Analytical applications of ultraviolet resonance Raman spectroscopy

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UV resonance Raman spectroscopy (UVRR) is a new analytical technique with a unique selectivity which is capable of speciating individual analytes in complex samples. The new instrumentation is discussed as are applications of this technique to studies of polycyclic aromatic hydrocarbons (PAHs) in coal liquids and in tissue. UVRR can also be used to speciate PAHs eluting from high-performance liquid chromatography columns. Other applications to studies of protein structure are also described.

The potential of Raman spectroscopy for analytical applications has been recognized for some time[1,2]. To a large extent, this potential has not been realized, owing to a lack of applications where Raman spectroscopy shows a clear advantage over other, usually more traditional, techniques. With few exceptions, Raman spectroscopy has not been usefully or routinely applied to the many areas of major interest to analytical chemists such as trace analysis, detection and identification of environmental pollutants, pesticide research, and coal and petroleum research. The recent development of ultraviolet resonance Raman spectroscopy (UVRR) promises to make Raman spectroscopy a much more attractive technique for the analytical chemist. Many of the problems inherent in using visible excitation Raman spectroscopy for analytical applications are overcome with ultraviolet excitation. In this article, we discuss the role that Raman spectroscopy can play in analytical chemistry.

A brief discussion of the work that has been done with visible excitation will serve to illustrate some of the problems which may be solved by UVRR spectroscopy. Historically, Raman spectroscopy has been classified as non-resonant (normal) or resonant depending upon whether the excitation wavelength is outside or within an absorption band of the analyte

