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High-sensitivity chromatographic detector incorporating three-dimensional charge coupled device fluorimetry

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Abstract

This article describes a detector for liquid chromatography that provides three-dimensional, excitation–emission-intensity data of fluorescent compounds. The instrument achieves multi-wavelength excitation by employing a polychromator that disperses radiation along the vertical portion of a quartz capillary flow cell. The ensuing sample fluorescence is collected in the horizontal plane and dispersed using an emission polychromator to form a focused two-dimensional excitation–emission image at a charge coupled device (CCD) pixel array. Each vertical pixel equates to a narrowly defined excitation wavelength band, whilst horizontal pixels equate to corresponding emission wavelengths, with the intensity of light encroaching onto the CCD pixel elements providing the third-dimensional variable in the measurement. It is shown that the extreme sensitivity of CCD detection coupled to the dual polychromator arrangement makes possible the collection of fluorescence excitation–emission-intensity data of polycyclic aromatic hydrocarbons in a flow stream in less than 0.05 s with nanogram per millilitre limits of detection.

Keywords: Detection, LC; Charge coupled device detection; Fluoresence detection; Polynuclear aromatic hydrocarbons

1. Introduction

The inherent sensitivity of spectrofluorimetry makes it an ideal tool for the on-line analysis of chromatographically resolved fluorescent compounds [1–3]. Fluorescence methods can be up to three orders of magnitude more sensitive than UV–visible absorption techniques, yielding spectra that are ideally suited to analytical purposes. HPLC detectors conventionally measure the emission intensity of an eluting fluorophore at a selected wavelength, arising from a fixed excitation wavelength [4–6]. Whilst this approach is satisfactory for the analysis of groups of compounds exhibiting very similar fluorescence properties, problems arise when analysing compounds with widely differing excitation–emission characteristics. In such cases, it is often preferable to increase the dimensionality of the data so that further information can be obtained.

A number of routes have been used to address this problem. A common approach is to decrease the time taken to acquire fluorescence data using either fast scanning instruments or multi-channel detection devices, such as diode array detection (DAD) systems and charge coupled devices (CCDs) [7–16]. The multi-channel approach allows the simultaneous monitoring of fluorescence emission over a wide wavelength range for a given fixed excitation energy. A further data dimension can be added by examining both the excitation and emission wavelengths, with

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corresponding emission intensities, and to combine these into an excitation–emission matrix (EEM) [17–22]. These rapid scanning or multi-channel detector array techniques typically involve the collection of an entire emission spectrum for each incremental increase in excitation wavelength. The process is repeated until an entire EEM is created. Whilst such a method is acceptable for quiescent samples using mechanically driven monochromators, the time taken to acquire the data necessary for the generation of a complete EEM makes this approach incompatible with flowing samples of fluctuating composition, as is the case in chromatography. A solution to this problem was proposed by Johnson et al. [23,24], in which a novel polychromator optical arrangement simultaneously irradiates a sample across a wide excitation energy range followed by the collection of the entire emission spectrum. A silicon-intensified target (SIT) vidicon incorporating a two-dimensional array of detector elements was used to collect the emitted radiation. Chromatographic applications of the video fluorimeter have been examined by other researchers [25,26].

Given the benefits of CCD detection over the vidicon and other multi-channel detector arrays (high sensitivity, reduced read-out noise levels and wide spectral response range) it is surprising that there are no reports concerning their usage for the real-time generation of EEMs for chromatographic and other applications. The primary aim of this study has, therefore, been to demonstrate the analytical capability of such a device using state-of-the-art CCD technology coupled to a spectrofluorimeter incorporating holographic excitation–emission gratings. It is shown that this detector is able to capture the entire EEM signature of a passing compound in a flow cell within the time constraints of liquid chromatography detection. It is demonstrated that this detection technique is sensitive enough to capture radiation emission from compounds present at very low concentrations and having a short residence time within the flow cell. The approach is based on an optical arrangement that permits simultaneous multi-wavelength excitation and instantaneous collection of the broad band sample emission using orthogonally positioned polychromatic reflection gratings. This produces a spatially resolved two-dimensional spectral image of the fluorophore in the flow cell that is focussed onto a highly sensitive CCD detector for visualisation and quantification. The performance of the instrument as an on-line chromatographic multi-wavelength detector was assessed using polycyclic aromatic hydrocarbons (PAHs), a class of priority pollutant exhibiting strong fluorescence by virtue of their condensed multi-ring benzenoid structure.

2. Experimental

2.1. Simultaneous multi-wavelength detection

Multi-wavelength sample illumination and resulting multi-wavelength fluorescence is achieved using an optical design that was first reported in the 1970s by Johnson et al. [23, 24]. However, modern optical components employing holographic reflection gratings are used in place of quarter meter Ebert type monochromators to disperse the incident and emitted radiation. Light from a xenon excitation source is dispersed onto a vertical portion of a quartz capillary flow cell. Molecular fluorescence of analytes passing through the flow cell is collected and dispersed horizontally by an orthogonally positioned emission reflection grating. For both excitation and emission, light is dispersed such that its component wavelengths are spatially separated. This produces a two-dimensional (2D) spectral image that is focused onto the CCD detector array. Each vertical pixel in the array equates to a narrowly defined excitation wavelength band covering a 250–550 nm range, whilst each horizontal pixel equates to a correspondingly narrow emission wavelength band (250–750 nm). A shutter arrangement is used to illuminate the sample for the desired exposure time, after which the photogenerated charge stored within the pixel array is collected and processed using the CCD read-out operation. The principle of multi-wavelength operation is summarised in Fig. 1.

