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Note

In situ identification of paper chromatogram spots by surface enhanced Raman scattering

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Color reactions and R_F values are often used in the identification of paper chromatogram spots. They are, however, unreliable, time consuming and unsuitable for the analysis of unknowns. Optical spectroscopic techniques appear to be suitable methods for the direct identification of paper chromatogram substances. Among those techniques, diffuse reflectance infrared fourier transform (DRIFT) spectrometry is probably the most popular detection technology because it provides detailed, fine structure IR spectra[1]. There are, however, disadvantages to this technique such as it uses a costly apparatus, has a low detector sensitivity, has a high background absorption, and it requires a long time to acquire a spectrum[1].

The Raman scattering technique is considered promising because of its independence from moisture and background absorption. It is not, however, practical as an analytical method because Raman cross sections are normally too weak to be detected. Any technique capable of enhancement and hence, detection of the Raman scattering of chromatographic fractions at nanogram levels would, therefore, be a major contribution to analytical methodology.

Raman scattering enhancements can sometimes occur when molecules are adsorbed on specific metal surfaces. These remarkable enhancements, which are frequently five or six orders of magnitude higher than in the absence of metal surfaces, have been observed for molecules adsorbed on Au, Ag and Pt on either rough electrode or deposited surfaces and also on colloidal hydrosols[2]. The latter provide a convenient system for surface enhanced Raman scattering (SERS) since they can be prepared easily[3–5]. Such considerations prompted initiation of the present study to use SERS as an analytical method for the *in situ* identification of chromatographic fractions at very low concentrations. It will be demonstrated in this communication that silver colloidal hydrosols enhance the Raman scattering of paper chromatogram spots and that this enhancement is dependent on the interaction between the substrate, silver particles and paper fibers, and therefore, on the type of paper used. Using this technique, nanogram amounts of various dyes (crystal violet, malachite green, and basic fuchsin) separated by paper chromatography were detected and identified directly with an argon ion laser of only 4 mW.

EXPERIMENTAL

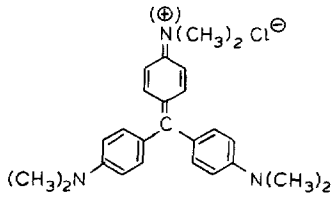
The 0.33-mm thick (3MM), P-81 cation-exchange and DE-81 anion-exchange chromatographic papers (Whatman) were employed. Crystal violet (Aldrich), malachite green (Eastman) and basic fuchsin (Fisher) were used as received. A stock solution was made by dissolving these three dyes together in methanol to give a final concentration of $3 \cdot 10^{-5}$ *M* for each dye. A volume of 7 μl of this stock solution was spotted on the chromatographic papers and then were dried by an air dryer. All ascending chromatograms were developed in an air-tight tank in which the vapor phase was saturated with solvent mixture containing water–*n*-butanol–formic acid (125:9:1). Development time took about 2 h. The developed paper chromatogram was dried by an air dryer and then was sprayed to wetness with silver colloidal hydrosols by a spray atomizer, Raman spectra of the wet chromatogram were then taken. By taking the Raman spectra at different positions within the chromatograms, the locations and identifications of each dye were established.

Silver colloidal hydrosols were prepared by reducing silver nitrate aqueous solution with sodium borohydride, according to the procedure described earlier[3–5].

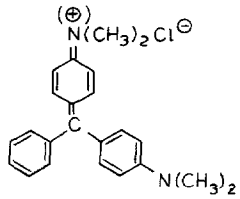
Raman spectra were taken with a coherent CR-15 argon ion laser as an excitation source. The 5145-Å laser line intensity, after passing the beam through a series of neutral density filters, and an interference filter to remove unwanted plasma lines, was 4 mW. The unfocused laser beam was parallel to the slit of the double monochromator (Spex Model 1403). The Raman scattered light (90°) was collected by a 6:1 90° off-axis ellipsoidal mirror focused onto the slit of the monochromator, and was detected by a dry-ice cooled EMI S-20 extended photomultiplier and a conventional photon counting device. Generally the monochromator slits were set at 5 cm^{-1}, integration time was 10 sec at 3000 count scale, and scanning speed was 0.5 cm^{-1} sec^{-1}.