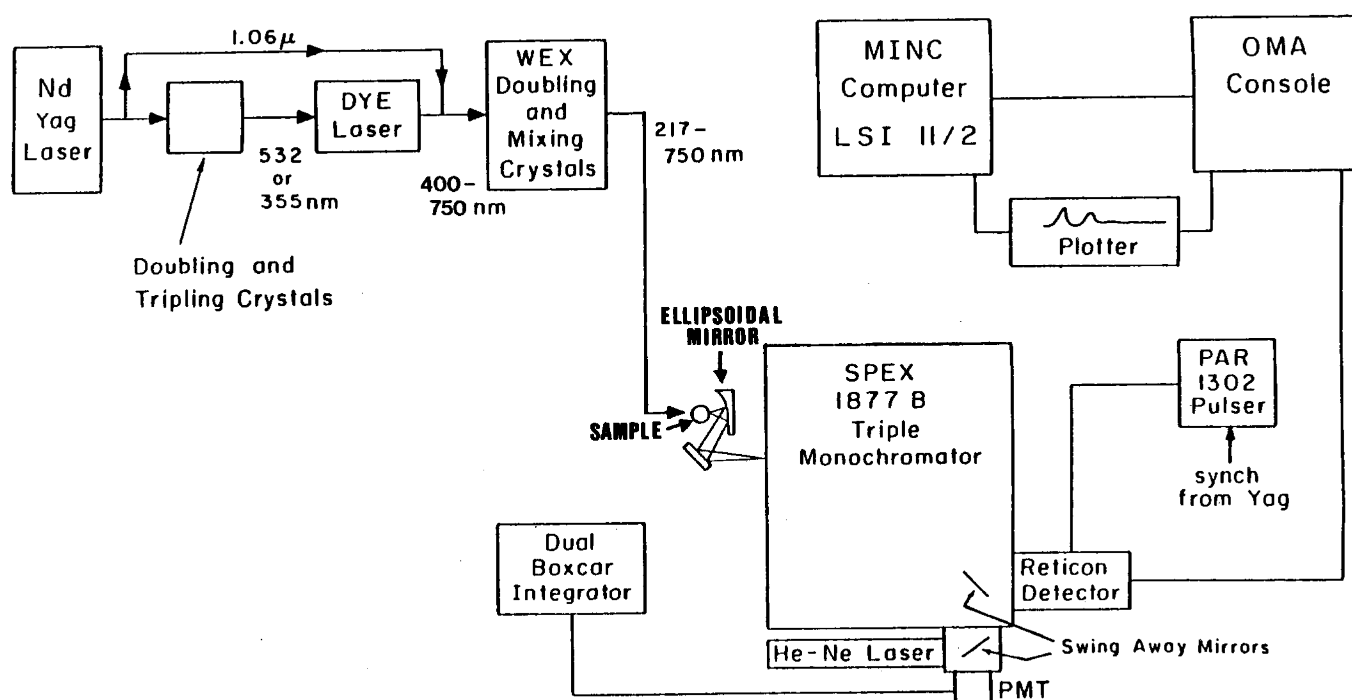

Fig. 1. Block diagram of the UVRR instrument. The laser system consisting of the Nd–YAG and pulsed dye laser is shown to the left. The WEX unit houses the doubling and mixing crystals used for the generation of UV light. The light scattered from the sample is collected by the ellipsoidal mirror and dispersed by a SPEX 1877B Triplemate spectrograph. The spectrum is obtained with the Reticon detector (gated by the pulser) or, optionally, by a photomultiplier and boxcar integrator. The spectra are stored digitally with the OMA control and data acquisition circuitry. A Minc computer is used for later plotting and analysis.

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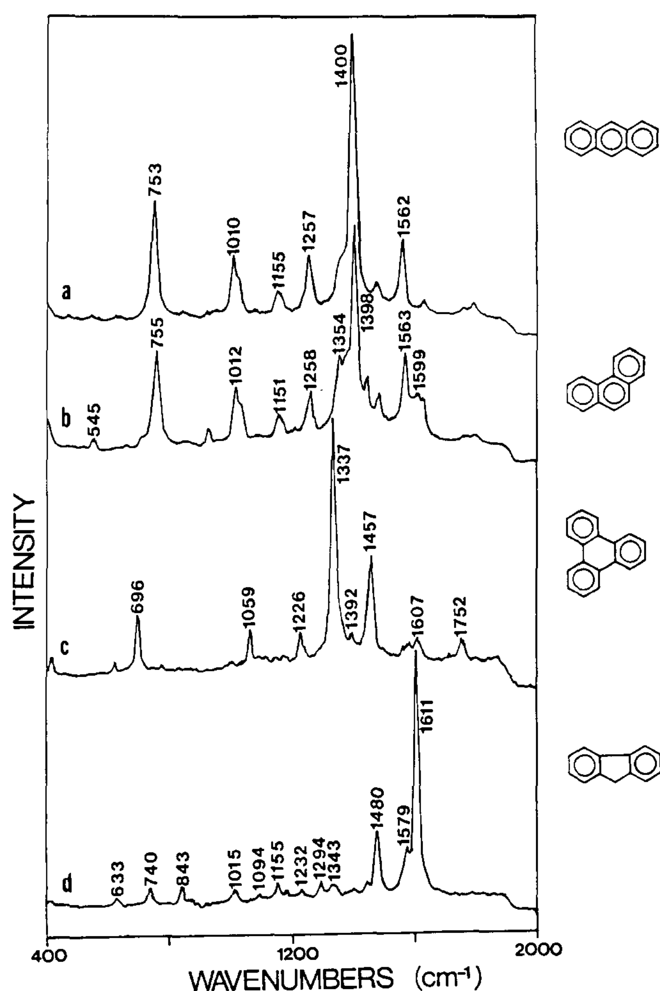

Fig. 2. *UVRR spectra of several PAHs. (a) Anthracene; (b) phenanthrene; (c) triphenylene; (d) fluorene. Each spectrum is of a* 5×10^{-3} *M solution of the PAH in acetonitrile.* $\lambda_{ex} = 235$ *nm; average power = 5.0 mW. Each spectrum was obtained in 15 min.*