2.2. Instrument configuration

A prototype SPEX 3D spectrofluorimeter was supplied by Instruments SA (Stanmore, UK). An excitation spectrograph focused the 75 W xenon excitation source onto a $<$10 mm vertical portion of the sample chamber via a narrow rectangular excita-

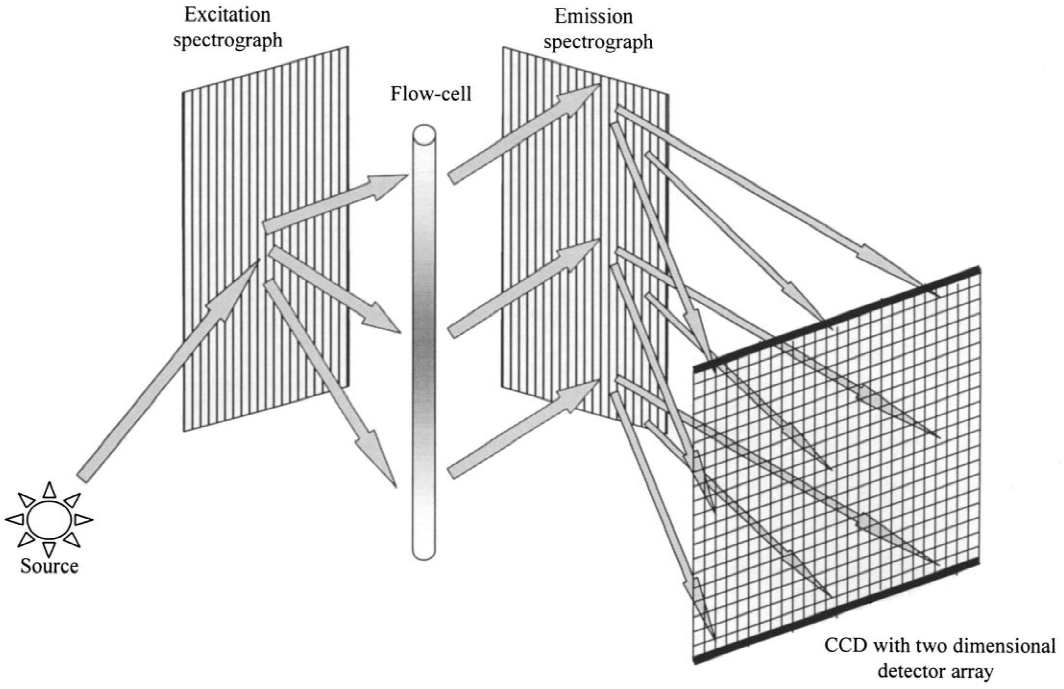

Fig. 1. Principle of three-dimensional spectrofluorimetry with CCD detection. Light from the excitation source is dispersed in a vertical manner by an excitation spectrograph and focused on the sample housing. Emitted fluorescence is dispersed in a horizontal manner by the emission spectrograph and collected on a CCD 2D detector array.

tion slit, collimating concave mirror, flat mirror and concave holographic reflection grating (133 grooves/mm). Resultant fluorescence emissions were collected via an emission slit, spherical mirror, corrected for aberration using a toroidal mirror and directed, via a flat mirror onto a concave holographic emission grating (133 grooves/mm), that focused the fluorescence radiation onto the CCD detector array. Excitation and emission slit widths were 0.05 mm. A high-speed emission shutter (MDC Uniblitz shutter), located in the emission spectrograph, determined the integration time for CCD acquisition. The instrument was programmed to sequentially illuminate the CCD detector at a uniform pre-defined time interval of 10 s as required for on-line monitoring purposes.

The EEM was collected using a backthinned UV enhanced multichannel CCD silicon photodiode detector head (Spectrum One CCD Detection System). The pixel array was in the form of a 2D square of 512×512 individual detector elements, each with dimensions of 24×24 μm, yielding a total active chip area of 12.3×12.3 mm. The system was designed to allow imaging of the entire excitation (250–550 nm) and emission (250–750 nm) EEM. The CCD specifications included 16-Bit A/D conversion precision with a chip dynamic range of $1–6.5\cdot10^4$ photon counts per second. The dark current level was specified as <5 electrons per pixel per second. A quantum efficiency of $>40\%$ across the wavelength range 250–750 nm is quoted by the manufacturer with a read-out noise of 10–20 e^- RMS (electrons root mean square) per pixel or binned pixel. The read-out rate was 20 kHz, which was deemed suitable for general purpose spectroscopy applications. The device was maintained within temperature using an air cooled Peltier element.

On illumination, each pixel integrates the charge arising from the photoelectric response of the semiconductor. The detector interface unit allowed transfer of each pixel charge to the serial register for formatting as individual pixel datapoints or ''binned'' into units of several pixels for data manipulation purposes. The data was passed to the CCD controller for amplification and digitisation and

accessed by personal computer-controlled Datamax software (Grams/32 software, Galactic Industries) for appropriate processing, display and import/export capabilities. The system was able to acquire a full 300×500 nm EEM acquisition in <1 s. Shorter acquisition times could be obtained by specifying a particular pixel ''window'' within which relevant EEM activity could be recorded. The set-up of the complete detection system is shown in Fig. 2.

