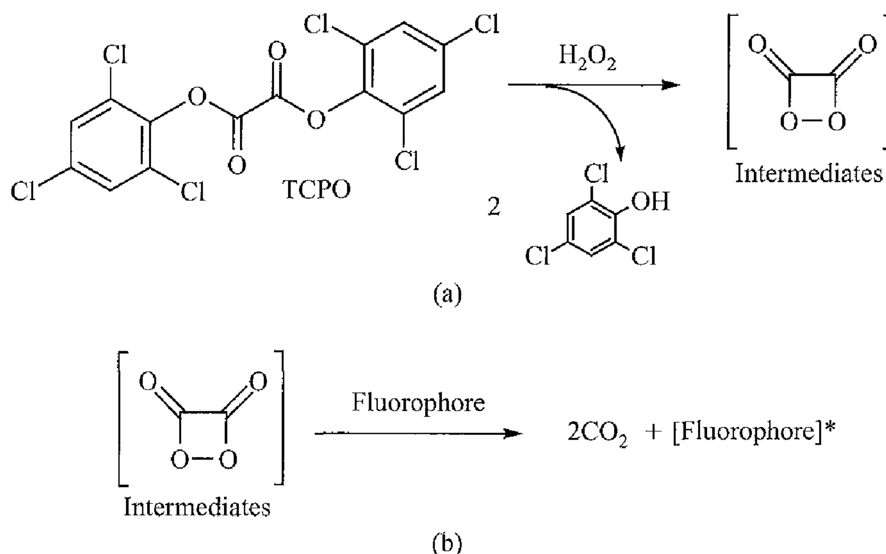

Figure 11.3 (a) The reaction of bis(2,4,6-trichlorophenyl)oxalate (TCPO, a popular peroxyoxalate chemiluminescence reagent) with hydrogen peroxide to produce reactive intermediates; (b) decomposition of the intermediates and energy transfer to an efficient fluorophore from which the emission of light occurs.

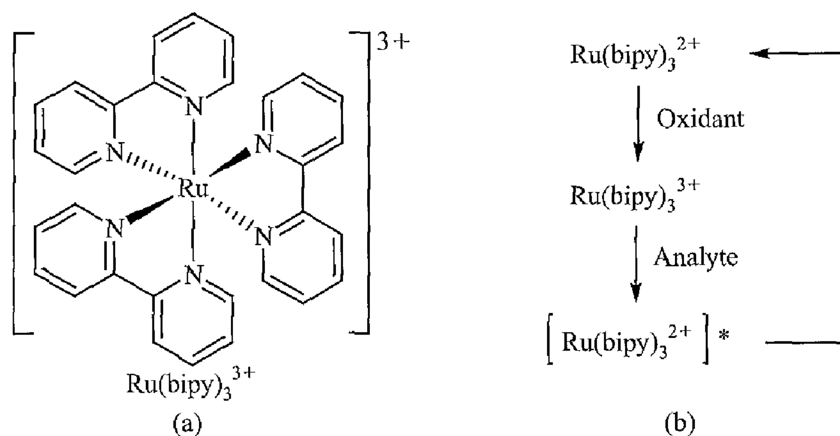

Figure 11.4 (a) Tris(2,2′-bipyridine)ruthenium(III); (b) its preparation and subsequent reaction to form the corresponding ruthenium(II) species in an electronically excited state.

pharmaceuticals [8, 9]. The reagent is only moderately stable in acidic aqueous solutions and therefore is normally generated from the corresponding ruthenium(II) complex by oxidation: chemical [often using lead dioxide or cerium(IV)], electrochemical (at an electrode surface), or photochemical (using potassium persulphate). Subsequent reaction with a suitable analyte produces the characteristic orange luminescence ($\lambda_{max} = 610\text{–}620$ nm) from an excited metal-to-ligand charge-transfer (MLCT) triplet state (^{3}MLCT) of tris(2,2′-bipyridine)ruthenium(II). The active ruthenium(III) complex can be regenerated and there has been extensive investigation into stable immobilised forms of this reagent.

The reaction of potassium permanganate with a wide variety of organic compounds (particularly phenols, polyphenols, catechols, indoles, antioxidants and selected alkaloids and pharmaceuticals) and a few inorganic substances in acidic solution produces an emission with maximum intensity at 734 ± 5 nm, which emanates from an excited manganese(II) species formed in the reaction [10, 11]. The addition of either formaldehyde or sodium hexametaphosphate to the reaction mixture has been used to increase the emission intensity and improve the limits of detection in flow-analysis applications. The presence of hexametaphosphate also shifts the wavelength of maximum intensity to 689 ± 5 nm.

Although new flow-analysis applications that involve these well-established reagents continue to emerge, there has been considerable growth in the chemiluminescence detection of organic compounds with oxidants such as cerium(IV), hexacyanoferrate(III), hypohalites and periodate. The emission from these reactions tends to be fairly weak, but can often be enhanced significantly by adding sensitisers such as rhodamine B, fluorescein, quinine, or lanthanide ions.

11.3 Chemiluminescence and Selectivity

Chemiluminescence is generally more selective than other spectroscopic modes of detection because of the limited number of compounds that produce a significant emission of light with any particular reagent. A simple example of this inherent

selectivity that involves the determination of morphine in a commercially available pharmaceutical preparation is shown in Figure 11.5.

Sample analysis using RP-HPLC and UV-absorbance detection produced a chromatogram with four peaks (Figure 11.5a). In contrast, only the morphine peak was observed when the same chromatographic separation was coupled with chemiluminescence detection that consisted of a post-column reaction with acidic potassium permanganate (Figure 11.5b). The same method of detection was then performed using FIA, without physical separation of the sample components, which reduced the analysis time for each sample from minutes to seconds. As shown in Table 11.1, the determination of morphine in the commercial formulation using FIA with chemiluminescence detection was in good agreement with the HPLC procedures.

