New Method for the Microscopic, Nondestructive Acquisition of Ultraviolet Resonance Raman Spectra from Plant Cell Walls

ANDREW D. CZAJA,* ANATOLIY B. KUDRYAVTSEV, and J. WILLIAM SCHOPF

Department of Earth & Space Sciences, University of California, Los Angeles, California 90095-1567 (A.D.C., J.W.S.); Institute of Geophysics & Planetary Physics (Center for the Study of Evolution and the Origin of Life), University of California, Los Angeles, California 90095-1567 (A.D.C., A.B.K., J.W.S.); NASA Astrobiology Institute, Center for Astrobiology, University of California, Los Angeles, California 90095-1567 (A.D.C., A.B.K., J.W.S.); and Molecular Biology Institute, University of California, Los Angeles, California 90095-1570 (J.W.S)

Raman spectroscopy has long been used for the chemical analysis of organic matter, including natural products, using excitation wavelengths in the visible, infrared, or ultraviolet portions of the spectrum. The use of ultraviolet resonance Raman spectroscopy (UVRR) to study bulk samples of plant tissue has typically been carried out by rotating homogeneous macro-samples beneath the laser beam in order to minimize the amount of UV radiation impinging on any one spot, thereby avoiding its potentially damaging effects on the organic matter analyzed. This paper extends the use of UVRR to the study on a microscopic scale of individual plant cell walls by use of the controlled micro-displacement of a sample.

Index Headings: **Ultraviolet resonance Raman spectroscopy; UVRR; Plant cell walls; Xylem; Lignin.**

INTRODUCTION

Raman spectroscopy is widely used for the molecular-structural analysis of organic materials. However, the damage to a sample that can result from the use of an excitation laser focused on a micrometer-sized spot is a well-known problem that must be overcome during analysis of such materials, one that has been addressed by many authors, especially those studying individual living cells[1,2] and other sensitive biological materials, such as proteins.[3,4] This problem is especially significant in the analysis of samples having a weak Raman response (such as those analyzed here) and that therefore require relatively long acquisition times to produce an adequate signal-to-noise ratio. Such damage results from excessive laser energy (which is equal to the laser power multiplied by the duration of exposure of the sample)† that, as it impinges on and is absorbed by the sample, leads to thermal breakdown of chemical bonds in the material analyzed. When such damage occurs, the spectra acquired do not accurately reflect the molecular structure of the material studied.

Three types of laser excitation are currently used for Raman spectroscopy: infrared (IR), visible (VIS), and ultraviolet (UV). Of these, Raman systems using IR excitation would appear to be optimal for analysis of organic materials because their wavelengths are lowest in energy and therefore radiation damage of an analyzed sample is unlikely to occur. However, IR Raman is not always the method of choice because each type of excitation has its strengths and limitations as applied to various materials. This paper deals with the acquisition of UV resonance Raman (UVRR) spectra from the organic matter making up plant cell walls, and discussion will therefore be limited largely to such material.

Visible Raman spectroscopy has been used effectively for the analysis of individual plant cell walls[5–9] and paper pulps[10] because the visible wavelengths typically used are, like those of IR Raman, of sufficiently low energy to typically not cause thermal damage to such materials. Such studies, however, have required special experimental conditions (i.e., use of either particular microscope objective apparatus[5–8] or a Kerr gate[9,10]) to minimize the fluorescence produced when aromatic structures, such as those present in lignin, a major component of secondary plant cell walls, are excited by the wavelengths typically used for VIS Raman. This very strong fluorescent response serves to "swamp out" the much weaker Raman signal. Because lignin is present in wood, and residual lignin and other similarly fluorescent chromophores are present in processed paper pulp, IR Raman and UVRR (use of which does not induce fluorescence in the spectral range of analytical interest) have been routinely used by those studying plant and/or paper chemistry.[11–21] In such analyses, UVRR has proven particularly useful because the resonance effect produced by UV light on aromatic structures greatly enhances the spectral components resulting from such structures relative to those from non-aromatic structures (the most prevalent being those from cellulose).[13] This effect permits the detection of minute quantities of lignin in paper pulp, providing extremely sensitive means for evaluating the efficacy of various methods of lignin removal.[13] However, because UV radiation is more highly energetic than IR or VIS radiation, bulk samples are rotated during analysis under the laser beam so that no single spot receives laser energy sufficient to cause degradation.[13,22,26]

