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Detectors: Fluorescence Detection

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The process whereby molecules are excited by electromagnetic radiation to produce luminescence is termed photoluminescence. If the release of energy is delayed, or persists after the removal of the exciting radiation, then the substance is said to be *phosphorescent*. Signal persistence (even with a short but significant lifetime) limits the use of phosphorescence for liquid chromatography (LC) detection, because signal continuance will produce apparent peak broadening and consequent loss of resolution. If the release of electromagnetic energy is immediate, or stops on the removal of the excitation radiation, the substance is said to be *fluorescent*. In contrast to phosphorescence, fluorescence has been shown to be extremely useful for LC detection, and has provided some of the highest sensitivities available.

When light is absorbed by a molecule, a transition to a higher electronic state takes place and this process is highly specific for each substance. This is because radiation of a particular wavelength, or energy, will be absorbed by specific molecular structures. If electrons are raised, due to absorption of light energy, to an upper excited singlet state, and the excess energy is not dissipated rapidly by collision with other molecules or by other means, the electron will return to the ground state with the emission of light at a lower frequency. Under such circumstances the substance is said to fluoresce. In reality, some energy is always lost before emission occurs and thus, in contrast to Raman scattering, the wavelength of the fluorescent light emitted is always greater than the incident light. For further information on the theory of fluorescence the reviews by Guilbault, Undenfriend and Rhys-Williams are recommended (see Further Reading section).

With the exception of certain electrochemical detectors and the mass spectrometer, the fluorescence detector affords greater sensitivity to sample concentration than other devices. In addition, the fluorescence sensor is less sensitive to changes in ambient conditions, e.g. temperature and pressure. The high sensitivity that is achieved is also partly due to the very low background light level and the consequent low noise level. The low noise level of the fluorescent detector is in contrast to those detectors that involve light absorption measurements, where the signal is superimposed on a strong background signal with a high noise level. The major disadvantage of fluorescence detection is that relatively few compounds fluoresce in a practical range of wavelengths. However, the scope of fluorescence detection can be extended by forming derivatives. For example, the reagents fluoropa (*o*-phthalaldehyde) and fluorescamine (4-phenyl-spiro(furan-2-(3H),1′-phthalan)-3′,3′-dione) are both commercially available derivatizing reagents that can react with primary amines to produce fluorescent derivatives. One other minor disadvantage is the effect of molecular oxygen which, if present in the mobile phase, can cause significant fluorescent quenching. It is essential, therefore, for maximum and constant response, to degas the solvents by helium sparging before use.

Most fluorescent detectors are configured so that the fluorescent light that is sensed is emitted at an angle (usually at right angles) to the direction of the exciting incident light beam. This arrangement minimizes the amount of incident light that may provide a background signal to the fluorescent sensor. It follows that the fluorescent signal is sensed against

a virtually black background and hence provides the maximum signal-to-noise ratio. If necessary, the background signal can be further reduced by the use of an appropriate filter to remove any stray scattered excitation light that might be received by the sensor.

The fluorescence signal (I_f) is given by

$$I_f = \phi I_o(1 - e^{-\varepsilon c l})$$

where ϕ is the quantum yields (the ratio of the number of photons emitted and the number of photons absorbed), I_o is the intensity of the incident light, c is the concentration of the solute, ε is the molar absorbence and l is the path length of the cell.

It is clear that the solute concentration is a somewhat complex function of the intensity of the emitted fluorescent light. As a consequence, the signal from the photocell must be electronically modified to produce an output that is linearly related to solute concentration.

Fluorescence detectors vary widely in complexity. The simplest consists of a single wavelength excitation source in conjunction with a sensor that responds to light at all wavelengths (UV and visible). For certain applications, this simple form of fluorescence detector can be very sensitive and inexpensive. However, by restricting the excitation light to a single wavelength, and with no means of selecting the emission wavelength, the system has limited versatility. At the other extreme is the fluorescence spectrometer that has been fitted with a sensor cell of appropriate dimensions. This comprehensive fluorescence monitoring system is highly complex and versatile and allows both the excitation and emission wavelengths to be chosen. Furthermore, excitation spectra can be obtained at any fixed emission wavelength, or an emission spectrum can be obtained for any fixed excitation wavelength.