Many papers on the determination of pharmaceuticals in commercial formulations using FIA with chemiluminescence detection have emerged [12]. In several cases it has been stated that these FIA procedures are sufficiently selective for the analysis

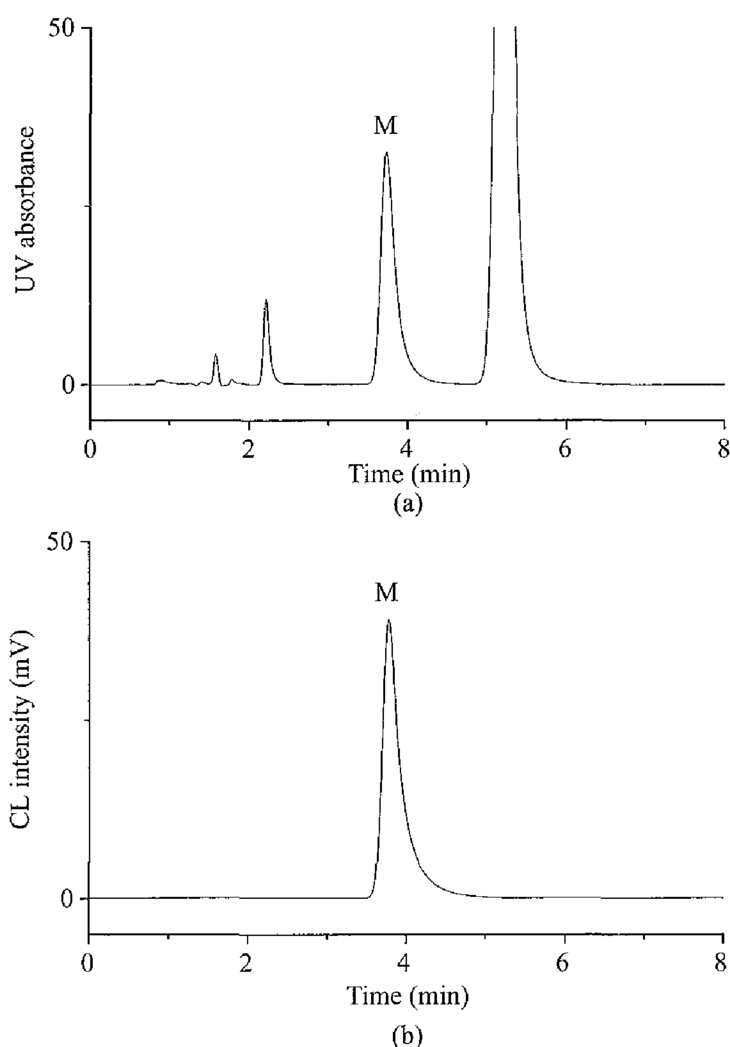

Figure 11.5 Chromatograms from the analysis of morphine in a pharmaceutical preparation using HPLC with (a) UV absorbance; (b) chemiluminescence detection, with a monolithic column (Chromolith Flash) and acidic potassium permanganate as the chemiluminescence reagent.

Table 11.1 The determination of morphine in a pharmaceutical preparation using HPLC and FIA with UV-absorbance and chemiluminescence detection.

Instrument	Detection	Concentration (mg/mL)
HPLC	UV absorbance	1.92
HPLC	Chemiluminescence	1.91
FIA	Chemiluminescence	1.93

of more complex samples, such as biological fluids. These claims are often supported by an examination of the interference from selected individual compounds and percentage recoveries using spiked samples, but not by a direct comparison of the absolute values with established procedures.

Analysis of a typical urine sample using HPLC with most commonly used chemiluminescence reagents reveals that many biomolecules present in the matrix produce light (see Figure 11.6 for an example) [13]. When attempting to use a simple FIA manifold to determine a pharmaceutical in urine without any sample pre-treatment, the 'blank' response for urine (that does not contain the target analyte) is significantly higher than the response for deionised water [14].

Some compounds produce a relatively intense emission with a particular reagent, and it could be argued that the light from the biomolecules present in the matrix would be insignificant if the analyte concentration is relatively high. Nevertheless, FIA

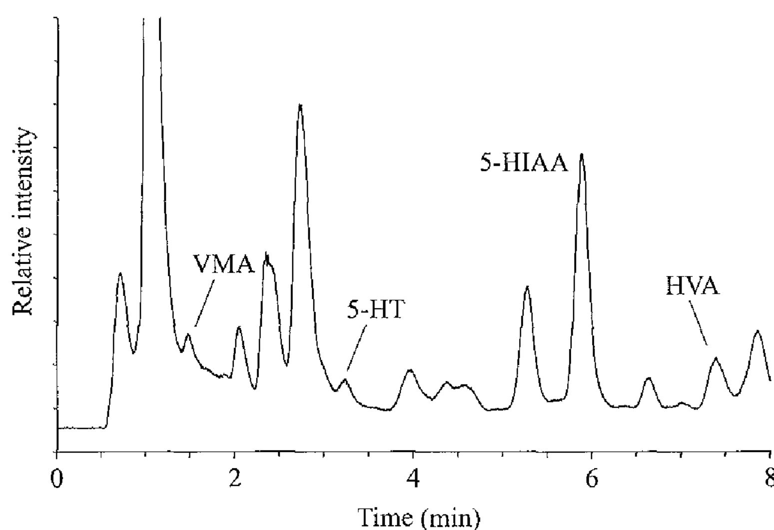

Figure 11.6 Chromatogram from the analysis of a urine sample (diluted ten-fold with water) using LC with acidic potassium permanganate chemiluminescence detection (VMA = vanilmandelic acid; 5-HT = serotonin; 5-HIAA = 5-hydroxyindole acetic acid; HVA = homovanillic acid).

(Reprinted from *Anal. Chim. Acta*, 600, Adcock, J.L., Francis, P.S., Agg, K.M., Marshall, G.D., Barnett, N.W., A hybrid FIA/HPLC system incorporating monolithic column chromatography, 136, Copyright (2007), with permission from Elsevier.)

methodology with selectivity based solely on the chemiluminescence reagent does not discriminate between the target analyte and other pharmaceuticals that may produce light with the same reagent and that may have also been ingested by the patient. Furthermore, metabolism of the target analyte may produce compounds that elicit a less or more intense chemiluminescence signal.