RESULTS AND DISCUSSION

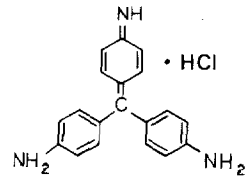
The three structurally similar dyes, crystal violet (CV), malachite green (MG) and basic fuchsin (BF) (Scheme 1) were chosen in order to show the potential of the Raman scattering technique for the direct identification of chromatogram spots. Since the absorption spectra of these three dyes are similar and their fluorescence quantum yields very low, *i.e.*, 10^{-4} for CV[6] and MG[7], and 10^{-2} for BF[8], make it very difficult to use these techniques for identification. A 7-μl mixture containing the three dyes at $3 \cdot 10^{-5}$ *M* each were separated well on 3MM and on P-81 cation-



CRYSTAL VIOLET (CV)



MALACHITE GREEN (MG)



BASIC FUCHSINE (BF)

Scheme 1.

exchange chromatographic papers using water–*n*-butanol–formic acid (125:9:1) as developing solvents. No Raman scattering was observed from these separated dye spots on the developed chromatographic papers when excited with a 4-mW, 514.5-nm laser. This is probably due to the low Raman cross section of the three dyes. Increasing the dye concentrations on the paper over a wide range did not result in any observed Raman signal. Since these dyes are such good absorbers in the visible region, there will be self-absorption of any Raman scattering at high concentration, leading to this observation. Strong Raman scattering signals were measured on a 3000 count scale when the developed chromatogram was sprayed with silver colloidal hydrosols. The Raman spectra of the three spots on the chromatogram, together with the blank consisting of silver colloidal hydrosol on paper are shown in Fig. 1. Each spot of the chromatogram was identified by comparing its SERS with the SERS obtained on a developed chromatogram containing a single dye, and also by comparing it with the published Raman spectra[8,9]. To the investigator's knowledge, this is the first application of SERS for the *in situ* detection and identification of chromatogram spots of nanogram levels excited with a 4-mW unfocused laser. Furthermore, the observed spectra are relatively free of any background scattering from residual solvents or from the paper itself (baseline of silver colloids on blank developed 3MM paper is shown in Fig. 1d). This is because these silver colloids enhanced resonance Raman spectra were obtained by the exclusive excitation at the 514.5-nm absorption line of the dyes ($\lambda_{514.5} = 5.0 \cdot 10^3\ M^{-1}\ cm^{-1}$, $1.0 \cdot 10^3\ M^{-1}\ cm^{-1}$ and $6.8 \cdot 10^3\ M^{-1}\ cm^{-1}$ for CV, MG and BF, respectively). The continuum background (Fig. 1d) is probably due to the fluorescence of impurities in the chromatographic paper as suggested by other workers[10]. These explanations are also based on the observation that this background disappeared when the excitation wavelength was changed from 514.5 nm to 632.8 nm. As expected from their similar structures, the SERS of CV, MG and BF are very similar. Generally the SERS of these dyes are similar to their reported Raman spectra in water. The band at 210 cm^{-1} of CV is of particular interest because it is due to the breathing vibration of the central bonds[9]. This breathing of the central bonds shifts from 210 cm^{-1} in CV to 230 cm^{-1} in MG.