2.3. Chromatographic system

The Gilson high-performance liquid chromatographic system (Anachem, Luton, UK) consisted of two model 306 pumps, an 811C dynamic mixer and an 805 manometric pressure regulator. Samples were injected using a Gilson 231 XL autoinjector via a rheodyne valve and 50-μl injection loop. The needle was washed between injections with a 401C dilutor. A 125×4 mm I.D. Envirosep-PP column (Phenomenex, Macclesfield, UK), with guard column (30×4 mm I.D.) of the same material was used. The column stationary phase was believed to be polymerically bonded C_{18}-silica. The column was equilibrated in 40% (v/v) acetonitrile in water, flow-rate of 1 ml min^{-1}. After sample injection, the system was run isocratically for 4 min in 40% (v/v) acetonitrile followed by a linear gradient increase to 100% (v/v) acetonitrile over 50 min at the same flow-rate. The water and acetonitrile mobile phases were degassed daily by ultrasonication and helium purging.

The flow-cell consisted of a quartz tube (1.0 mm I.D.) maintained in position using a cuvette with a pre-cut window. Narrow bore (0.25 mm) polyether ether ketone (PEEK) tubing with HPLC-compatible PEEK ferrules (Alltech, UK) were used to connect the flow-cell to the downstream end of the chromatographic column. PEEK tubing was connected to the quartz capillary tubing using epoxy-resin based glue and heat-shrink tubing.

2.4. Reagents

HPLC-grade water, acetonitrile, acenapthene, anthracene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, 1,2:5,6-dibenzanthracene, fluoranthene, fluorene, 1-methylanthracene, 2-methylanthracene, napthalene, perylene, phenanthrene and pyrene were supplied by Sigma–Aldrich (Gillingham, UK). Standard solutions were prepared in acetonitrile.

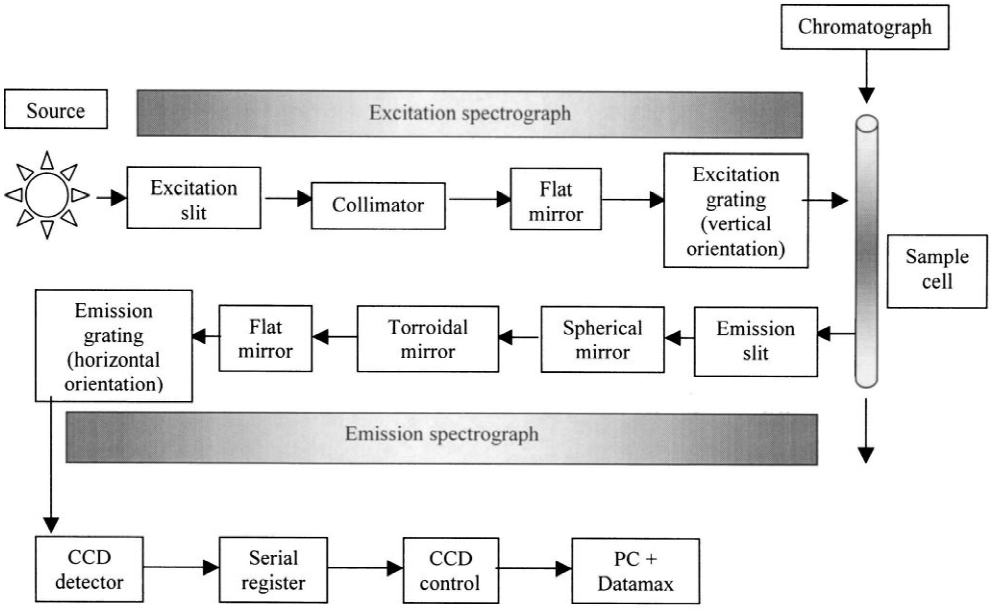

Fig. 2. Key components of the three-dimensional spectrofluorimeter with CCD detection.

3. Method development

3.1. Data display

The Grams software allowed the data to be observed in a variety of formats. Three-dimensional (3D) isometric representation of the data – whereby the *x*, *y* and *z* axes corresponded to the emission, signal intensity and excitation variables, respectively – proved a suitable means of peak identification due to the highly distinctive shape profiles of the standard PAHs analysed. Observation of the EEM spectra was also possible in a 2D format whereby the *x* and *y* axes corresponded to the emission and excitation variables, with signal intensity orientated into the plane of the paper. The closeness of the contour lines equated to signal intensity in the same manner as the contour lines on a geographical survey map. Excitation and emission values are reported in pixels, with work on going to develop a suitable mathematical relationship to allow expression of such values in terms of wavelength. In the 3D diagrams, signal intensity is reported in arbitary units.