However, FIA methodology can be used to couple chemiluminescence detection to additional operations that increase the overall selectivity of the analytical procedure. Examples include enzymatic reactions [4], immunoassay [15], dialysis, gas-diffusion, adsorption [16], or molecular recognition [14].

Alternatively, multiple analytes can be determined using chemiluminescence detection after chromatographic or electrophoretic separation [17, 18]. Traditional HPLC and CE approaches generally require far greater analysis time and result in poorer limits of detection than does FIA because of the lower volume of analyte that merges with the chemiluminescence reagent and (in the case of fast chemiluminescence reactions) the detrimental effect of lower flow rates. However, the introduction of monolithic columns for HPLC has allowed chromatographic separations to be performed at relatively high flow rates, without a significant decrease in efficiency [19–22], which has provided the opportunity to combine rapid separations with chemiluminescence detection for highly sensitive and selective analyses. Fundamental aspects, advantages and limitations of monolithic columns are described in detail elsewhere in this book, and therefore the following discussion focuses solely on applications that involve chemiluminescence detection.

11.4 Monolithic Column Chromatography With Chemiluminescence Detection

Beginning in 2001, Saito and co-workers published a series of papers on HPLC with Chromolith C_{18} RP silica monolithic columns purchased from Merck, and chemiluminescence detection [23–29]. Each procedure involved an isocratic mobile phase, a flow rate of between 0.5 and 0.8 mL/min, and detection based on post-column reaction with acidic tris(2,2′-bipyridine)ruthenium(III) using a commercially available detector (Comet 3000; Comet, Japan). The reagent was prepared on-line by photochemical [23] or electrochemical [24–29] oxidation (Figure 11.4b). Analytes included *t*-butyl hydroperoxide [23], tetramethylthiuram disulphide [24], secondary and tertiary amines [25, 26, 29], aliphatic conjugated dienes [25, 27] and catechins [28] (Figure 11.7).

To determine domoic acid (Figure 11.7a), a neurotoxic amino acid associated with amnesic shellfish poisoning, Saito and co-workers described a procedure that involved the injection of 20 μL of sample onto a Chromolith Performance column [100 mm length × 4.6 mm internal diameter (i.d.)] and separation using an isocratic mobile phase [10% acetonitrile:90% phosphate buffer (5 mM) adjusted to pH 2.7] and a flow rate of 0.5 mL/min [25]. The optimum pH to detect this analyte was found to be 6.5, and therefore the column eluate was merged with a suitable buffer solution prior to combination with the tris(2,2′-bipyridine)ruthenium(III) reagent within the chemi-

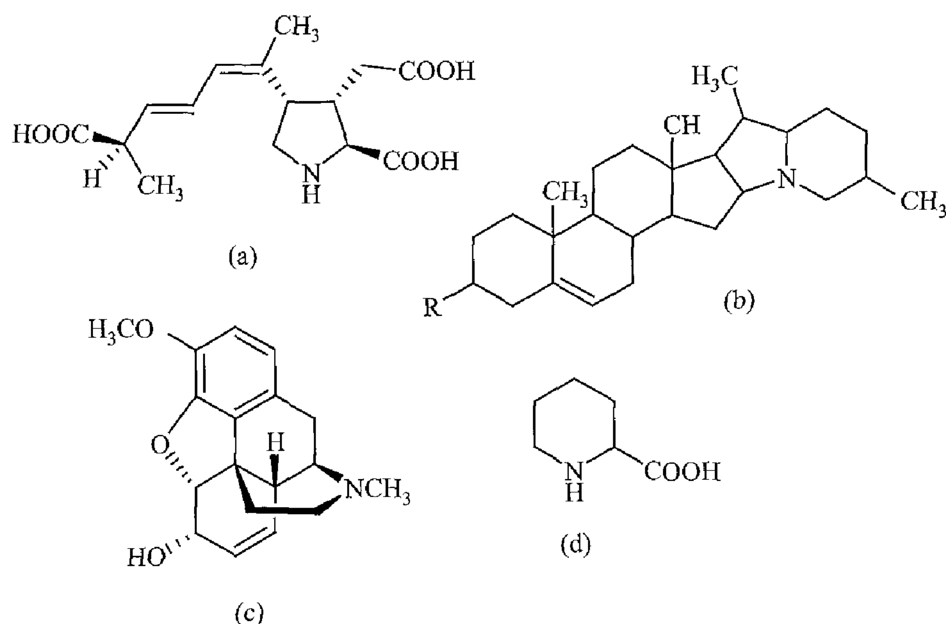

Figure 11.7 Selected compounds that have been detected with tris(2,2′-bipyridine)ruthenium(III) chemiluminescence: (a) domoic acid; (b) α-chaconine [R = β-D-glucose linked to two α-L-rhamnose]; (c) codeine; (d) pipecolic acid.

luminescence detector. Under these conditions, the retention time of the target analyte was 18 minutes and the limit of detection was 8 pg (1×10^{-9} M). The authors analysed a blue-mussel sample spiked with 2 μg/g domoic acid; a $106.2 \pm 2.1\%$ recovery was reported.

Similar procedures were developed to determine two glycoalkaloids, α-solanine and α-chaconine (Figure 11.7b), in potato tubers, which are considered unsafe for human consumption when they exceed concentrations of 20 mg per 100 g of tuber [26], and to determine pipecolic acid (Figure 11.7d), a biomedical marker for human peroxisomal disorders [29]. Typical chromatograms for pipecolic acid in serum, apple juice and milk are shown in Figure 11.8. Although these procedures clearly demonstrate the use of chemiluminescence for the sensitive detection of compounds that do not possess a suitable chromophore or fluorophore, they do not exploit the full potential of monolithic columns for rapid separation.