This was expected since there is one $-N(CH_3)_2$ group for each of the three benzene rings of CV (Schene 1) while there are only two benzene rings of the MG having $-N(CH_3)_2$ groups so that there is more freedom for the breathing in MG and hence shift in this band to the higher frequencies[3]. The effect is more pronounced

TABLE I

R_F VALUES OF VARIOUS DYES ON 3MM AND P-81 CATION-EXCHANGE CHROMATOGRAPHIC PAPERS AND DEVELOPED WITH MIXTURE OF WATER–*n*-BUTANOL–FORMIC ACID (125:9:1)

Compound	*R_F Values*	
	P-81 paper	*3MM paper*
Crystal violet	0.33	0.32
Malachite green	0.50	0.48
Basic fuchsin	0.40	0.07

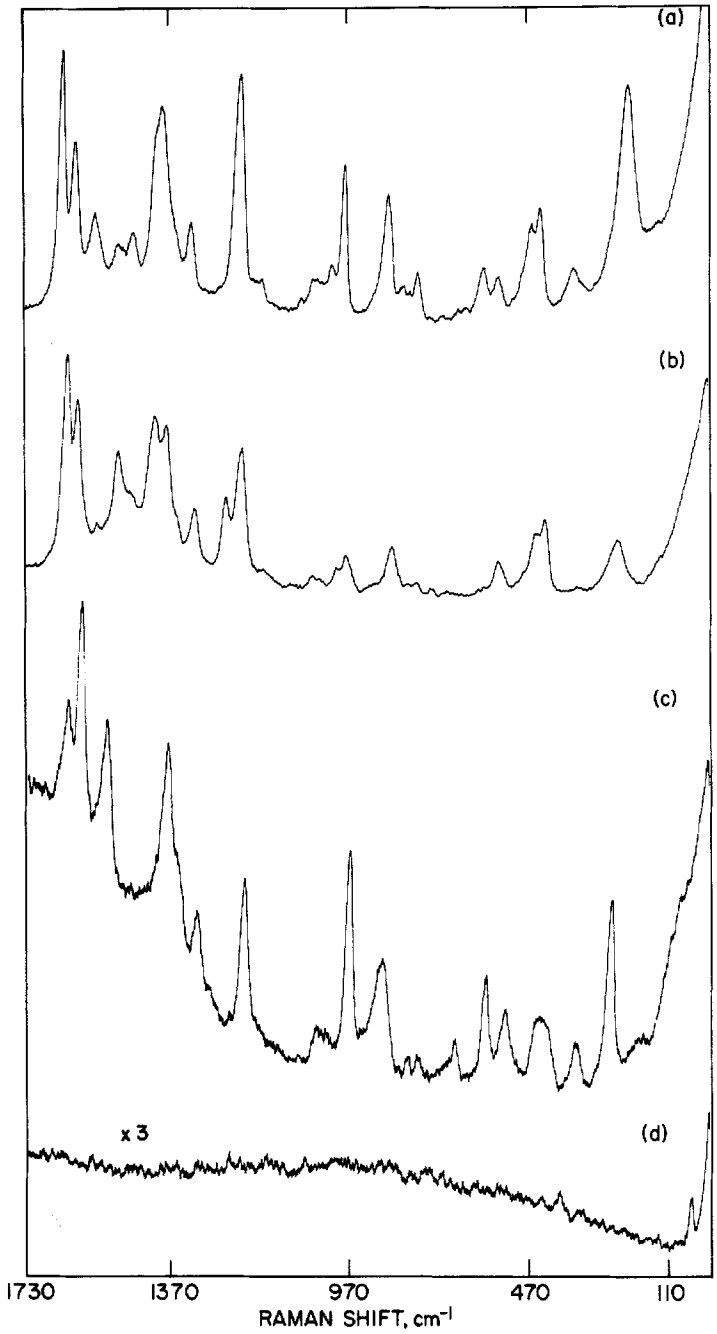


Fig. 1. SERS of 3MM chromatogram spot (85 ng/1.4 cm^2) of (a) crystal violet; (b) malachite green and (c) basic fuchsin. (d) is the baseline in the absence of the dye.