3.2. Optimisation of detection conditions

3.2.1. Binning

Binning describes the process whereby the individual charge packets associated with adjacent pixels on a 2D array detector are physically combined into an individual charge packet. Binning is an analogue rather than a digital summation process, the latter approach involving the individual collection and digitisation of individual charge packets, followed by storage and summation. Binning reduces the number of operations required to quantify the amount of charge associated with a group of charge packets, thereby increasing readout speed and reducing detector noise (improved detector sensitivity), although at a cost of decreased resolution. After initial experimentation, a binning number of 3 was selected for both the excitation and emission planes, equating to a 3×3 block of pixels, representing a compromise between readout speed, S/N ratio and resolution.

3.2.2. Detector window

The instrument was designed to function across the whole of the analytically significant portion of the fluorescence electromagnetic spectrum. Individual fluorophores may only be active in a small portion of this spectrum, thereby negating the need to address the complete CCD detector array. Consequently, standard PAH solutions were prepared in acetonitrile at the 1 μg ml^{-1} level and analysed in a quartz cuvette (1 cm path length). The optimised window selected was 120–240 pixels and 85–165 pixels for the excitation and emission planes, respectively (binned pixels).

3.2.3. Slit width

The selection of the optical slit width represents a compromise between the required signal intensity and spectral resolution. A wider slit width allows a greater light intensity to be incident upon the sample and detector, but at a cost of decreased spectral resolution. Of the three slit widths studied (0.05, 0.10 and 0.25 mm), the smallest slit width of 0.05 mm was found to provide an acceptable degree of instrument sensitivity and spectral resolution.

3.2.4. Blank subtraction

An artefact present in all of the EEMs but seen most clearly in those collected in the absence of an eluting species was a diagonal line of peaks ranging from the high excitation–emission corner of the matrix through to the low excitation–emission corner. This phenomenon is characteristic of the scattering of light by particles such as atoms and molecules where the dimensions of the scatterer are much smaller than the incident beam wavelength. The scattered radiation intensity varies inversely with the fourth power of the wavelength and, therefore, was more intense at the higher energy corner of the matrix. These peaks were removed using a blank subtraction facility and were performed during the sample collection and processing operation.

3.2.5. Integration time

The shutter speed had a range of 0.01–5.00 s. The longer shutter speeds are of benefit in the analysis of static samples where extreme instrument sensitivity is required. Operation of the instrument as an on-line detector necessitates short sample collection times so that the resolving power of the chromatographic column is not compromised. Integration times of 50

ms, 1 s and 5 s were selected for these studies, balancing the requirement for acceptable sample resolution and high sensitivity.

4. Results and discussion

The CCD fluorimeter has a number of applications. Samples may be housed within a cuvette for the rapid analysis of the complete fluorescence properties of a quiescent solution. The absence of moving parts, notably within the optical system results in an instrument that is potentially rugged and ideal for field-based analyses of fluorescent components in environmental and other samples. However, given the excellent performance characteristics of the CCD, the device lends itself to the role of chromatographic detector for the trace level analysis of fluorimentric compounds and the optical separation of non-chromatographically resolved compounds. The high sensitivity of the CCD detector also allows short sample integration times to be used, an important consideration in high-resolution chromatography. The performance of the device with respect to qualitative sample analysis, linear range, limit of detection and repeatability when operated as a chromatographic detector is presented in this section.

4.1. Analytical performance of device

A mixture containing 1 μg ml^{-1} of each of the PAHs listed in the reagents section was prepared in acetonitrile, ultrasonicated to aid solids dissolution and filtered through a 0.45-μm filter. The 3D fluorimetric analysis of the PAH standard mixture yielded a complex EEM signature that was not a simple summation of each individual PAH present (Fig. 3). This was attributed primarily to fluorescence quenching effects which, in the case of low