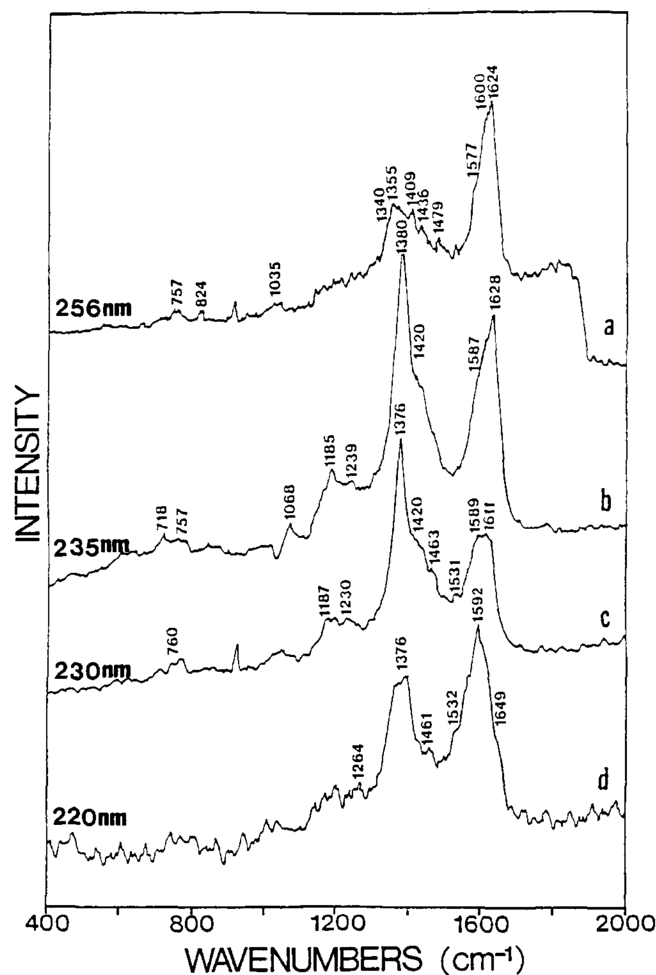

Fig. 3. *UVRR spectra of a coal liquid sample at several excitation wavelengths. The sample is a heavy distillate (340–510°C fraction) dissolved in acetonitrile.*

molecule. Normal Raman scattering cross sections are in general too low to provide useful detection limits for analytical applications for real samples; however, benzene[3] and ions[4,5] such as nitrate, phosphate, sulphate and carbonate can be detected at the 25–75 ppm level with the visible lines of an Ar^+ or He–Ne laser under ideal conditions for pure solutions. In real case applications, however, interferences, particularly broad fluorescence backgrounds, make such detection limits unobtainable.

The increase in the Raman scattering cross section which occurs with resonance excitation within a dipole-allowed absorption band can be a factor of 10^6 or more. Resonance Raman spectroscopy also offers the additional advantage of increased selectivity over normal Raman. Only the Raman bands of the chromophore which is in resonance at the wavelength of excitation are significantly enhanced. The Raman bands of non-absorbing species are not enhanced and do not interfere with the bands of the chromophore. Prior to the introduction of tunable UV lasers, the only molecules amenable to resonance Raman studies were those with absorption bands in the visible region. Examples of such molecules include long-chain polyenes such as carotenes, porphyrins (including those in heme proteins) and numerous transition metal complexes having strong visible charge-transfer transitions. The latter class includes non-heme iron–sulfur proteins and blue copper proteins. Many of these species are routinely studied at μM concentrations. The advantages of resonance Raman spectroscopy for studying these molecules has been summarized elsewhere[1,2]. Among the advantages are low detection limits ($< 10^{-6}$ M), structural sensitivity with high resolution, and lack of interference from non-chromophoric species.