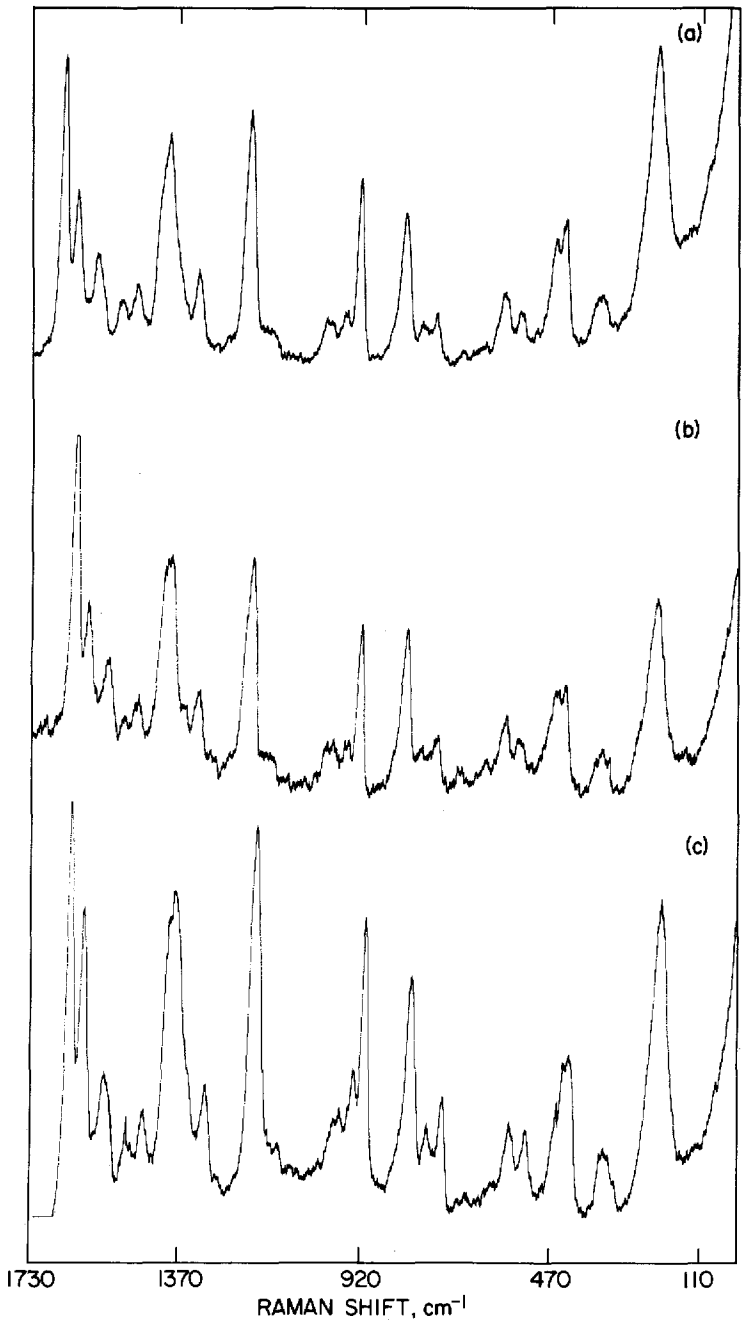


Fig. 2. SERS of a chromatogram spot of crystal violet on (a) 3MM; (b) P-81 cation exchange and (c) DE-81 anion-exchange papers.

in the case of BF: the band shift to 252 cm^{-1} because in BF the three $-N(CH_3)_2$ are replaced by three much smaller $-NH_2$ groups (Scheme 1).