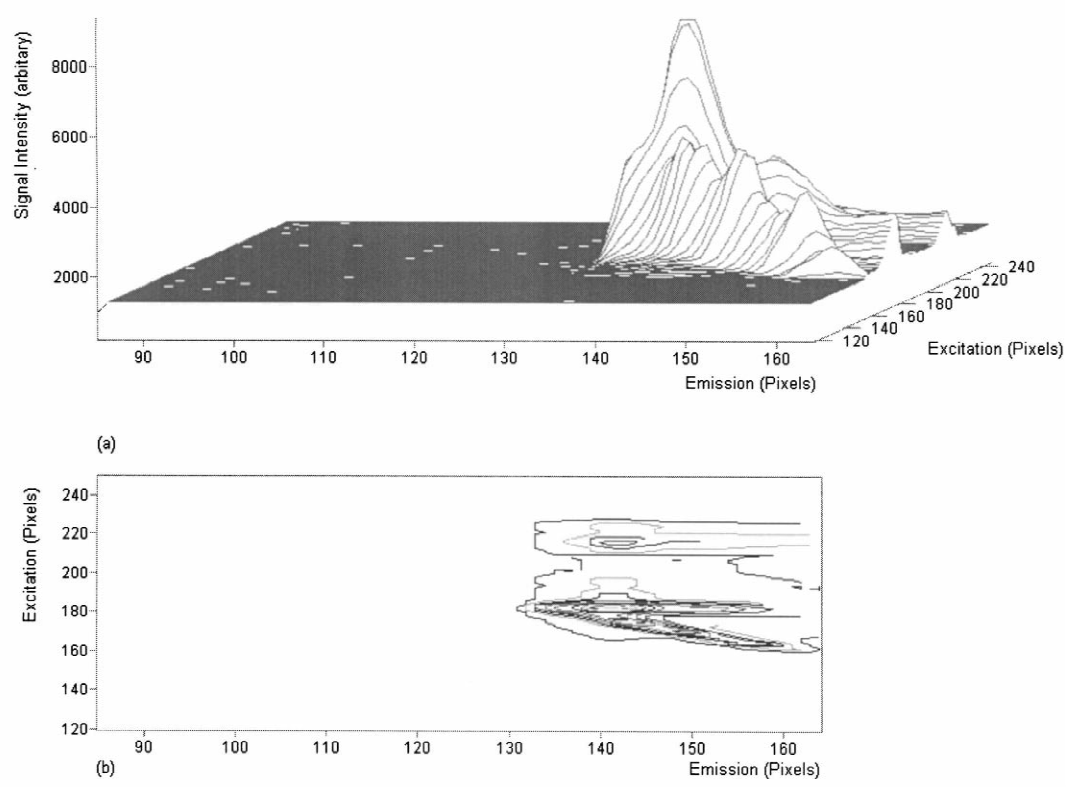

Fig. 3. Mixture of all PAH standards listed in Section 2.4, each with a concentration of 1 μg ml^{-1}. Tests were performed in a quartz cuvette with 50 ms sampling time, 0.05 mm excitation+emission slit widths, 3×3 excitation+emission pixel binning.

individual sample component concentrations, can be removed by resolving the mixture into its constituent parts. This was achieved in this study using the chromatographic procedure outlined in Section 2.

The instrument allowed collection, manipulation and storage of the data in under 1 s, thus not compromising the resolving power of the chromatographic process. In situations were it was desirable to have a real-time display of the eluent EEM spectra on the computer monitor, longer sampling intervals (approximately 5 s) were required. Significant EEM spectral data were obtained for all of the chromatographically resolved PAH standards studied. The EEM co-ordinates of these major peaks for each of the standard PAHs studied are given in Table 1 with the excitation co-ordinate recorded in bold type,

Table 1
Excitation (**bold**) and emission co-ordinates in binned pixels of major peak activity for 14 standard PAHs and mixture of 14 standard PAHs

PAH standard[a]	**Excitation**–emission pixel co-ordinates	t_R (min)[b]
Napthalene	**232.1**:95.0	19.2
Acenapthene	**226.2**:93.0, 96.8	22.6
Fluorene	**170.1**:88.8 **231.3**:88.8	24.0
Phenanthrene	**170.9**:100.8, 106.4, 112.5 **229.8**:100.8, 106.4, 112.5 **237.9**:100.8, 106.4, 112.5	26.6
1-Methylanthracene	**208.4**:113.8, 120.5, 128.5 **237.2**:113.8, 120.5, 128.5	27.8
Anthracene	**209.2**:113.4, 118.6, 126.6 **235.8**:113.4, 118.6, 126.6	29.0
Fluoranthene	**212.1**:138.8 **229.9**:138.8	30.0
Pyrene	**219.5**:109.4, 115.8, 122.2 **232.1**:109.4, 115.8, 122.2	32.2
2-Methylanthracene	**207.7**:120.9 **237.9**:120.9	32.5
Perylene	**204.8**:112.6	42.8
Benzo[*j*]fluoranthene	**219.5**:158.1	43.0
Benzo[*b*]fluoranthene	**198.0**:133.7, 141.0, 150.9 **224.7**:133.7, 141.0, 150.9	44.2
Benzo[*k*]fluoranthene	**201.8**:122.7, 129.5 **226.1**:122.7, 129.5	48.1
1,2:5,6 Dibenzanthracene	**227.6**:116.9, 124.4,132.7	52.4
Standard PAH mixture	Chromatographically resolved	

[a] A standard PAH concentration of 100 μg ml^{-1} was used in all studies.
[b] t_R refers to retention time of the sample component within the chromatographic system.

followed by the emission co-ordinates noted under the excitation conditions applied. The 3D isometric and contour diagrams relating to a number of these compounds are shown in Figs. 4–7. The applicability of the device as a qualitative identification tool is amply demonstrated by observation of the characteristic 3D isometric ''shape'' of the EEM (Figs. 4a–7a), which reflects the structural properties of that particular fluorescent analyte. Furthermore, the exact excitation–emission co-ordinates of the eluting sample component can be unambiguously determined through examination of the EEM in contour form (Figs. 4b–7b). In addition, viewing in contour mode allows the determination of co-eluting fluorophores.

4.2. Analytical range

The effective working range of the instrument was examined by injecting dilutions of standard PAHs into the chromatographic system and quantifying the resultant EEM response. Signal intensity was determined by measuring the height of the most prominent peak within the matrix. Calibration data for anthracene, phenanthrene and benzo[*j*]fluoranthene are shown in Table 2.

A linear response was observed between signal intensity and non-quenching analyte concentrations. The linear analytical range for chromatographically resolved anthracene was found to be 1.90 ng ml^{-1}, for phenanthrene: 25.3 ng ml^{-1} and for benzo[*j*-]fluoranthene: 8.11 ng ml^{-1} for an integration time of 50 ms. The minimum detection limit for anthracene was 67 pg ml^{-1} when using the maximum integration time of 5 s. The differences in the detection limits for these compounds are due to the differences in their fluorescently active fused-ring structures. The repeatability of the measurements is high as demonstrated by the relative standard deviation (RSD) values in Table 2.