Raman analyses have also been carried out on living microorganisms, both bacterial[27–34] and eukaryotic.[35,36] In order to avoid damage to such specimens studied using UV excitation, spectra were acquired from circulating concentrated suspensions of pure cultures.[27,28,30] Individual cells of such microorganisms have also been analyzed, but by use of lower energy visible excitation toward the red end of the spectrum.[32,34,36]

We report here the development of an additional solution to the problem posed by the damage that can be thermally induced in organic materials during UVRR spectroscopy. This new technique permits *in situ*, nondestructive spectroscopic analysis of microscopic structures, illustrated here by studies of the lignin-rich walls of individual fern xylem cells.

EXPERIMENTAL

Materials. We analyzed the walls of xylem cells (water-conducting tube-like structures) from the rhizome (underground stem) of the fern *Dennstaedtia cicutaria* (Fig. 1).

Received 9 December 2005; accepted 6 February 2006.

* Author to whom correspondence should be sent. E-mail: aczaja@ess.ucla.edu.

† The absorption efficiency of the material is also important, but since it is a function of the laser wavelength used and the given material, we will not consider it further.

0003-7028/06/6004-0352$2.00/0

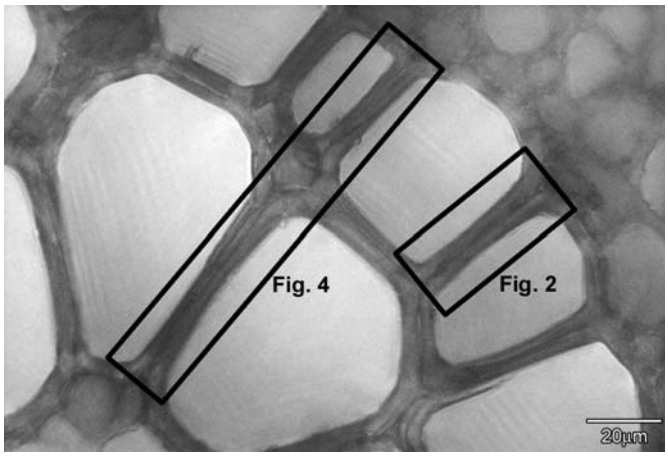

FIG. 1. Photomicrograph of the fern xylem cell walls studied here, in transverse section. The walls indicated by the rectangles at the right and center are those for which spectra are shown, respectively, in Figs. 2 and 4.

Rhizome specimens were cleaned of soil and sliced by hand with a methanol-cleaned razor blade to a thickness of ~0.5 mm. These thin slices were then soaked in Millipore-filtered water for ~30 min and rinsed five times in aliquots of such water to remove soluble material (e.g., cytosol and starch). The slices were dried and stored in a vacuum desiccator over Drierite while held between two glass microscope slides. For analysis, the slices were rehydrated with fresh Millipore-filtered water, supported on a fused silica microscope slide, and covered by a fused silica coverslip (use of fused silica being necessary due to its transmissibility to UV radiation).

Raman Spectroscopy. The Raman instrument used in these experiments was a UV-capable JY Horiba T64000 triple-stage laser-Raman system, having macro-Raman and confocal micro-Raman capabilities, coupled with an Innova 90C FreD frequency doubling Ar^+ laser (Coherent, Inc., CA) having a wavelength of 244 nm, a source output reduced to ~1 mW (calculated to be ~0.04 mW at the sample), and a spot size of ~2–3 μm. The cell walls were located and positioned by use of an Olympus BX41 microscope equipped with a 40× UV objective (OFR Inc., NJ) and a motorized high precision stage (SCAN 75 × 50, Märzhäuser GmbH & Co. KG).