Clearly a more widespread use of visible resonance Raman spectroscopy would be possible except: (1) the vast majority of known compounds ab-

sorbs strongly only in the UV region and (2) fluorescence interference is nearly ubiquitous in 'real life' samples. Several approaches have been adopted to circumvent these problems. Molecules which absorb only in the UV region can be derivatized to form visible absorbing chromophores. This method has been demonstrated for the detection of biogenic amines[6–8]. Biogenic amines, such as adrenaline, are easily oxidized to the corresponding aminochrome (λ_{max} 500 nm) and detected to 10^{-5} *M* concentrations with 488 nm excitation[6–8]. This approach is limited to a small number of molecules and is inconvenient in terms of increased sample preparation. Highly fluorescent samples, such as laser dyes and flavins, have been studied by coherent anti-Stokes Raman spectroscopy (CARS)[9]. This non-linear technique provides very high rejection of fluorescence. CARS has found fairly widespread application to the study of flames[10]. The general use of CARS in other areas has been limited. The technique requires the use of two powerful pulsed dye lasers, which increases the complexity of the experiment. In addition, the CARS signal, unlike Raman, can show dispersive bandshapes which severely complicate interpretation.

With the introduction of commercially available lasers tunable in the UV region, a new area has emerged for Raman spectroscopy. We recently detailed the construction of an instrument for UVRR research[11]. A block diagram of this instrument is shown in Fig. 1. This instrument is completely tunable from 217 to 750 nm. The system is based on a Nd–YAG laser. The Nd–YAG fundamental (1064 nm) is frequency doubled (532 nm) to pump a dye laser. A selection of common laser dyes provides tunability throughout the visible region. Laser output in the ultraviolet region is obtained with a set of doubling and mixing crystals. The dye laser output can be mixed with the Nd–YAG fundamental (420–300 nm), doubled (350–260 nm), or doubled and then mixed with the fundamental (260–217 nm). The Raman scattered light is collected with reflective optics (ellipsoidal mirror) and imaged onto the entrance slit of a triple-monochromator for dispersion. The Raman spectrum is detected with a gated intensified Reticon detector. The spectra are stored on floppy disks for later analysis.

Typical spectra obtained with this system are shown in Fig. 2. Polynuclear aromatic hydrocarbons (PAHs) are important environmental pollutants and many are potent carcinogens. A large amount of research has been conducted to find ways of detecting and quantifying PAHs in complex samples such as soot, soil, waste water and biological tissues. Many of the current methods require a significant amount of sample preparation. PAHs absorb strongly in the UV region and exhibit strongly enhanced resonance Raman scattering when excited in this region. As illustrated by Fig. 2, the PAHs have Raman spectra with numerous sharp features which easily distinguish one PAH from another. Compounds as similar as 2-methyl- and 9-methylanthracene can be differentiated by their Raman spectra[12].

We have demonstrated the detection of PAHs at the ppb level using UVRR[12]. Such detection limits