The Fluorescence Detectors

The Single Wavelength Excitation Fluorescence Detector

With the exception of the electrochemical detector, the single wavelength excitation fluorescence detector is probably the most sensitive detector generally available to LC but, as already stated, it is so at the cost of limited versatility. A simple form of the fluorescence detector excited by light from a single wavelength UV source is shown in **Figure 1.**

The UV excitation source is usually a low pressure mercury lamp which is comparatively inexpensive and provides relatively high intensity UV light at 253.7 nm. Many substances that fluoresce will, to a lesser or greater extent, be excited by light at this wavelength. The excitation light is focused by a quartz lens, through the cell. Another lens situated normal to the incident light focuses the fluorescent light through a circular mask on to a photocell. Typically, a fixed wavelength fluorescence detector will have a minimum detectable concentration at an excitation wavelength of 254 nm of *c.* 1×10^{-9} g mL^{-1} and a linear dynamic range of 1×10^{-9}–5×10^{-6} g mL^{-1}. One of the disadvantages of the fluorescence detector is this rather limited linear dynamic range.

Detectors have been designed as a compromise between the expensive fluorescence spectrometer and

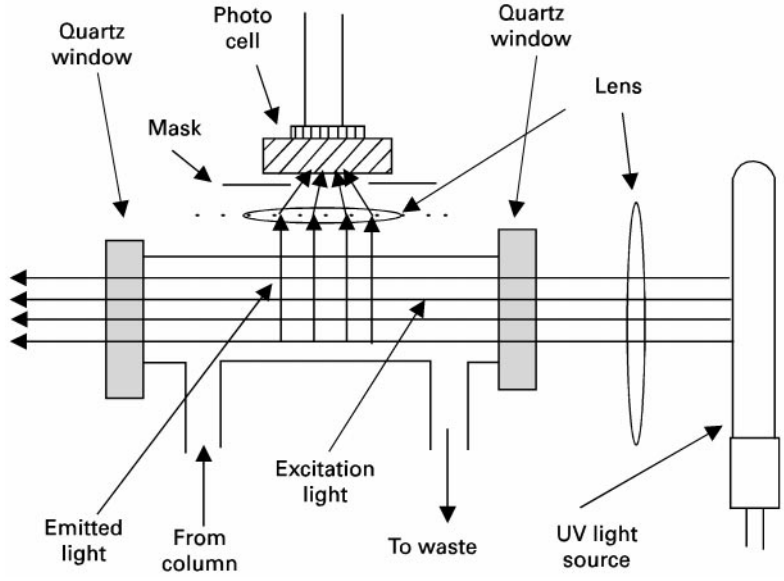

Figure 1 The single wavelength fluorescent detector.