RESULTS AND DISCUSSION

Traditional Raman spectroscopic data collection involves the acquisition of spectra for tens to hundreds of seconds at a single point in order to achieve an acceptably high signal-to-noise ratio (S/N). However, single-point UVRR analyses over such periods of time produce damage to the xylem samples studied here, even at low laser power (~0.04 mW at the sample). For example, spectra acquired from a single point on a cell wall before (Fig. 2a, upper spectrum) and after (Fig. 2a, lower spectrum) laser exposure for 50 s are dramatically different, the overall intensity of the latter spectrum being much lower. That this intensity change is caused by UV laser radiation damage is shown by photomicrographs of the analyzed cell wall (indicated in Fig. 1) obtained before (Fig. 2b) and after (Fig. 2c) exposure to the laser beam.

To develop a method of spectra acquisition that can be used to avoid such damage, we first determined experimentally that exposure to ~0.04 mW of focused laser power for ~1–2 s produces no damage at a given spot on a cell wall. However,

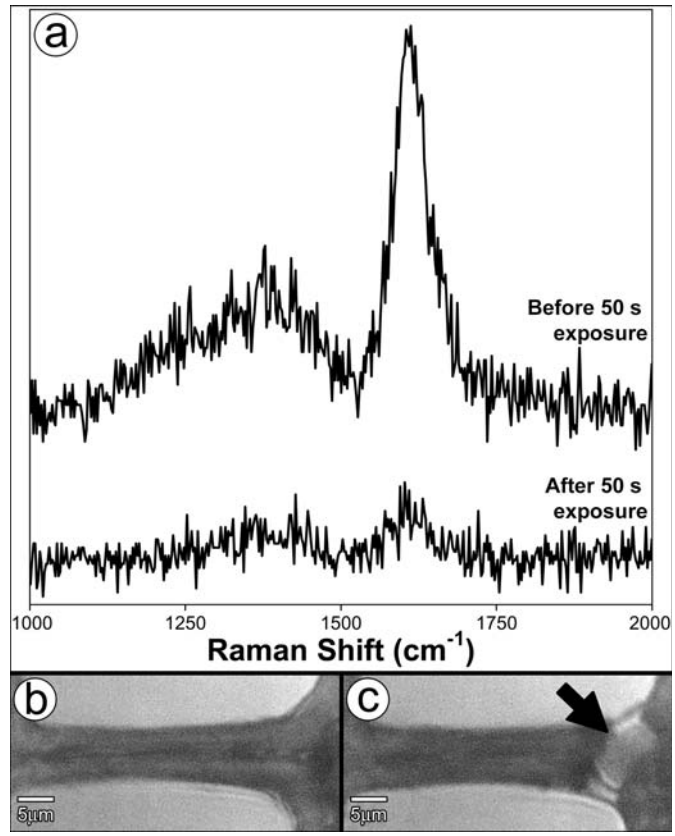

FIG. 2. (**a**) Typical UVRR spectra acquired using a standard technique. The upper spectrum in (**a**) was acquired for 1 s from a single spot on a xylem cell wall; the lower spectrum was acquired from the same spot for 1 s after prior exposure to the laser for 50 s, showing the results of excessive laser energy. No processing was performed on these spectra. (**b** and **c**) Photomicrographs of the cell wall analyzed using a traditional single-point technique, taken (**b**) before and (**c**) after acquisition of Raman spectra, showing the analysis spot (at arrow) and heat-induced physical damage.

single spectra, acquired at this low power and short duration, do not possess an adequate S/N (see the upper spectrum in Fig. 2a) because of the weak Raman response of plant cell walls. Hence, given the chemical homogeneity along the length of such walls at the scale of the laser beam spot-size, we then devised a technique by which to acquire a series of spectra along a given cell wall measured at low power for short duration that could be averaged to produce a single resultant mean spectrum having a high S/N that accurately represents the molecular-structural characteristics of the wall analyzed.