Saito and co-workers also explored a method to detect aromatic compounds that involves on-line photochemical degradation of the analytes before chemiluminescence reaction with tris(2,2′-bipyridine)ruthenium(III) [28]. The photochemical reactor consisted of a knitted polytetrafluoroethene (PTFE) tube (20 m length × 0.5 mm i.d.) rolled around a low-pressure mercury lamp, covered with aluminium foil and placed inside a plastic black box. The degradation products were found to include oxalic acid [28], which is known to produce light with this reagent [8]. This approach was applied to the determination of catechins [(−)-epicatechin and (−)-epigallocatechin gallate] in tea samples (Figure 11.9).

We have also reported several analytical procedures based on monolithic column chromatography with chemiluminescence detection [30–34]. These procedures were developed with a shorter monolithic column (Chromolith SpeedROD; 50 mm ×

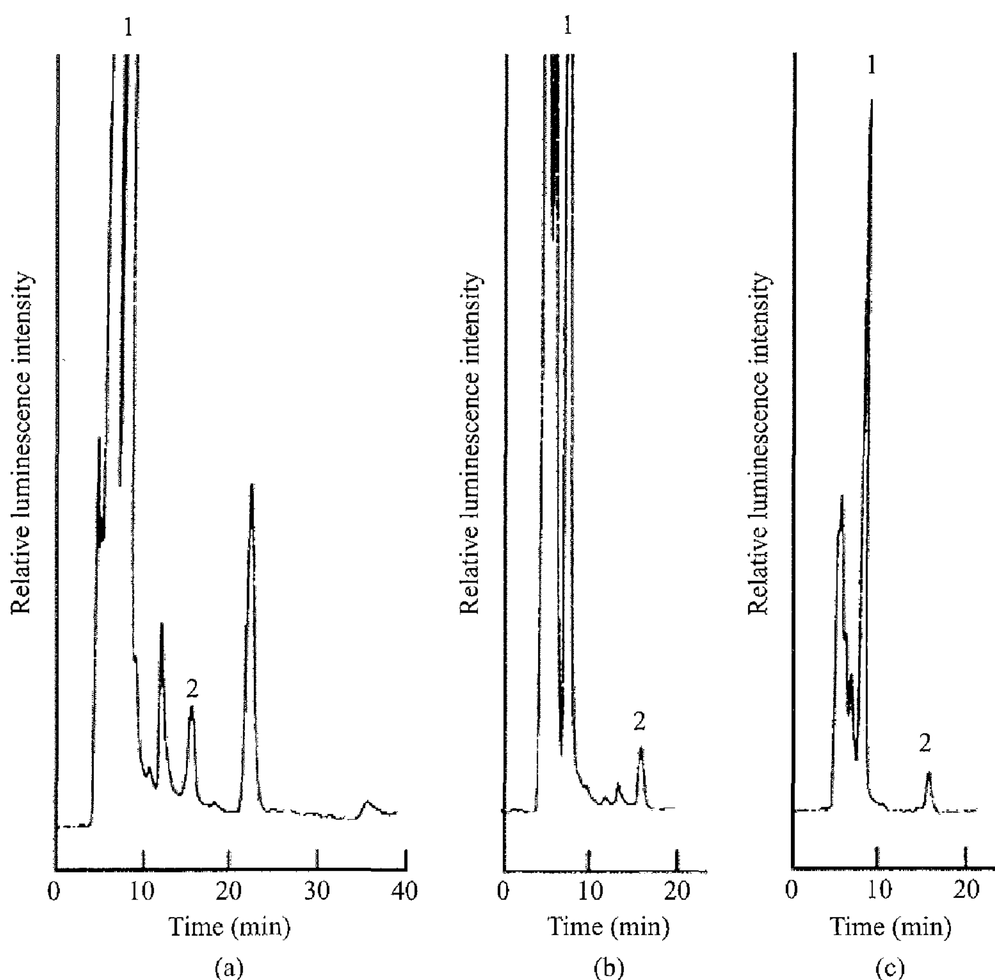

Figure 11.8 Chromatograms from the analysis of pipecolic acid in (a) human serum, (b) apple juice and (c) cow's milk (after ten-, 100- and 100-fold dilution, respectively). Peaks: 1 = proline, 2 = pipecolic acid.
(Reprinted from *J. Chromatogr. A*, 1140, Kodamatani, H., Komatsu, Y., Yamazaki, S., Saito, K., Highly sensitive and simple method for measurement of pipecolic acid using reverse-phase ion-pair high performance liquid chromatography with tris(2,2'-bipyridine)ruthenium(III) chemiluminescence detection, 88, Copyright (2007), with permission from Elsevier.)

4.6 mm i.d.), and in most cases we used gradient elution and relatively high flow rates. A custom-built flow-through luminometer that comprised a T-piece and coil of PTFE tubing mounted against a photomultiplier tube within light-tight housing was constructed and several different chemiluminescence reagents were employed.

Alkaline hypobromite chemiluminescence was used to detect the presence of arginine in oligopeptides [30], as an alternative to more conventional spectrophotometric approaches [35, 36]. A mixture of five peptides was separated within three minutes using a solvent gradient of 5% to 35% acetonitrile in an aqueous solution, which contained 0.1% v/v trifluoroacetic acid, and a flow rate of 3 mL/min, similar to the optimum flow rate for the chemiluminescence reaction with hypobromite that had previously been established using FIA [37].