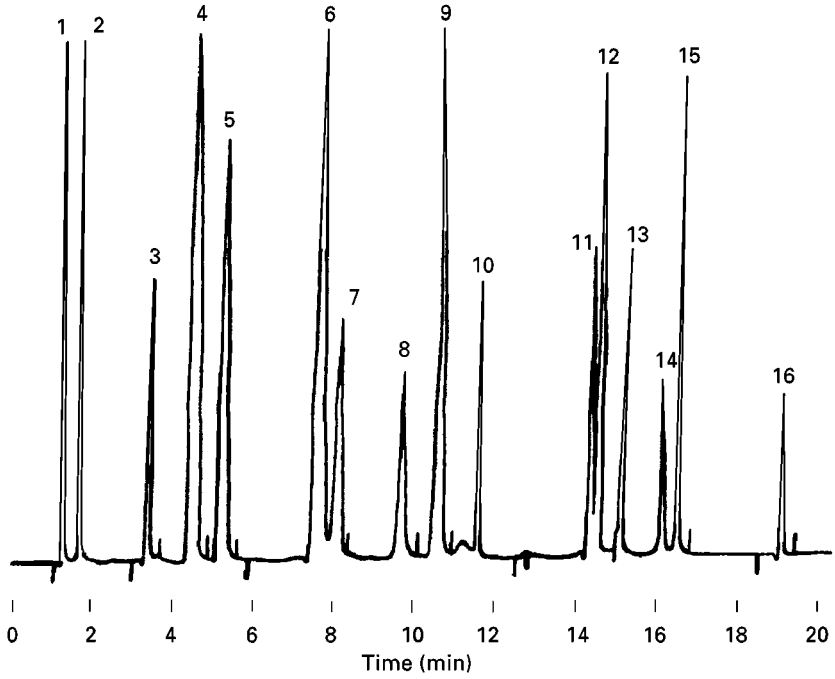

Figure 2 The seperation of some amino acids by monitoring their *o*-phthalaldehyde derivatives with a fluorescence detector. Column: Supelcosil LC-18, 5 cm × 4.6 mm, 5 μm particles. Mobile phase: methanol–tetrahydrofuran–0.02 mol L^{-1} sodium acetate (pH 5.9 with acetic acid) A, 22.5 : 2.5 : 77.5, B, 80 : 2.5 : 17.5. 2 min at 100% (A) to 100% (B) in 20 min. Flow rate: 2 mL min^{-1}. Sample: 50–100 pmol of each derivative in solvent A. Peak identification: 1, Aspartic acid; 2, glutamic acid; 3, asparagine; 4, serine; 5, glutamine; 6, glycine; 7, threonine; 8, arginine; 9, alanine; 10, tyrosine; 11, methionine; 12, valine, 13, phenylalanine; 14, isoleucine; 15, leucine; 16, lysine. (Courtesy of Supelco Inc.)

the fixed wavelength detector. A typical example of this compromise is the fluorescence detector that utilizes the monochromator of a dispersive UV spectrometer in conjuction with light filters. It consists of a UV dispersion spectrometer fitted with a special absorption cell having reduced dimensions.

The small sensor cell ensures that the narrow peaks produced by high efficiency LC columns can be monitored without loss of chromatographic resolution. The wavelength of the excitation light is selected by the monochromator which will be within the normal UV range of the spectrometer (*c.* 200–360 nm). The excitation light passes through the cell and the fluorescent light, emitted at right angles to the path of the excitation light, is focused on to a photocell. Up to this point, the sensor system is very similar to that of the fixed wavelength fluorescence.

In most of these types of compromise detectors, appropriate light filters can be inserted between the sensor cell and the lens that focuses the emitted fluorescent light on to the photocell. In this way, the wavelength of the light monitored by the sensor can be selected by the choice of an appropriate filter. This, in fact, is a rather primitive way of selecting the emission wavelength. Nevertheless, the arrangement can be quite effective, and certainly eliminates the need for a second monochromator and the added cost. The use of this type of detector in monitoring the separation of the *o*-phthalaldehyde derivatives of some amino acids is shown in **Figure 2**. It is seen that a very high sensitivity is realized and the integrity of the chromatographic resolution is well maintained.

The Multi-wavelength Fluorescence Detector

One form of multi-wavelength fluorescence detector consists of two monochromators: the first selects the wavelength of the excitation light, and the second disperses the fluorescent light, and provides a fluorescence spectrum, or allows the separation to be monitored at a selected fluorescence wavelength. The multi-wavelength fluorescence detector is shown in **Figure 3**. The detector comprises a fluorescent spectrometer fitted with a suitable absorption cell that can be used with high efficiency LC columns without degrading the resolution of the column. The spectrometer involves two distinctly different light systems. The function of the detector is easier to understand if the different light systems and the respective light paths are considered separately. The detector