To our knowledge, this multiple-point method of spectral acquisition, detailed below, has not previously been described. By use of the optical microscope attached to the Raman system, a cell cross-section oriented parallel to the plane of the microscope stage was selected for analysis, a procedure necessary in order for the wall to be in focus along its entirety during spectral acquisition. The imaging feature of the computer program that controls the Raman system (LabSpec v4.14, JY Horiba, Inc.) was then used to pre-select points for spectral acquisition. Either a straight line along a segment of a cell wall was designated by use of the "line function" of the program, or a series of points along the periphery of an entire wall was plotted by use of the "points function," denoting the locations where point spectra were to be collected. Data shown here were acquired by use of the "line function," illustrated in

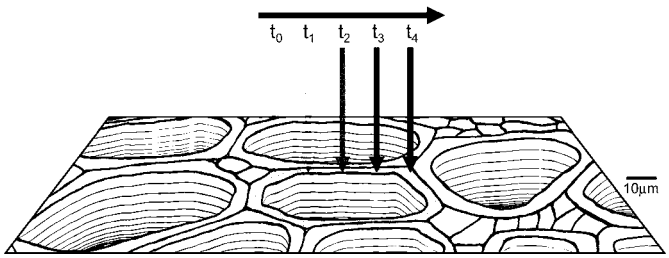

FIG. 3. Schematic drawing of fern xylem cells in a transverse section illustrating the LabSpec "line function" technique used for acquisition of UVRR spectra. The laser, represented by the vertical arrows, was focused on the left end of the cell wall segment and spectra were acquired from left to right at ~2 µm increments for 1 s each at times t_0 through t_4. The horizontal arrow shows the movement of the excitation laser beam relative to the cell wall.

Fig. 3. Once spectra accumulation began, the stage automatically moved a designated distance along the specified path. Each set of measurements acquired from multiple points along each cell wall was then averaged to produce a mean spectrum for that cell wall.

Our data (Fig. 4a) show that by use of this new technique UVRR spectra having a high S/N (~90) can be collected from individual plant cell walls (Fig. 4a, upper spectrum) and that such spectra have a much higher S/N than that of any of their component single-point spectra (Fig. 4a, lower spectrum). Figures 4b and 4c show photomicrographs of a cell wall (indicated in Fig. 1) obtained, respectively, before and after acquisition of the upper Raman spectrum shown in Fig. 4a, illustrating that the cell wall was not damaged during these measurements.

For the materials here studied by this new technique, three parameters define the laser energy absorbed at each analyzed point: (1) the laser power, (2) the duration of laser exposure at each analyzed point, and (3) the spacing of the points analyzed. This last parameter is important because if the points analyzed are not spaced sufficiently far apart, laser-induced heat can "bleed" into the immediate vicinity of an analyzed spot causing localized excessive heating and alteration of the cell wall. In the present study, the optimal settings for the three parameters were determined experimentally to be a laser power of ~0.04 mW (at the sample), a total exposure time of 1 s at each analyzed point, and a spacing between sequential points of ~2 µm. Several sets of conditions were investigated before this optimal set was determined. It should be noted, however, that these settings are not optimal for all materials and that they must be determined experimentally on a case-by-case basis. For example, data collected (not shown) indicate that in a single fern axis, the cell walls of different tissues (e.g., xylem and cortical parenchyma), having different biochemical make-ups, can withstand different amounts of UV laser radiation before the onset of thermal damage.

Other equipment, e.g., the Dilor XY (JY Horiba, Edison, NJ) 0.8 m triple-stage system with confocal line-scanning capabilities, is available that could help avoid the damaging effects of UV radiation while recording Raman spectra of organic materials. However, as implied by the name, line-scanning is limited to measurement of spectra along a straight line, whereas the new technique described here provides means to measure not only along a line but also along any complex curve using the "points function." In the present study, measurement of spectra along straight segments of xylem cell walls of sufficient length to provide reasonable S/N ratios was possible. Such long, straight cell wall segments are not universal features of plant tissues, however, necessitating the use of the "points function" in certain circumstances. Another advantage of this technique is that it can be implemented on existing Raman systems by the addition of a motorized, high-precision microscope stage and appropriate software, a solution that is simpler than adding line-scanning optics.