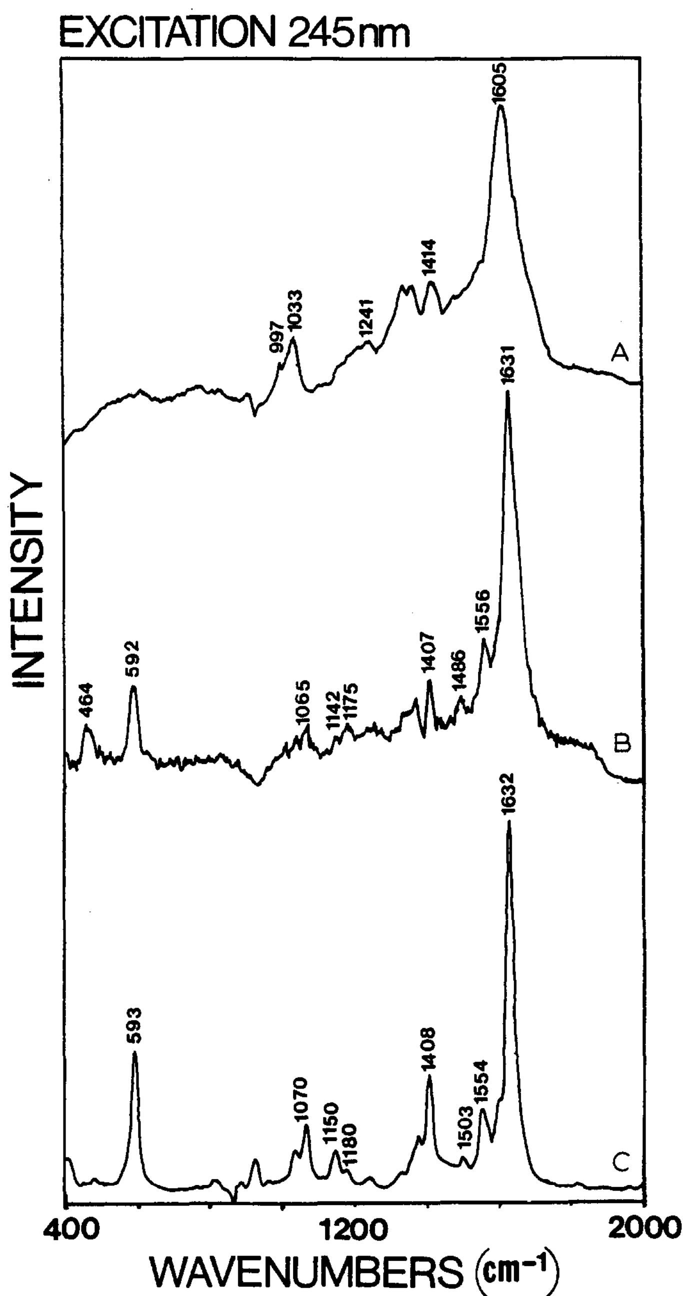

Fig. 4. (A) UVRR of calf liver homogenate excited at 245 nm. Whole calf liver was homogenized in a tissue grinder. (B) UVRR of the same sample after adding pyrene (10^{-3} M final concentration), and further disruption in the tissue grinder. The features of spectrum B all derive from pyrene. (C) Raman spectrum of pyrene (10^{-3} M in acetonitrile) at 245 nm excitation.