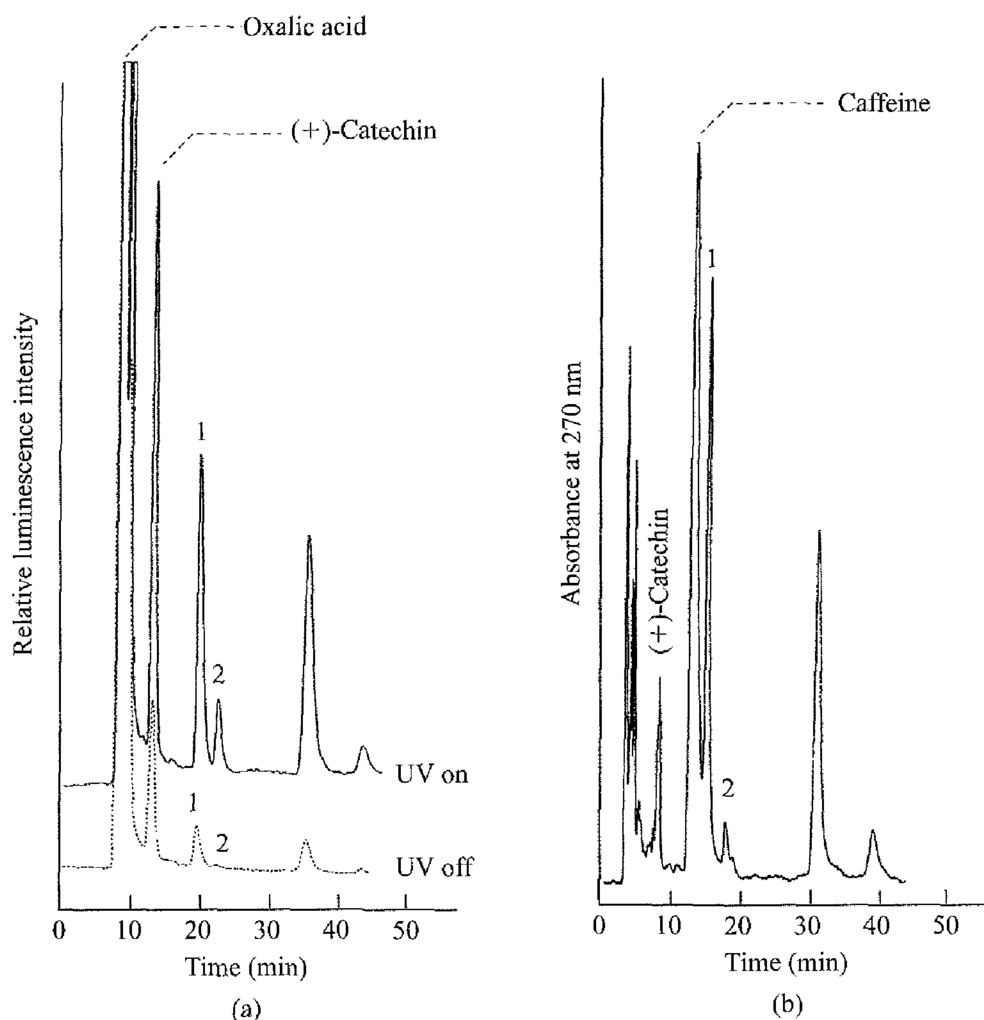

Figure 11.9 Chromatograms from the analysis of catechins in tea using monolithic column LC with (a) chemiluminescence (with and without photochemical decomposition of the sample); (b) UV-absorbance (270 nm) detection. Peaks: 1 = (−)-epigallocatechin gallate, 2 = (−)-epicatechin.

(Reprinted from *J. Chromatogr. A*, 1102, Kodamatani, H., Shimizu, H., Saito, K., Yamazaki, S., Tanaka, Y., High performance liquid chromatography of aromatic compounds with photochemical decomposition and tris(2,2'-bipyridine)ruthenium(III) chemiluminescence detection, 200, Copyright (2006), with permission from Elsevier.)

To determine urinary neurotransmitter metabolites (such as homovanillic acid and 5-hydroxyindole-3-acetic acid; Figures 11.10a and 11.10b), 20 μL of sample was injected onto a Chromolith SpeedROD column and separated using gradient elution (with methanol as the organic modifier) and a flow rate of 3 mL/min [31]. The target analytes were detected using acidic potassium permanganate chemiluminescence. Although not all of the neurotransmitter metabolites were completely resolved (Figure 11.11), this approach was performed with minimal sample preparation and provides a convenient option for screening urine samples for elevated levels of these biomolecules, which is important in the diagnosis of several disorders. Greater resolution could potentially be attained through further method development, particularly the use

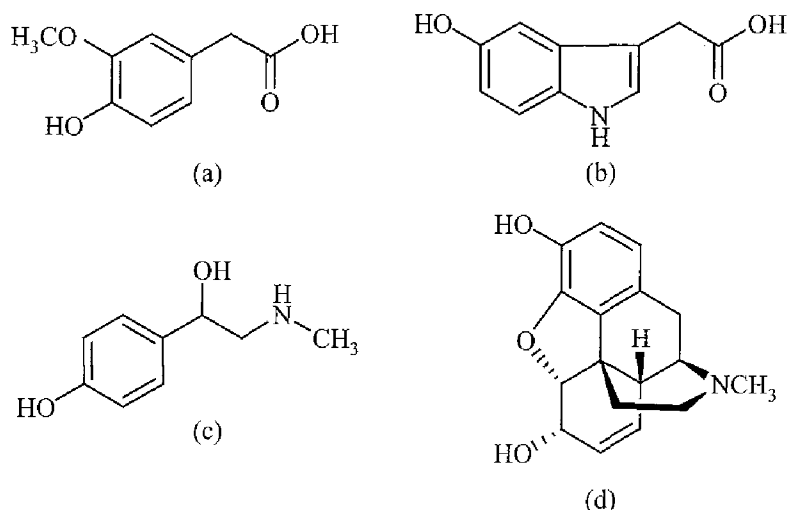

Figure 11.10 Selected compounds that have been detected with permanganate chemiluminescence: (a) homovanillic acid; (b) 5-hydroxyindole-3-acetic acid; (c) synephrine; (d) morphine.

of longer monolithic columns. Increases in analysis time could be minimised by the relatively high flow rates that can be passed through the monolithic material. Further experimentation is required to realise the true potential of this approach for diagnostic screening applications. The permanganate chemiluminescence reagent has also been applied to determine synephrine (an adrenergic amine; Figure 11.10c) in weight-loss products [34].

Monolithic column HPLC with chemiluminescence detection has been used to monitor the concentration of opiate alkaloids at various stages of the industrial extraction process [32]. Acidic potassium permanganate is a very effective chemiluminescence reagent for the detection of certain phenolic opiate alkaloids, such as morphine (Figure 11.10d) and oripavine, but produces a relatively weak emission with closely related non-phenolic alkaloids. Tris(2,2′-bipyridine)ruthenium(III) exhibits a somewhat complementary selectivity and produces a relatively intense emission with certain non-phenolic opiate alkaloids, such as codeine (Figure 11.7c) and thebaine.