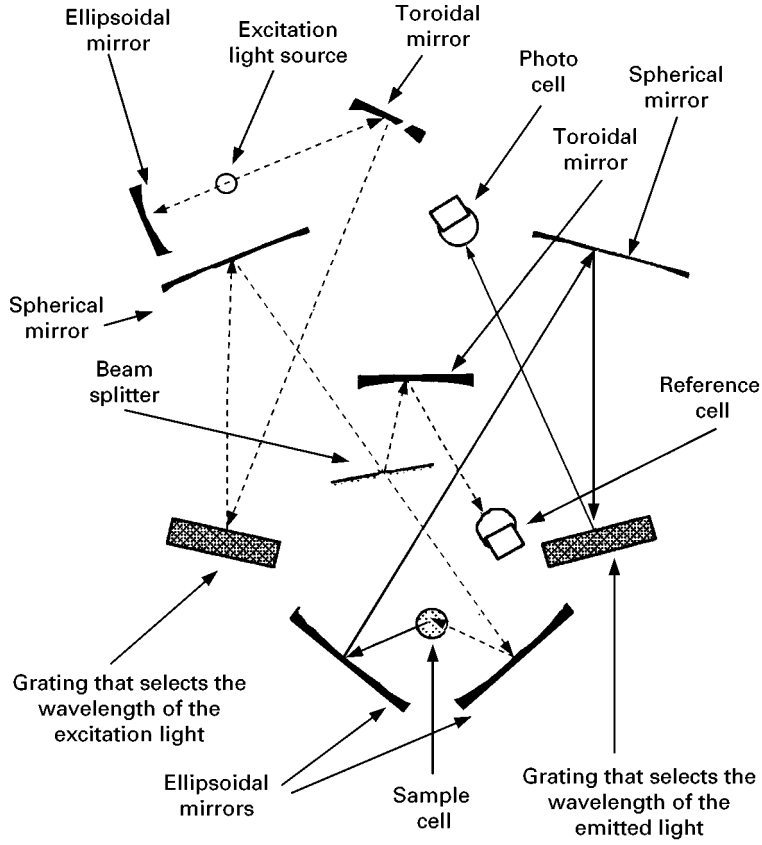

Figure 3 The fluorescence spectrometer detector. - - →, excitation light; ——→, fluorescent light.

comprises an excitation light system and fluorescent light system.

The excitation source (emitting light over a wide wavelength range, such as a deuterium lamp) is situated at the focal point of an ellipsoidal mirror, shown at the top left-hand corner of the diagram. The parallel beam of light is collimated to fall on to a toroidal mirror, which then focuses it on to the grating, shown on the left-hand side of the diagram. This grating is used to select the wavelength of the excitation light or it can be used to scan the complete range of excitation wavelengths and provide a corresponding excitation spectrum that is monitored at a specific fluorescent wavelength. The selected wavelength then passes to a spherical mirror and then to a ellipsoidal mirror, shown at the base of the diagram, which focuses it on to the sample. The excitation light path is mostly depicted on the left-hand side of the diagram.

In the centre of the diagram, between the spherical mirror and the ellipsoidal mirror, is a beam splitter that diverts a portion of the incident light on to another toroidal mirror. This mirror focuses the light on to the reference photo cell. The reference photo cell provides an output that is proportional to the intensity of the excitation light. The path of the fluorescent light is depicted on the right-hand side of the diagram. Fluorescent light, emitted from the cell, is focused by an ellipsoidal mirror on to a spherical mirror at the top right-hand side of the diagram. This mirror focuses the light on to a grating which is situated at about centre right of the diagram. This grating selects a specific wavelength of the fluorescent light to monitor, or can scan the fluorescent light produced by excitation light of a given and selected wavelength, and provide a fluorescent spectrum. Fluorescent light from the grating passes to a photoelectric cell which monitors the intensity. The instrument is complex and relatively expensive; however, for measuring fluorescence, it is extremely versatile.

The optical system allows the wavelength of the excitation light and that of the fluorescent light to be chosen to provide the maximum selectivity for a given solute or its fluorescent derivative. The use of this optimization procedure is demonstrated by the high sensitivity detection of the fluoropa derivative of neomycin shown in **Figure 4**. It is an excellent example of the selection of a specific excitation light wavelength and the complementary emission light wavelength to provide maximum sensitivity.