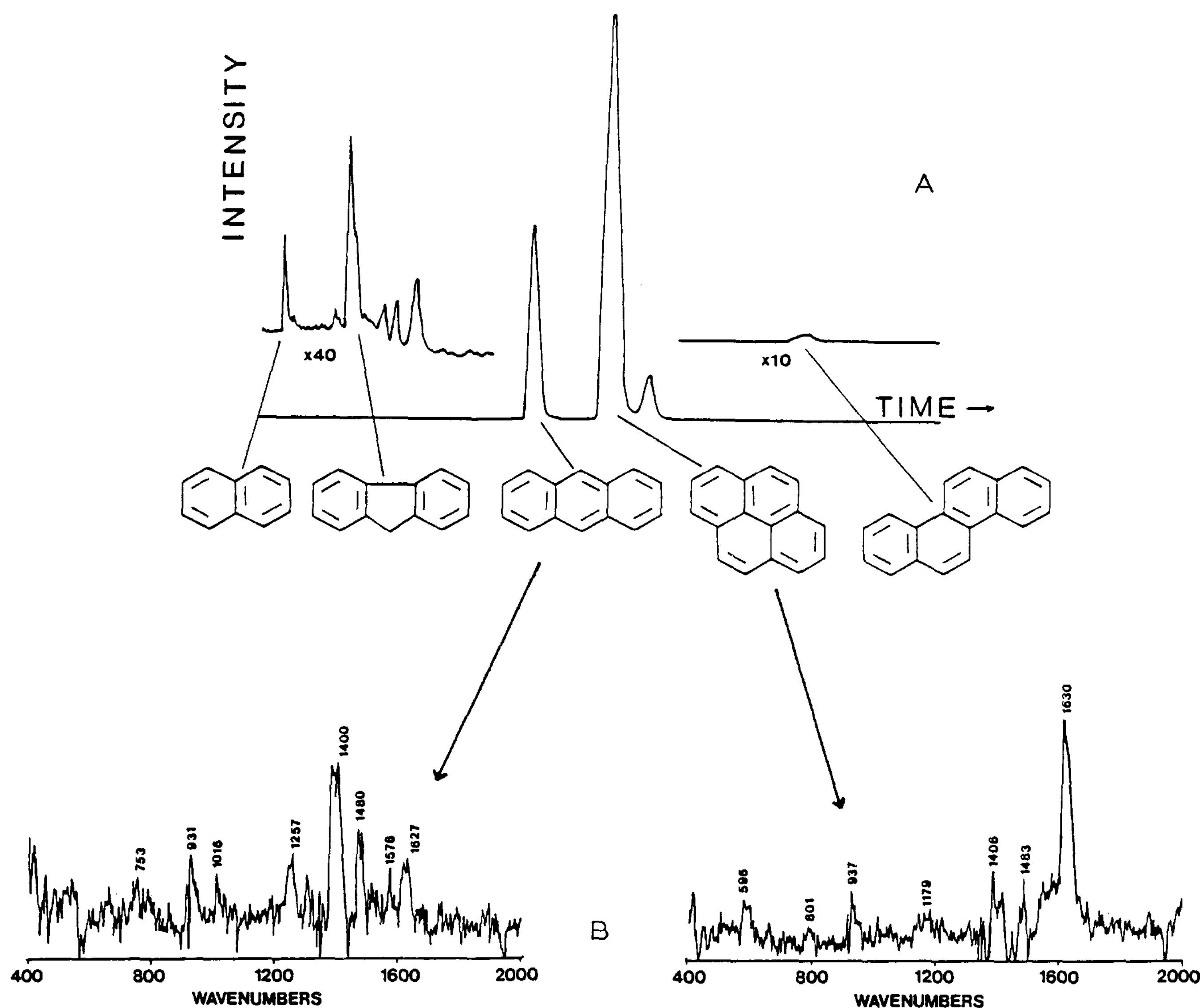

Fig. 5. HPLC separation of a PAH mixture. (A) Conventional recorder output using fluorescence detection. PAHs in the mixture are indicated under the corresponding peak. (B) Resonance Raman spectra obtained for several components of the mixture as they eluted from the column. λ_{ex} = 230 nm; laser power ca. 5 mW.

are achieved with relatively low laser power (*ca.* 5 mW), a single pass of the beam through the sample, and with a 10-min scan time. Clearly the detection limits can be pushed much lower. The spectra are obtained with no interference from fluorescence. Fluorescence has plagued the study of these compounds with visible excitation. In general, there is no interference from fluorescence for any sample with excitation below *ca.* 250 nm[13]. All of the fluorescence occurs at lower energies than the Raman scattered light when exciting this far into the UV.

UVRR may be used to study PAHs in very complex samples. The PAHs in a coal liquid fraction have been studied[14]. Spectra indicative of these fused ring systems have been obtained from the coal liquid sample without any prior separation or treatment. Spectra of the coal liquid sample at different excitation wavelengths are shown in Fig. 3. Different PAHs are selectively enhanced at different excitation wavelengths because each PAH has a distinct excitation profile. In addition, the relative intensities of individual Raman bands of a given PAH change dramatically with different excitation wavelengths. Thus, changes which occur in the spectra of the coal liquid as a function of excitation wavelength (Fig. 3) aid in the identification of the PAHs present in the sample. A sample such as this contains hundreds of different PAHs and complete identification of all of them is not possible, but the different ring classes are easily distinguished.

Another example of PAH detection in a complex matrix[15] is illustrated in Fig. 4. The carcinogenicity of certain PAH derivatives has led to an intense interest in their study in biological matrices. Fig. 4A

shows the UVRR spectrum of a calf liver homogenate. Sample preparation consisted only of grinding the liver in a blender and of cell disruption in a tissue grinder. The spectral features may be due to proteins, nucleic acids, riboflavin and other UV absorbing species in the sample. Fig. 4B shows the UVRR spectrum of the liver homogenate which contains 1 mM pyrene. The Raman bands are easily identified as pyrene vibrations (compare to Fig. 4C which is a spectrum of pyrene in acetonitrile). Possible interferences from the liver components (Fig. 4A) are not evident. This demonstrates the feasibility of detecting and identifying PAHs in complex biological matrices with minimal sample preparation. Such analytical procedures using UVRR may have an impact on areas such as PAH metabolism studies in cancer research. We are currently exploring these possibilities[15].