Using a Chromolith SpeedROD column, gradient elution (with methanol as the organic modifier), a flow rate of 3 mL/min and permanganate chemiluminescence detection, both morphine and oripavine were determined in less than two minutes (Figure 11.12a). Codeine and thebaine were quantified using a similar procedure, except with acetonitrile as the organic modifier and tris(2,2′-bipyridine)ruthenium(III) chemiluminescence (Figure 11.12b). These procedures provided limits of detection for the four opiate alkaloids that were similar to the best values obtained with FIA and sequential injection analysis (SIA) methodology (10^{-10} to 10^{-9} M). Samples were taken from various points in the process line and diluted between 250- and 125,000-fold to adjust their alkaloid concentrations into the linear calibration ranges. The results were in good agreement with those obtained using a more conventional procedure based on ion-pairing HPLC with UV-absorbance detection (with 25–30 minutes of separation time) [32].

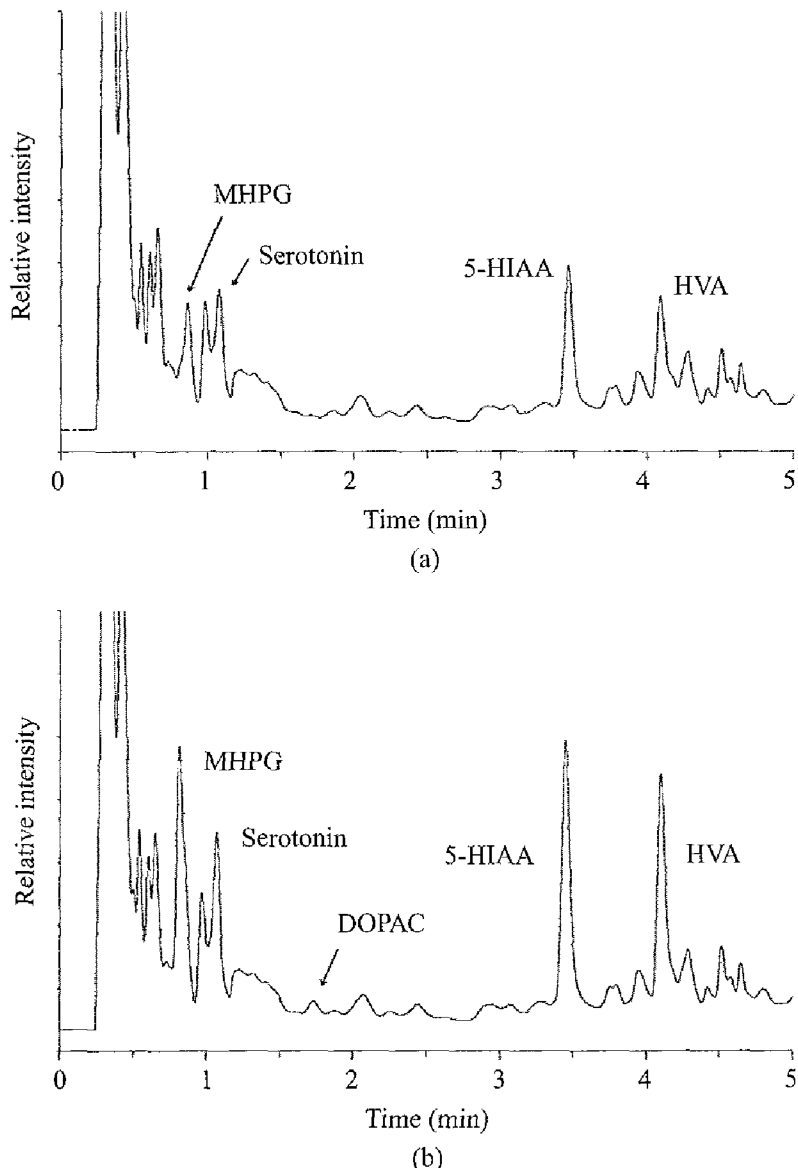

Figure 11.11 Chromatograms from the analysis of (a) urine; (b) spiked urine, using acidic potassium permanganate chemiluminescence detection. Peaks: MHPG = 4-hydroxy-3-methoxyphenylglycol; 5-HIAA = 5-hydroxyindole-3-acetic acid; HVA = homovanillic acid; DOPAC = 3,4-dihydroxyphenylacetic acid.
(Reprinted from *Talanta*, 67, Adcock, J.L., Barnett, N.W., Costin, J.W., Francis, P.S., Lewis, S.W., Determination of selected neurotransmitter metabolites using monolithic column chromatography coupled with chemiluminescence detection, 585, Copyright (2005), with permission from Elsevier.)

A wider range of opiate alkaloids were detected using a chemiluminescence reagent system that consisted of an acidic solution of manganese(IV) and formaldehyde, although the limits of detection ($\sim 5 \times 10^{-7}$ M) were less impressive [33]. In the first demonstration of soluble manganese(IV) as a reagent for post-column chemiluminescence detection, six opiate alkaloids were separated within four minutes using a monolithic column, gradient elution (using methanol as the organic modifier) and a flow rate of 2 mL/min.