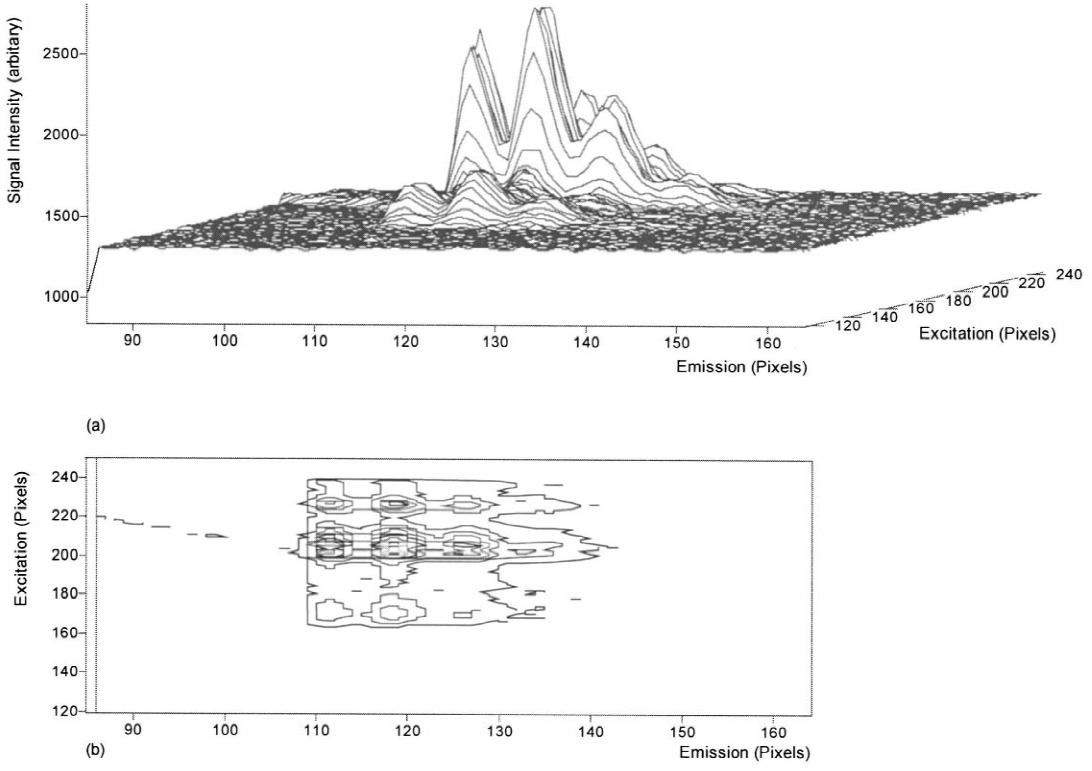

Fig. 4. Three-dimensional isometric (a) and contour (b) diagrams of PAH standard samples after chromatographic resolution. Sampling time: 50 ms; excitation+emission slit widths: 0.05 mm; excitation+emission pixel binning: 3×3; 2 μg ml^{-1} anthracene.

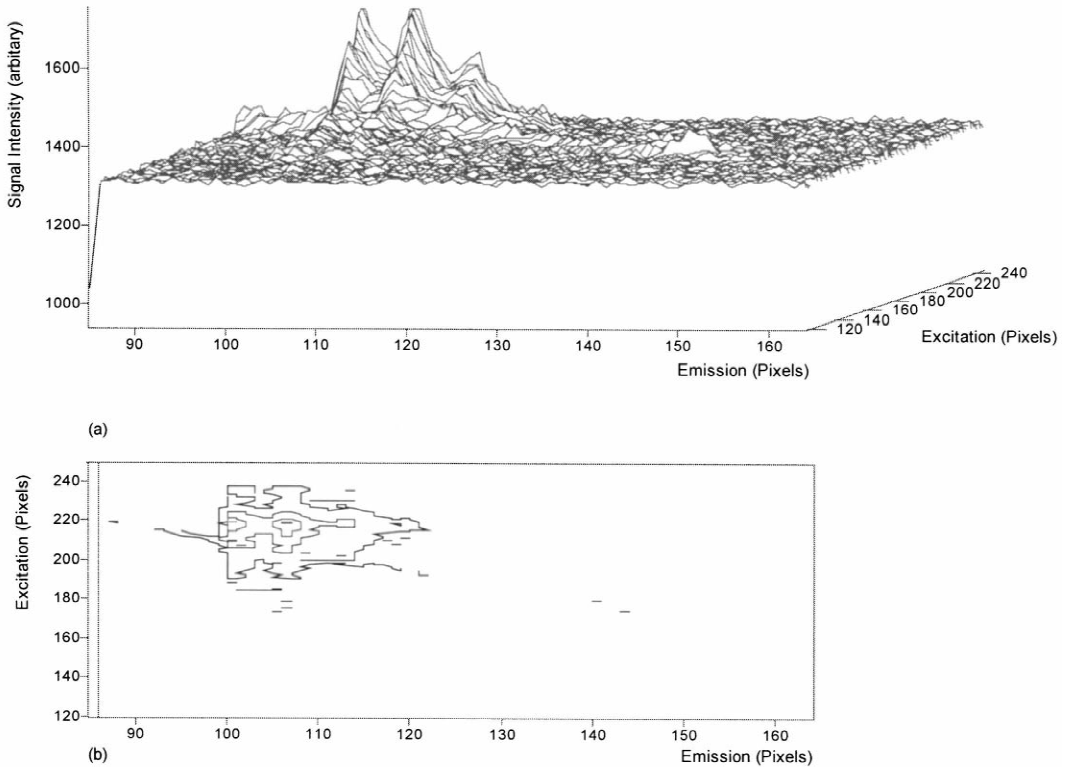

Fig. 5. As Fig. 4; 10 μg ml^{-1} phenanthrene.