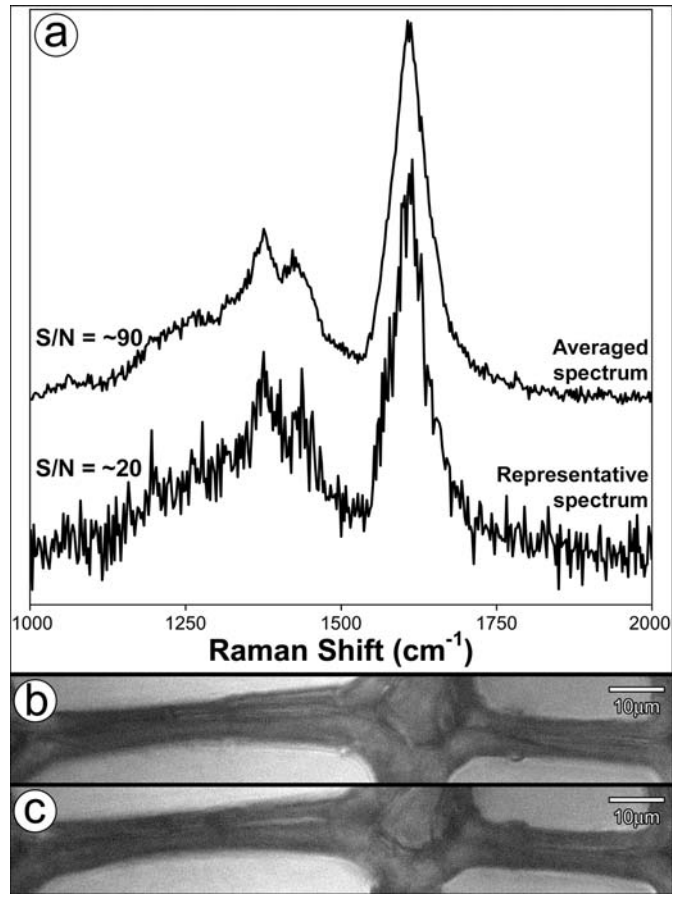

FIG. 4. (**a**) UVRR spectra acquired using the new method described here. The lower spectrum (S/N ~ 20) in (**a**) is a representative example of 51 spectra acquired for 1 s each along a single cell wall segment; the upper spectrum (S/N ~ 90) is the average of these 51 spectra. No processing was performed on these spectra other than being normalized to the strongest band (centered at ~1600 cm^{-1}). (**b** and **c**) Photomicrographs of the cell wall analyzed using the new technique described here taken (**b**) before and (**c**) after acquisition of Raman spectra.

ACKNOWLEDGMENTS

For assistance in preparing this manuscript, we thank A. B. Tripathi and J. Shen-Miller. This research was supported by NASA Exobiology Grant NAG5-12357 (to JWS). ADC is an NSF Pre-doctoral Fellow and a Fellow in CSEOL, the UCLA/IGPP Center for the Study of Evolution and the Origin of Life. We also thank Clinton Morse of the University of Connecticut Department of Ecology and Evolutionary Biology for providing the fern specimen.

1. C. Favard, P. Valisa, M. Egret-Charlier, S. Sharonov, C. Herben, M. Manfait, E. Da Silva, and P. Vigny, Biospectroscopy **5,** 101 (1999).
2. F. Sureau, L. Chinsky, C. Amirand, J.-P. Ballini, M. Duquesne, A. Laigle, P.-Y. Turpin, and P. Vigny, Appl. Spectrosc. **44,** 1047 (1990).
3. Q. Wu, G. Balakrishnan, A. Pevsner, and T. G. Spiro, J. Phys. Chem. A **107,** 8047 (2003).
4. G. Niaura, A. K. Gaigalas, and V. L. Vilker, J. Raman Spectrosc. **28,** 1009 (1997).