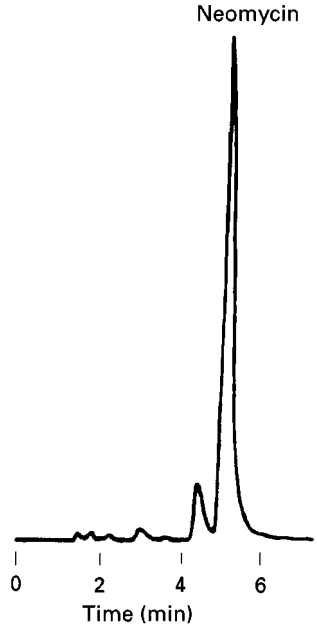

Figure 4 Detection of neomycin OPA derivative at an excitation wavelength of 365 nm and an emission wavelength of 418 nm. Column: Supelcosil LC-8, 15 cm × 4.6 mm, 5 μm particles. Mobile phase: tetrahydrofuran: 0.0056 mol L^{-1} sodium sulfate–0.007 mol L^{-1} acetic acid–0.01 mol L^{-1} pentanesulfonate, 3 : 97. Flow rate: 1.75 mL min^{-1}. Post-column reagent: 1 L 0.4 mol L^{-1} boric acid–0.38 mol L^{-1} potassium hydroxide containing 6 mL 40% Brij-35, 4 mL mercaptoethanol, 0.8 g *o*-phthalaldehyde. Flow rate 0.4 mL min^{-1}. Mixer 5 cm × 4.6 mm column packed with glass beads. Reactor 10 ft × 0.5 mm knitted Teflon capillary tubing. Reaction temperature 40°C. Sample: 20 mL of a mobile-phase extract of a commercial sample. Excitation wavelength 365 nm; emission wavelength 418 nm. (Courtesy of Supelco Inc.)

The principle of optimizing excitation and emission light wavelengths to obtain maximum sensitivity for a multi-component mixture can be quite complex, as shown by the separation of some priority pollutants depicted in **Figure 5**. The separation was carried out on a column which was 25 cm long, 4.6 mm in diameter and packed with a C_{18} reversed phase. The mobile phase was programmed from 93 : 7 acetonitrile–water to 99 : 1 acetonitrile–water over a period of 30 min. The gradient was linear and the flow rate was 1.3 mL min^{-1}. All the solutes were separated and the compounds, numbered from the left, are given in **Table 1**. The separation illustrates the clever use of wavelength programming to obtain the maximum sensitivity. The programme used is shown in Table 1.

The wavelength of the excitation light and that of the emission light was changed during chromatographic development to provide optimum fluorescent conditions, and thus maximum sensitivity, for each solute. This ensured that each solute, as it was eluted, was excited at the most energetic wavelength and then monitored at the strongest fluorescent wavelength.

It is seen that the analysis involves a somewhat elaborate wavelength programme; nevertheless, if the analysis is sufficiently important, it is readily justified. The system also provides fluorescence and excitation spectra, by arresting the flow of mobile phase when the solute resides in the detecting cell, and scanning the excitation and/or fluorescent light. (This is the