The current system compares favourably with the only truly comparable detector reported in the literature, namely the video fluorimeter, based on the silicon-intensified target (SIT) vidicon. Hershberger et al. [25], using a video fluorimeter composed of a 64×64 detector element array, reported a limit of detection of 50 ng ml^{-1} for perylene (1 ng detected, 20-μl injection loop) with a 16.7 ms EEM collection time. Given the excitation radiation is dispersed along the vertical plane and the dispersion of emitted radiation in the horizontal plane, the resultant EEM is dispersed across a relatively large area. It is, therefore, to be expected that these systems do not have the absolute detection limits associated with the one-dimensional detector array and single-point detector systems reported in the literature [27–29], many of which employ high-energy excitation sources. However, the dual polychromator systems have certain key advantages: the optimal excitation–emission wavelength characteristics of the analyte can always be seen. A component's identity can be confirmed by three-parameter identification (excitation spectrum, emission spectrum and chromatographic retention time). Finally, sample co-elution can be easily observed. It is worth noting that the two-dimensional deuterium lamp–CCD system of Jalkian and Denton [16] yielded detection limits of 8.9 and 44.6 pg ml^{-1} for anthracene and phenanthrene, respectively, but with integration times of 25 and 20 s.

There are a number of ways of improving the sensitivity of the system further. Approaches that are common to all systems include the use of higher-energy excitation sources, longer integration times and optimised slit widths, although the latter parameters may reduce the resolving power of the detector. CCD detector performance can be enhanced by optimisation of the binning configuration and cooling the detector head to a low temperature where dark current effects are almost negligible allowing longer sample integration times if required, but again at a cost of reduced component resolution.

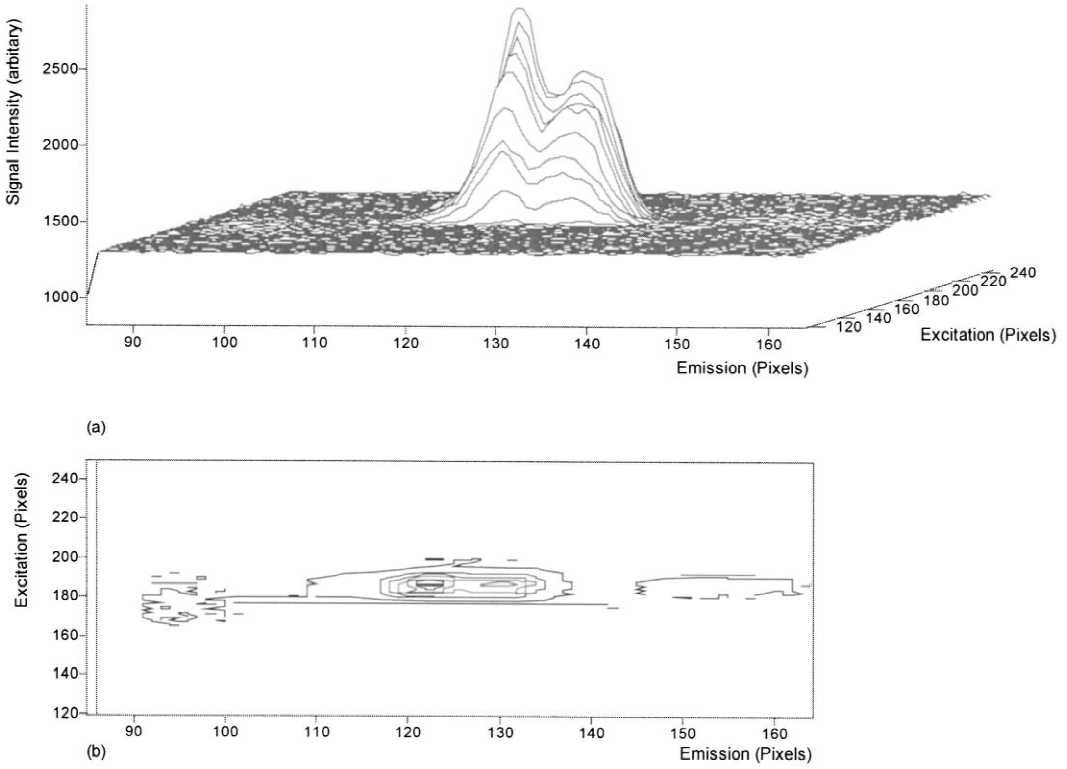

Fig. 6. As Fig. 4; 10 μg ml^{-1} napthalene.