5. U. P. Agarwal and R. H. Atalla, Planta **169,** 325 (1986).
6. R. H. Atalla and U. P. Agarwal, "Raman microprobe optimization, and sampling technique for studies of plant cell walls", in *Microbeam Analysis,* A. D. Romig, Jr., and J. I. Goldstein, Eds. (San Francisco Press, San Francisco, 1984), pp. 125–126.
7. R. H. Atalla and U. P. Agarwal, Science (Washington, D.C.) **227,** 636 (1985).
8. R. H. Atalla and U. P. Agarwal, J. Raman Spectrosc. **17,** 229 (1986).
9. S. Barsberg, P. Matousek, and M. Towrie, Macromol. Biosci. **5,** 743 (2005).
10. A.-M. Saariaho, A.-S. Jääskeläinen, P. Matousek, M. Towrie, A. W. Parker, and T. Vuorinen, Holzforschung **58,** 82 (2004).
11. U. P. Agarwal and S. A. Ralph, Appl. Spectrosc. **51,** 1648 (1997).
12. P. A. Evans, Spectrochim. Acta, Part A **47,** 1441 (1991).
13. M. Halttunen, J. Vyörykkä, B. Hortling, T. Tamminen, D. Batchelder, A. Zimmermann, and T. Vuorinen, Holzforschung **55,** 631 (2001).
14. A. H. Kuptsov, Vib. Spectrosc. **7,** 185 (1994).
15. I. R. Lewis, N. W. Daniel, Jr., N. C. Chaffin, and P. R. Griffiths, Spectrochim. Acta, Part A **50,** 1943 (1994).
16. L. M. Proniewicz, C. Paluszkiewicz, A. Wesełucha-Birczyńska, A. Barański, and D. Dutka, J. Mol. Struct. **614,** 345 (2002).
17. T. Röder, G. Koch, and H. Sixta, Holzforschung **58,** 480 (2004).
18. T. Röder and H. Sixta, Macromol. Symp. **223,** 57 (2005).
19. C. F. B. Séné, M. C. McCann, R. H. Wilson, and R. Grinter, Plant Physiol. **106,** 1623 (1994).
20. Q. Shen, H. Rahiala, and J. B. Rosenholm, J. Colloid Interface Sci. **206,** 558 (1998).
21. D. Stewart, H. M. Wilson, P. J. Hendra, and I. M. Morrison, J. Agric. Food Chem. **43,** 2219 (1995).
22. M. Nuopponen, T. Vuorinen, S. Jämsä, and P. Viitaniemi, J. Wood Chem. Technol. **24,** 13 (2004).
23. M. Nuopponen, H. Wikberg, T. Vuorinen, S. L. Maunu, S. Jämsä, and P. Viitaniemi, J. Appl. Polym. Sci. **91,** 2128 (2004).
24. M. Nuopponen, S. Willför, A.-S. Jääskeläinen, A. Sundberg, and T. Vuorinen, Spectrochim. Acta, Part A **60,** 2953 (2004).
25. M. Nuopponen, S. Willför, A.-S. Jääskeläinen, and T. Vuorinen, Spectrochim. Acta, Part A **60,** 2963 (2004).
26. A.-M. Saariaho, A.-S. Jääskeläinen, M. Nuopponen, and T. Vuorinen, Appl. Spectrosc. **57,** 58 (2003).
27. M. Baek, W. H. Nelson, and P. E. Hargraves, Appl. Spectrosc. **43,** 159 (1989).
28. K. A. Britton, R. A. Dalterio, W. H. Nelson, D. Britt, and J. F. Sperry, Appl. Spectrosc. **42,** 782 (1988).
29. R. A. Dalterio, W. H. Nelson, D. Britt, J. Sperry, and F. J. Purcell, Appl. Spectrosc. **40,** 271 (1986).
30. R. A. Dalterio, W. H. Nelson, D. Britt, and J. F. Sperry, Appl. Spectrosc. **41,** 417 (1987).
31. W. F. Howard, Jr., W. H. Nelson, and J. F. Sperry, Appl. Spectrosc. **34,** 72 (1980).
32. G. J. Puppels, F. F. M. de Mul, C. Otto, J. Greve, M. Robert-Nicoud, D. J. Arndt-Jovin, and T. M. Jovin, Nature (London) **347,** 301 (1990).
33. P. Rösch, M. Schmitt, W. Kiefer, and J. Popp, J. Mol. Struct. **661–662,** 363 (2003).
34. K. C. Schuster, E. Urlaub, and J. R. Gapes, J. Microbiol. Methods **42,** 29 (2000).
35. S. K. Brahma, P. E. Hargraves, W. F. Howard, Jr., and W. H. Nelson, Appl. Spectrosc. **37,** 55 (1983).
36. Y.-S. Huang, T. Karashima, M. Yamamoto, T. Ogura, and H. Hamaguchi, J. Raman Spectrosc. **35,** 525 (2004).