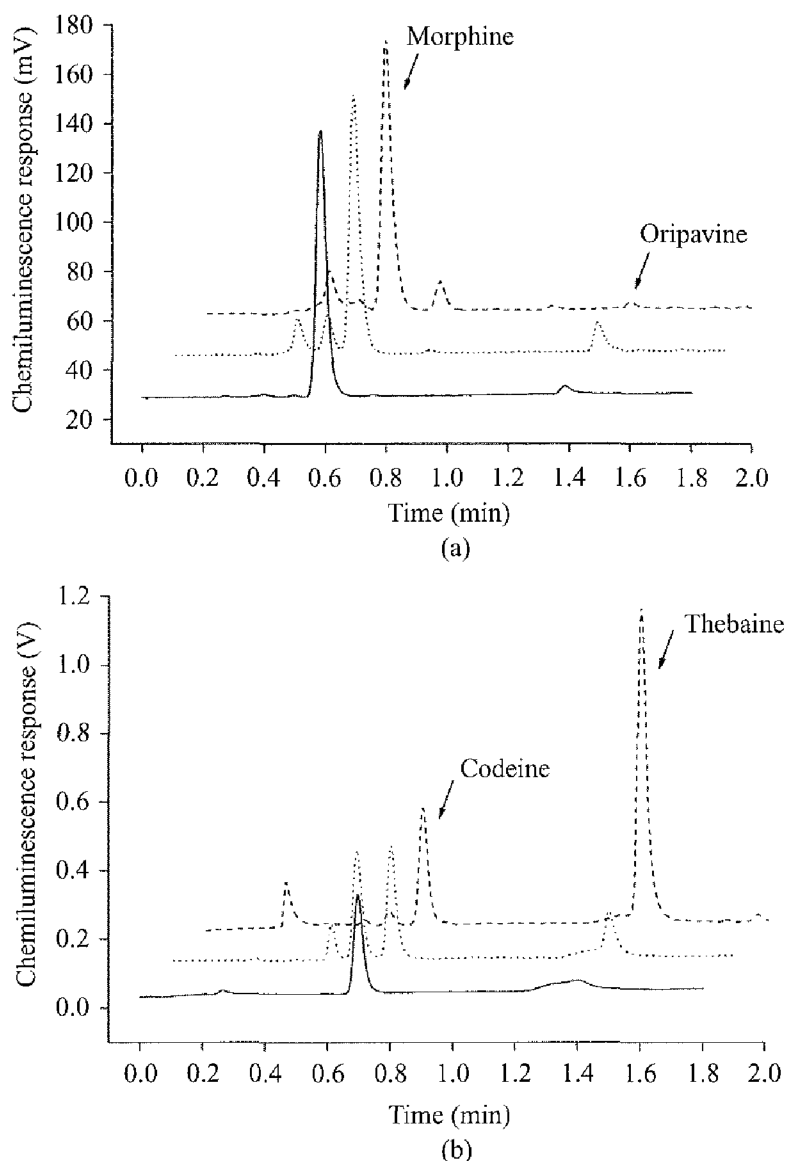

Figure 11.12 Chromatograms from the analysis of opiate alkaloids in various process liquors using (a) acidic potassium permanganate; or (b) tris(2,2′-bipyridine)ruthenium(III) chemiluminescence detection.

(Reprinted from *Anal. Chim. Acta*, 597, Costin, J.W., Lewis, S.W., Purcell, S.D., Waddell, L.R., Francis, P.S., Barnett, N.W., Rapid determination of *Papaver somniferum* alkaloids in process streams using monolithic column high-performance liquid chromatography with chemiluminescence detection, 19, Copyright (2007), with permission from Elsevier.)

A survey of the HPLC procedures that involved Chromolith monolithic columns and chemiluminescence detection, published by Saito and co-workers and the authors' research group, is shown in Table 11.2.

As previously discussed, FIA procedures with chemiluminescence detection often require other operations (such as dialysis, gas diffusion, IE or absorption) to provide sufficient selectivity to analyse complex samples such as biological fluids. For example, Tyrrell *et al.* incorporated a commercially available chelating monolithic disc (modified with dipicolinic acid) for the selective extraction of copper(II) from

Table 11.2 Selected analytical applications of monolithic column chromatography with chemiluminescence detection.

Chemiluminescence reagent	Analytes	Elution time (min)	Detection limit (M)	Matrix	Separation conditions	Ref.
Electrogenerated $Ru(bipy)_3^{3+}$ in 1 mM H_2SO_4	Domoic acid	18	1×10^{-9}	Shellfish tissue extract	Chromolith Performance. Isocratic (eluent: 5 mM phosphate buffer (pH 2.7)/acetonitrile 9:1 v/v). Flow rate of 0.5 mL/min.	25
Electrogenerated $Ru(bipy)_3^{3+}$ in 10 mM H_2SO_4	2,4-hexadien-1-ol 2,4-hexadiene 2,5-dimethyl-2,4-hexadiene α-terpinene α-phellandrene Abietic acid	3 6 10 19 20 –	– 5×10^{-8} 3×10^{-8} 1×10^{-7} 5×10^{-8} 3×10^{-7}	Deionised water	Chromolith Performance. Isocratic (eluent: water/methanol 3:7 v/v). Flow rate of 0.8 mL/min.	27
Electrogenerated $Ru(bipy)_3^{3+}$ in 10 mM H_2SO_4	α-solanine α-chaconine	12 15	1×10^{-9} 2×10^{-9}	Potato tuber skin and pith extract	Chromolith Performance. Isocratic (eluent: 20 mM phosphate buffer (pH 7.8)/acetonitrile 65:35 v/v). Flow rate of 0.6 mL/min.	26
Electrogenerated $Ru(bipy)_3^{3+}$ in 1 mM H_2SO_4	Proline Pipecolic acid	9 16	– 1×10^{-9}	Human serum, cow's milk, apple juice, beer	Chromolith Performance (×2). Isocratic RP-IP-HPLC. Eluent: 20 mM acetic acid and 15 mM sodium octanesulphonate (pH 3.5). Flow rate of 0.7 mL/min.	29
Electrogenerated $Ru(bipy)_3^{3+}$ in 10 mM H_2SO_4	(−)-epigallocatechin gallate (−)-epicatechin	20 22	4×10^{-8} 6×10^{-8}	Tea	Chromolith Performance. Post-column photochemical reactor. Isocratic (eluent: 20mM phosphoric acid/methanol 80:20 v/v). Flow rate of 0.5 mL/min.	28

(continued)

Table 11.2 (*continued*)