Table 2
Calibration and reproducibility data for anthracene, phenanthrene and benzo[*j*]fluoranthene at sample integration times of 0.05, 1.00 and 5.00 s. Data was collected using 3×3 binned pixels

Sample	Integration time (s)	Calibration equation	r^2	Limit of detection (ng ml^{-1})[a]	RSD range (%)[b]	Upper limit of linear range (ng ml^{-1})
Anthracene	0.05	$y=5.51x+895.4$	1.000	1.90	0.45–1.76	>1
	1.00	$y=106.6x+987.1$	1.000	0.24	1.04–2.40	>100
	5.00	$y=1051.2x+1296.0$	1.000	0.07	2.17–4.05	>1
Phenanthrene	0.05	$y=0.414x+895.4$	0.999	25.27	0.47–1.39	>1000
	1.00	$y=7.78x+1065.7$	0.999	3.26	0.60–1.10	>1000
	5.00	$y=41.82x+1691.4$	0.999	1.68	0.70–2.99	>1000
Benzo[*j*]-fluoranthene	0.05	$y=1.29x+888.0$	1.000	8.11	0.47–0.61	>1000
	1.00	$y=22.83x+922.1$	0.998	1.11	1.04–4.27	>100
	5.00	$y=105.3x+1116.0$	0.999	0.67	1.58–5.55	>100

[a] Based on 2.5×standard deviation of zero response value ($n=5$).
[b] Real standard deviation; equal to standard deviation divided by mean ($n=5$) and expressed as a percentage.

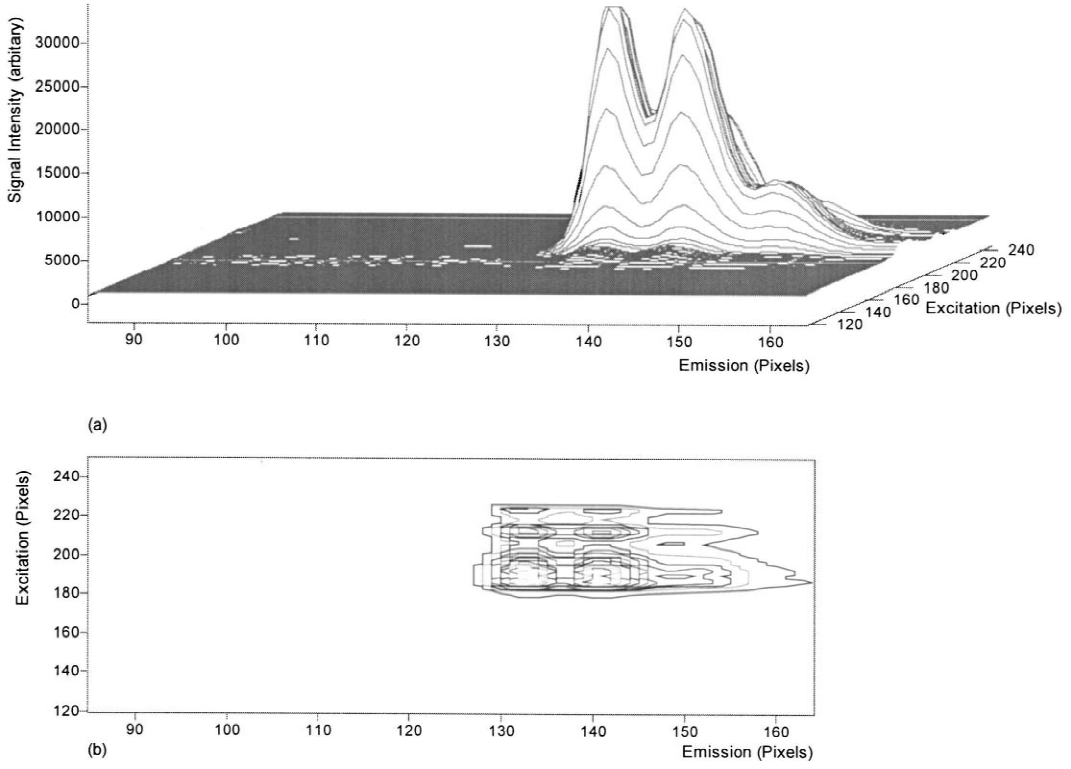

Fig. 7. As Fig. 4; 10 μg ml^{-1} perylene.

5. Conclusions

In this study, a 3D spectrofluorimeter, incorporating holographic excitation and emission polychromators was linked to a CCD detector and used to examine the EEM characteristics of chromatographically resolved polyaromatic hydrocarbons within a quartz capillary flow-cell. The inherently superior properties of the CCD, namely higher sensitivity and S/N ratio, superior resolution and wide dynamic range proved an ideal means of generating the high quality EEMs of these compounds. The sensitivity of the detection method, in addition to providing an acceptable limit of detection allowed very short (0.05 s) sample collection times to be employed, an important consideration where system resolution is of critical importance. The instrument had detection limits of 1.90 ng ml^{-1} and 67 pg ml^{-1} at integration times of 50 ms and 5 s, respectively, for anthracene, although with further optimisation of the instrument variables such as slit width, pixel binning and collection time, still lower detection limits are expected. A complete EEM could be sampled, collected and stored by the device in under 1 s, allowing the effective on-line monitoring of a fluctuating sample stream. The potential applications of this device are widespread, particularly in the trace analysis of fluorescent compounds in samples of unknown composition.

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