The SERS is so sensitive that it can be used to locate the exact size and location of developed spots. The R_F values of CV, MG and BF on 3MM and on P-81 cation-exchange chromatograms are shown in Table I. Each value was the average of at least three independent experiments. CV, MG and BF were separated well on a 3MM or on P-81 cation-exchange papers. No separation was observed when three dyes were applied on a DE-81 anion-exchange paper. The developed dye spots on 3MM and P-81 papers spread from a applied spots of 0.5 cm to slightly tailed spots of about 0.7 cm × 2 cm. The amount within the chromatographic dye spots shown in Fig. 1 was 7 μl of a $3 \cdot 10^{-5}$ M solution or 85 ng on a 1.4-cm^2 area.

It is of interest to study the effect of different chromatographic papers on SERS. The three SERS spectra shown in Fig. 2 were taken on chromatograms with the same silver and CV concentrations and under the same conditions. The only difference is that the type of chromatographic papers used: 3MM paper (Fig. 2b), P-81 cation-exchange paper (Fig. 2b) and DE-81 anion-exchange paper (Fig. 2c). The SERS intensities are highest on DE-81 paper and lowest on P-81 paper. A variety of reasons might account for this observation but the most likely one is the difference in the average distance between the CV dye and silver particle. As SERS is a short range interaction process[2,7], the closer the dye to the silver particles, the higher are the SERS intensities. In the P-81 paper, the cellulose fibers possess negative charges so that their electrostatic interactions with the positive CV dye may be stronger than the dye–silver interactions, resulting in less dye adsorption on silver particles, *i.e.*, the average distance between dye and silver particle increases leading to a lower SERS. The hydrogen bonding interactions of dye-paper fibers in the 3MM paper are probably not as strong as the electrostatic interactions in the P-81 paper. Consequently, the SERS in 3MM are higher than in P-81 but lower than in DE-81. The highest SERS observed on DE-81 paper is probably due to the repulsion between the positive paper fibers and the positive dye allowing the dye–silver particles interaction to remain unchanged.

It should be noted that there are reports on using the Raman scattering technique for the analysis of thin layer chromatogram spots[11,12]. Reported limits of detection (LOD) are relatively high, *i.e.*, in the order of micrograms. This high LOD is probably due to the necessity of the relatively long time for signal averaging to achieve a reasonable spectrum, due to a low normal Raman cross section, and also to a great deal of background scattering. Consequently, a subtraction is required to obtain the Raman spectrum of the chromatographic spot. Furthermore, there is also concern about sample heating and photodecomposition from the high power lasers that were probably used (laser power and focusing length were not given[11,12]).

The technique reported here employs silver colloidal hydrosols to enhance resonance Raman scattering of chromatogram spots so that the observed Raman cross sections are approx. 9 to 10 orders of magnitude higher than those observed with non-enhanced normal Raman scattering system[13]. Consequently, only a small amount of sample is required and the excitation laser can be unfocused and of very low intensity (4 mW). Thus, any concerns about effects due to heating and sample photodecomposition can be dismissed.

The limit of detection for this technique, defined as the dye spotted amount

that yields a signal to noise ratio of two when excited with a 4-mW, 514.5-nm laser, is estimated to be about 2 ng/cm^2 for these three dyes. As the sensitivity of the SERS depends on the number of analyte molecules per unit area of chromatographic surface, a smaller in size the spot size would yield a higher concentration and thereby a higher SERS signal. One disadvantage of paper chromatography is that the spots usually spread to a greater diameter during the development, thereby reducing the potential sensitivity of the capital SERS technique. This spreading is even greater in the case of the three dyes as the developed chromatogram contained three separated slight tails of about 0.7 cm × 2 cm dimension. Thus, it is expected that the excellent 2-ng LOD can be still further improved by using an analyte which yields a smaller developed spot.

It has been demonstrated that by using a simple and inexpensive method, chromatogram spots can be identified directly in a relatively short time. The low laser power employed (4 mW) suggests that a cheap air-cooled portable argon ion or an optical alignment He–Ne laser can be used in this technique. Furthermore, by tuning the excitation wavelength, this technique can be used for a variety of substrates. Experiments are now in progress to expand the application of this technique to other substrates separated by thin-layer chromatography.

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