Chemiluminescence reagent	Analytes	Elution time (min)	Detection limit (M)	Matrix	Separation conditions	Ref.
Alkaline hypobromite (0.4% v/v bromine in 0.4 M NaOH)	Arginine $[Arg_{10}]$-angiotensin I Bradykinin Angiotensin III Angiotensin I	0.3 2.0 2.3 2.5 2.8	– – 4×10^{-6}	Deionised water	Chromolith SpeedROD. 5% to 35% acetonitrile in an aqueous solution of trifluoroacetic acid (0.1%) over four minutes. Flow rate of 3 mL/min.	30
$KMnO_4$ in 1% sodium polyphosphate (pH 2.5)	5-HIAA Homovanillic acid	3.5 4.1	2×10^{-8} 1×10^{-7}	Human urine	Chromolith SpeedROD. 3% methanol in an aqueous solution of trifluoroacetic acid (pH 2) for two minutes, then linear increase to 80% methanol over 8 minutes. Flow rate of 3 mL/min.	31
$KMnO_4$ in 1% sodium polyphosphate (pH 2.5)	Synephrine Octopamine Tyramine Hordenine	1.1 1.5 2.4 4.0	5×10^{-6} –	Dietary supplements	Chromolith SpeedROD. Aqueous solution of trifluoroacetic acid (pH 2.5) for 5 minutes, then with 30% methanol for 15 minutes to remove other sample components from the column. Flow rate of 1 mL/min.	34
$KMnO_4$ in 1% sodium polyphosphate (pH 2)	Morphine Oripavine	0.6 1.4	1×10^{-10} 5×10^{-10}	Process liquors	Chromolith SpeedROD. 10% to 30% methanol in an aqueous solution of trifluoroacetic acid (pH 2) over 1.2 minutes, then held at that ratio for 0.8 minutes. Flow rate of 3 mL/min.	32

(*continued*)

Table 11.2 (*continued*)

Chemiluminescence reagent	Analytes	Elution time (min)	Detection limit (M)	Matrix	Separation conditions	Ref.
$Ru(bipy)_3^{3+}$ in 40 mM HNO_3	Codeine Thebaine	0.7 1.4	5×10^{-10} 1×10^{-9}	Process liquors	Chromolith SpeedROD. 10% to 30% acetonitrile in an aqueous solution of trifluoroacetic acid (pH 2) over 1.2 minutes, then held at that ratio for 0.8 minutes. Flow rate of 3 mL/min.	32
Soluble Mn(IV) in aqueous phosphoric acid solution and formaldehyde	Morphine Pseudomorphine Codeine Oripavine Thebaine Papaverine	1.0 1.3 1.8 2.0 2.8 3.2	all $\sim 5 \times 10^{-7}$	Deionised water	Chromolith SpeedROD. 10% to 50% methanol in an aqueous solution of trifluoroacetic acid (pH 2) over 3 minutes, then held at that ratio for 1 minute. Flow rate of 2 mL/min.	33

seawater matrices. The analyte was eluted from the disc by changing the carrier solution and detected using a chemiluminescent reaction with 1,10-phenanthroline and hydrogen peroxide [38].

Although the determination of multiple analytes using chromatographic separation prior to chemiluminescence detection has been performed successfully using HPLC instrumentation, the high hydrodynamic resistance of conventional particulate columns has prohibited their use in FIA systems. However, monolithic columns generate a far lower back pressure, and therefore could potentially be used in systems of relatively low pressure, such as those constructed from FIA components. Šatínský and co-workers demonstrated the use of a short monolithic column (Chromolith Flash; 25 mm length × 4.6 mm i.d.) in SIA systems (with UV-absorbance detection) [39]. We subsequently utilised the same type of monolithic column to determine opiate alkaloids and neurotransmitter metabolites (with chemiluminescence detection) using FIA components [13]. A typical chromatogram for the analysis of urine is shown in Figure 11.6. This 'hybrid FIA–HPLC' approach was not without problems: the pumps were found to heat the solutions after long periods of operation, and the flow rate (and thus the speed of separation) was limited by the relatively low maximum pressure of the manifold. Nevertheless, the study has contributed to the recent erosion of boundaries between FIA and HPLC methodologies [13].

Monolithic stationary phases are also an attractive option for capillary electrochromatography and for separation on microfluidic devices [19]. In these areas, where relatively small volumes of solution are analysed, there is also a great deal of interest in the development of sensitive detection systems, including chemiluminescence. Some preliminary applications that involve both separation on monolithic materials within capillaries or microfluidic channels and chemiluminescence detection have emerged. Chen and co-workers described the determination of two underivatised amino acids (L-threonine and L-tyrosine) using pressurised capillary electrochromatography with an *in situ* polymerised monolithic stationary phase (based on isobutyl methacrylate and ethylene dimethacrylate), and chemiluminescence detection that involved the reaction with luminol, copper(II) and hydrogen peroxide, using a custom-made coaxial-flow detection interface [40]. Xu *et al.* reported the determination of binary mixtures of underivatised amino acids (such as L-phenylalanine and L-arginine) using an *in situ* polymerised monolithic anion-exchange material, based on [2-(methylacyloyloxy)ethyl] trimethylammonium chloride on a poly(methyl methacrylate) microfluidic chip, and chemiluminescence detection that involved the reaction with luminol and hydrogen peroxide [41]. In both procedures, the analytes were separated successfully within a few minutes, but limits of detection were not reported.

11.5 Concluding Remarks

Monolithic column chromatography has been coupled successfully with chemiluminescence detection for rapid and highly sensitive analyses. The inherent selectivity of chemiluminescence detection reduces the number of interfering compounds that must be separated from the target analytes and therefore enhances the advantages of the

monolithic column approach to LC. To date, the fastest separations have involved solvent gradients, but bear in mind that these procedures require additional time to return the mobile phase to its initial composition, which contributes to the overall analysis time. In addition, the effect of the mobile phase solvents on the chemiluminescence reaction must be considered. Chemiluminescence detectors for HPLC are simple to construct and relatively inexpensive. The construction of detectors for capillary electrochromatography and microfluidic devices is more challenging, but a range of suitable instrumentation has already been developed over the past few decades for CE. Numerous chemiluminescence detection systems have been explored, but the true potential of this mode of detection is yet to be realised. Further investigation is required to create a better understanding of the light-producing pathways of known reactions, develop new reagents and extend established chemiluminescence systems to new analyte classes.

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