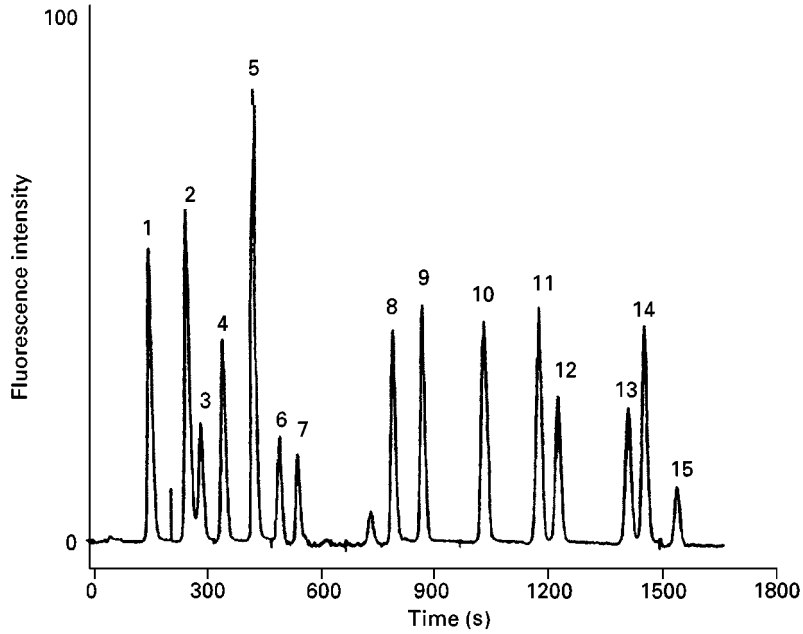

Figure 5 Separation of a series of priority pollutants with programmed fluorescence detection. 1, Naphthalene; 2, acenaphthene; 3, fluorene; 4, phenanthrene; 5, anthracene; 6, fluoranthene; 7, pyrene; 8, benz(*a*)anthracene; 9, chrysene; 10, benzo(*b*)fluoranthene; 11, benzo(*k*)fluoranthene; 12, benzo(*a*)pyrene; 13, dibenz(*a*,*h*)anthracene; 14, benzo(*ghi*)perylene; 15, indeno(123-*cd*)pyrene. (Courtesy of the Perkin Elmer Corporation.)

Table 1 Fluorescence detector programme

Time (s)	*Wavelength of excitation light (nm)*	*Wavelength of emitted light (nm)*
0	280	340
220	290	320
340	250	385
510	260	420
720	265	380
1050	290	430
1620	300	500

same technique as that used to provide UV spectra with the variable wavelength UV detector.) In this way, it is possible to obtain excitation spectra at any chosen fluorescent wavelength, or fluorescent spectra at any chosen excitation wavelength. Consequently, even with relatively poor spectroscopic resolution, many hundreds of spectra can be produced, any or all of which (despite many spectra being very similar) can be used to help confirm the identity of a compound.

The above spectrometric arrangement can be considerably simplified and much of the mechanical systems eliminated by employing a diode array sensing device for the fluorescent light. This allows the fluorescence spectrum to be recorded continuously throughout the development of the chromatogram. A specific excitation wavelength must be selected and this is achieved by employing the usual mechanical monochromator. Excitation spectra still need to be obtained by stopping the mobile-phase flow and scanning the excitation light.

Due to the high sensitivities achieved by fluorescence detection, the technique has proved very useful as a detection system in capillary electrochromatography and capillary electrophoresis. High sensitivity is achieved by employing a high energy excitation source such as a laser, emitting light at an appropriate wavelength. A typical optical system for fluorescent detection in capillary electrophoresis and capillary electrochromatography is shown in **Figure 6**. A window is opened in the quartz capillary tube, by removing the polyimide coating from about a millimetre length of capillary tube. The laser beam is arranged to pass through the window and the fluorescent light, emitted normal to the laser beam and the capillary tube, is focused on to a photoelectric cell or photodiode array. A filter can be interposed between the capillary window and the sensor measuring the fluorescent light, to eliminate scattered incident light. The signal from the photo cell is electronically modified in the same way as the normal LC fluorescence detector.

Unfortunately, lasers which have suitable wavelengths for this purpose are somewhat limited. However, lasers of various types are continuously being developed and this offers great promise for the future development of this type of detector.

An example of the use of fluorescence to monitor an electrophoretic separation of the AQC fluorescent derivatives of phenylalanine, methionine and serine are show in **Figure 7**. In this separation vancomycin was used as the chiral additive. The separation was carried out on a 30.5 cm fused silica capillary, 50 μm i.d., containing 0.1 mol L^{-1} phosphate buffer and 5 mmol L^{-1} vancomycin. The pH of the buffer was 7.0 and the electrophoretic voltage 5 kV.

Fluorescence detection is the most popular high sensitivity detection method presently in use in LC, and will continue to be so for the foreseeable future. The system is basically simple, easy to use and provides at least an order more sensitivity than the generally popular UV detector.