Fluorescence detection is commonly used in conjunction with high-performance liquid chromatography (HPLC). This method provides a sensitive means for detecting an eluant as it comes off the column, but does not spectroscopically identify it. The coupling of other analytical techniques to HPLC has been an active area of research. UVRR–HPLC detection[16] is illustrated in Fig. 5. A representative mixture of PAHs at 10^{-3} M was prepared and separated on a C_{18} silica column. The peaks observed by standard fluorescence detection are shown in Fig. 5A. Several corresponding UVRR spectra are shown in Fig. 5B. Spectra were obtained from 1- or 2-min scans taken as the analyte eluted from the column through a quartz capillary placed in the laser beam. The data are of sufficient quality to identify the PAH eluting. If a bank of UVRR reference spectra for different excitation wavelengths were available, complex mixtures could be separated and the components identified by this method. Such an approach could be applied to coal liquids and petroleum fractions, for example.

UVRR also has direct applications for biochemical analysis. Nucleic acids are amenable to study by UVRR[17]. The aromatic amino acids phenylalanine, tyrosine and tryptophan, have also been studied with UV excitation[18,19]. A UVRR spectrum of myoglobin with 230 nm excitation is shown in Fig. 6. The main features of this spectrum are assignable to tyrosine and tryptophan residues of the protein. Raman bands due to the heme, observed with visible excitation, do not contribute to or interfere with the aromatic amino acid Raman spectra. Similarly, Raman bands due to the protein backbone are also not observed. Changes in the excitation wavelength result in different relative enhancements of tryptophan or tyrosine residues. Useful information is available

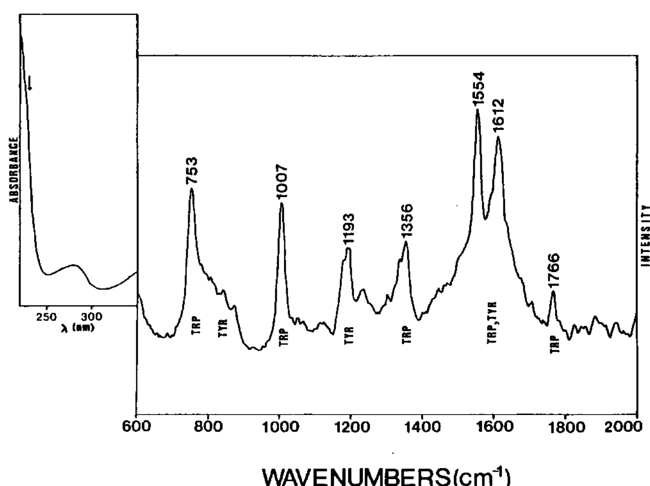

Fig. 6. UVRR spectrum of sperm whale metmyoglobin. Metmyoglobin (0.2 mM) in 0.01 M phosphate buffer (pH 6.8); λ_{ex} = 230 nm; laser power ca. 3 mW. An absorption spectrum of metmyoglobin is shown to the left with an arrow indicating the excitation wavelength. The bands in the Raman spectrum are assigned to tryptophan or tyrosine residues as indicated.

from the spectra of these residues because certain Raman bands of tryptophan and tyrosine have been shown to be environmentally sensitive. These bands can be used to estimate the degree to which the residue is buried within the protein or exposed to the solvent. The degree of ionization of the phenol group of tyrosine can also be probed by UVRR spectroscopy. Very sharp features in the excitation profiles of the aromatic amino acids also indicate the potential for selective enhancement of individual residues of a protein.