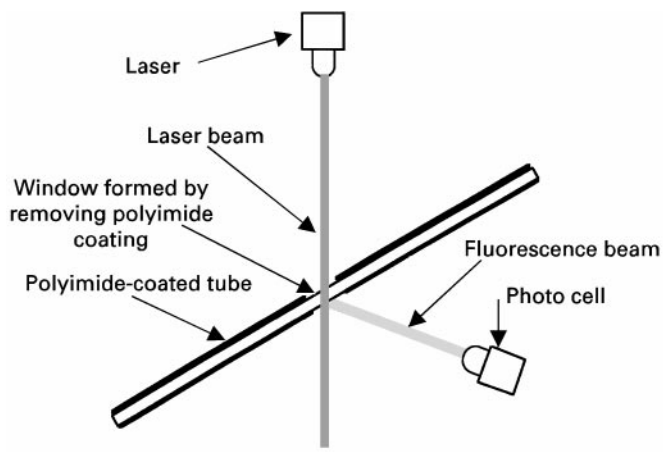

Figure 6 The laser system for fluorescence detection in capillary electrochromatography.

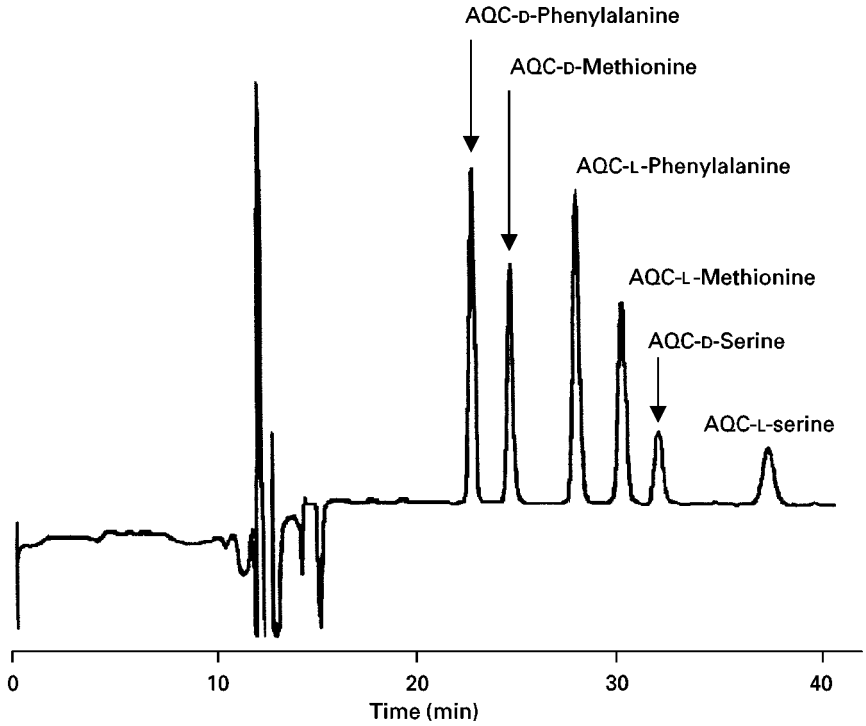

Figure 7 The separation of the enantiomers of the AQC fluorescent derivatives phenylalanine, methionine and serine. Courtesy of LC/GC. (T. L. Bereufer, *LC-GC*, Vol. 12 No. 10 (1994) 748).

Conclusion

Although relatively few substances are inherently fluorescent, most can be made to form fluorescent derivatives by relatively simple derivatization procedures. In addition, with the aid of a suitable laser, the natural high sensitivity of the device can be greatly enhanced. The main disadvantage of the fluorescence detector is its limited linear dynamic range but since in most LC analyses high sensitivity is usually required for trace analysis, in practice this limitation is not found to be so important.

See Colour Plate 22.

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Detectors: Infrared

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Introduction

Infrared (IR) light is the term given to electromagnetic radiation having a wavelength lying between 1 and 15 micron. In contrast to UV light, which is adsorbed when the light energy at a particular wavelength is equal to specific electronic transitions in the molecule, IR light is adsorbed when its energy is equal to changes in the vibrational and/or rotational energy of a molecule. A molecule can be considered as being made from a number of spheres (atoms) joined by springs (chemical bonds) and thus can vibrate in a very complex manner. As a general rule, a polyatomic molecule containing (n) atoms will exhibit ($3n - 6$) modes of vibration and a characteristic fundamental frequency (or wavelength) will be associated with each vibration mode. Both UV and IR spectra can be used for substance identification but, due to the many vibration modes that are possible,