UVRR is still in its infancy. As UV lasers become more available and (hopefully) lower in cost, UVRR spectrometers will be accessible to more laboratories. We have illustrated but a few of the potential analytical applications of this spectroscopic tool. Beyond solely analytical uses, UVRR studies, particularly excitation profiles, will lead to a new fundamental understanding of the excited states of many molecules. UVRR studies are expected to become commonplace among practicing Raman spectroscopists in the near future. The potential of this technique for analytical applications will make it a routine analytical tool.

Acknowledgements

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References

1 M. D. Morris and D. J. Wallan, *Anal. Chem.*, 51 (1979) 182A.
2 A. J. Melveger (Editor), *Resonance Raman Spectroscopy as an Analytical Tool*, Franklin Institute Press, 1977.
3 F. B. Bradley and C. A. Frenzel, *Water Research*, 4 (1970) 125.
4 S. F. Baldwin and C. W. Brown, *Water Research*, 6 (1972) 1601.
5 K. M. Cunningham, M. C. Goldberg and E. R. Weiner, *Anal. Chem.*, 49 (1977) 70.
6 M. D. Morris, *Anal. Chem.*, 47 (1975) 2453.
7 M. S. Rahaman and M. D. Morris, *Talanta*, 23 (1976) 65.
8 M. D. Morris, *Anal. Lett.*, 9 (1976) 469.
9 P. K. Dutta, J. R. Nestor and T. G. Spiro, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (1977) 4146.
10 J. F. Verdieck, R. J. Hall, J. A. Shirley and A. C. Eckbreth, *J. Chem. Educ.*, 59 (1982) 495.
11 S. A. Asher, C. R. Johnson and J. Murtaugh, *Rec. Sci. Instrum.*, 54 (1983) 1657.
12 S. A. Asher, *Anal. Chem.*, 56 (1984) 720.
13 S. A. Asher and C. R. Johnson, *Science*, 225 (1984) 311.
14 C. R. Johnson and S. A. Asher, *Anal. Chem.*, 56 (1984) 2258.
15 C. M. Jones and S. A. Asher, in preparation.
16 T. A. Naim, C. M. Jones and S. A. Asher, in preparation.
17 M. Pézolet, N. T. Yu and W. Peticolas, *J. Raman Spec.*, 3 (1975) 55.
18 C. R. Johnson, M. Ludwig, S. O'Donnell and S. A. Asher, *J. Amer. Chem. Soc.*, 106 (1984) 5008.
19 M. Ludwig and S. A. Asher, in preparation.

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Porphyrin analysis by reversed-phase high-performance liquid chromatography: biomedical applications

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Reversed-phase high-performance liquid chromatography has considerable advantages in porphyrin analysis since many of the extraction and esterification steps required for normal-phase high-performance liquid chromatography can be eliminated. This technique is of increasing importance for the diagnosis of porphyrias and in other clinical and experimental studies of porphyrins.

Since before Hans Fischer commenced his Nobel prize-winning synthesis of protohaem, the analysis and separation of porphyrins have presented chemists and biochemists with severe problems. Indeed it is extremely impressive that the steps of haem biosynthesis were elucidated by workers who had only the most rudimentary of chromatographic techniques. Less than a decade ago the problem was eased considerably by the demonstration in several laboratories that porphyrins could be analysed as their methyl esters using normal-phase high-performance liquid chromatography (HPLC) on silica columns with organic eluents. This was of particular importance for the diagnosis of porphyrias in patients by examination of excreted porphyrins. Porphyrias are caused by hereditary or sometimes acquired disorders in haem biosynthesis.

Although normal-phase HPLC has been most useful in its application to the analysis of synthetically and biologically derived porphyrins, the latter require time-consuming extraction and esterification steps with the risks of sample loss and selective destruction. In the last few years the use of reversed-phase HPLC for porphyrin analysis has become increasingly attractive since it obviates the necessity for many of the extraction and derivatization stages. Porphyrins in aqueous solution can be injected directly onto the hydrophobic columns and the components eluted with mixtures of aqueous and polar organic solvents. In this article we shall describe a

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