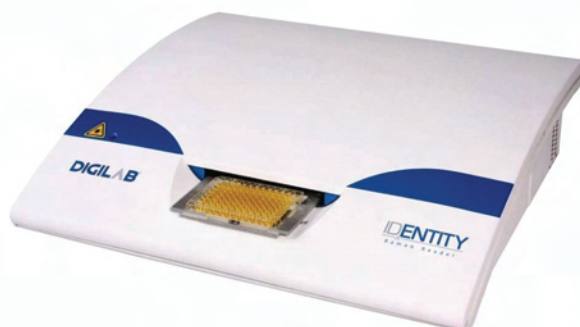




APPLICATION NOTES

IDENTITY
R a m a n R e a d e r



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- 1. Sampling Procedures with the Identity**
 - 2. High Sensitivity Trace Detection using SERS-Active Microtiter Plates with the Identity**
 - 3. Quantitative Analysis of Fructose in Water with the Identity**
-

Sampling Procedures with the Identity Raman Plate Reader

INTRODUCTION

The Identity Raman plate reader can easily accommodate samples in various physical forms, for analysis of liquids, gels, powders, tablets and solids. Switching between sample types is as easy as changing an adaptor in the plate holder of the Identity. This application note will describe the sampling methods, with example spectra, of many of these sample types.

Liquid samples

The default setup of the Identity is for analysis of liquids in the wells of microtiter plates. Simply fill the wells with approximately 200 μ l of liquid (for a 96-well plate), place the plate in the plate holder and load it into the reader. For this configuration the laser beam will focus approximately 2 mm above the plate bottom into the liquid, thereby discriminating against the spectrum of the plate material, as shown in Figure 1. Only material within the focus volume as indicated in

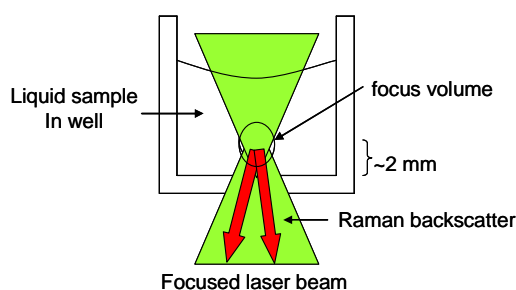


Figure 1. Laser focus into the well for analysis of liquids. The laser beam is focused approximately 2 mm into the liquid, with subsequent collection of the Raman backscatter from material in the focal volume. The 2 mm displacement of the laser focus from the plate material is sufficient to discriminate against the spectrum of the plate.

Figure 1 will contribute to the Raman spectrum. Routine collection of spectra of liquids in the wells can be performed with just a few seconds of integration time per well. Example spectra of organic solvents recorded on the Identity are displayed in Figure 2.

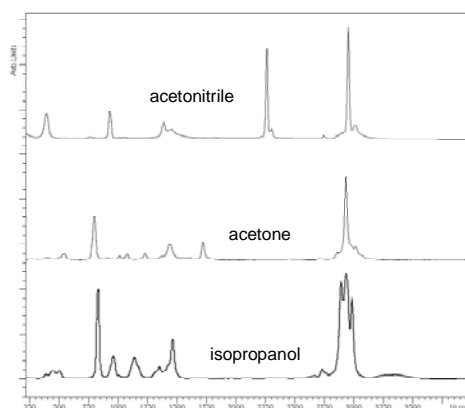


Figure 2. Raman spectra of isopropanol, acetone and acetonitrile (bottom to top) recorded with the Identity Raman plate reader in polystyrene 96-well plates with 532 nm laser excitation. The spectra are free from any contribution from the plate material.

APPLICATION NOTE

Powder samples

Powder samples as well can be analyzed in microtiter plates with the use of a powder adaptor (as shown in Figure 3) to raise the height of the plate, bringing the powder coating on the bottom of the plate into the laser focus as displayed in Figure 4.

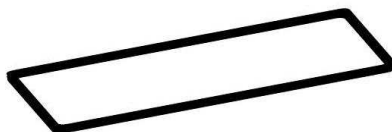


Figure 3. Powder adaptor for Identity Raman plate reader. The adaptor is a plastic frame with outside dimensions that match the format of standard microtiter plates. It is available in a range of thicknesses, from 1.8 to 2.2 mm, which when matched to a specific plate is used to raise the top surface of the well bottom to the focal point of the laser. Glass bottom plates are recommended for use with the powder adaptor to minimize the contribution of the plate material from the sample spectrum.

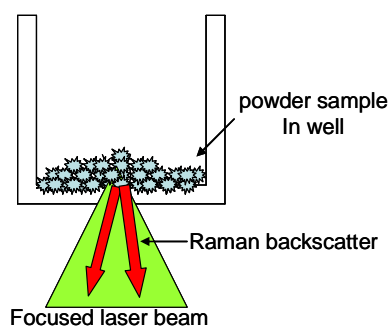


Figure 4. Schematic diagram of the laser focus into the powder at the bottom of the well with the use of the powder adaptor. The powder adaptor raises the height of the plate relative to the laser beam, bringing the top surface of the well bottom into the focus of the laser.

The plate height adjustment with the use of the powder adaptor is necessary because the Raman signal is highly attenuated with a focus 2 mm into the powder sample. The powder adaptor is used by placing it into the plate holder with the plate placed on top of it. Spectra of powdered materials obtained with and without the powder adaptor are shown in Figure 5, demonstrating the enhancement of the spectrum obtained with the powder adaptor.

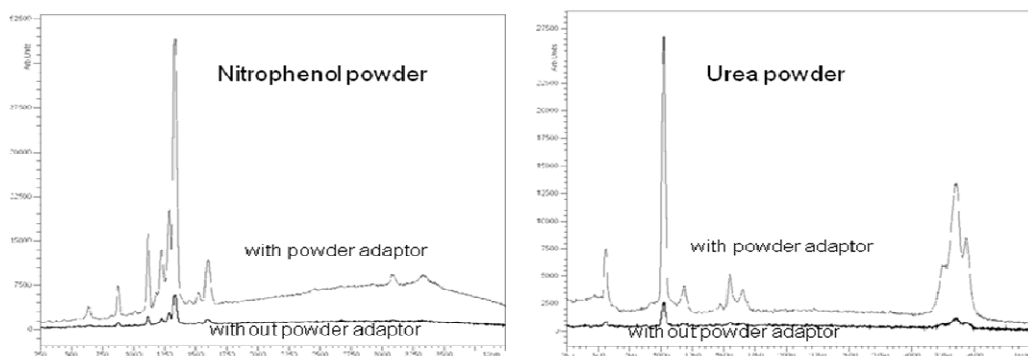


Figure 5. Spectra of nitrophenol powder (left) and urea powder (right) collected with (top) and without (bottom) the powder adaptor in the Identity plate reader with 532 nm laser excitation. Spectra of powdered samples obtained with the powder adaptor are enhanced by approximately a factor of 10 compared to those obtained without it.

APPLICATION NOTE

Tablets

For the measurement of compressed tablets, a tablet plate is available for the Identity reader. Instead of focusing the beam to the top of the bottom well, the tablet plate is designed with a set of machined dimples in a plastic plate, with holes in the center of each dimple, allowing the laser to focus to the bottom surface of the tablet without passing through any plate material. The tablet plate and a schematic cross-section showing the focus of the laser onto the tablet surface is displayed in Figure 6. The tablet holder can hold 32 tablets of either circular or oblong shape.

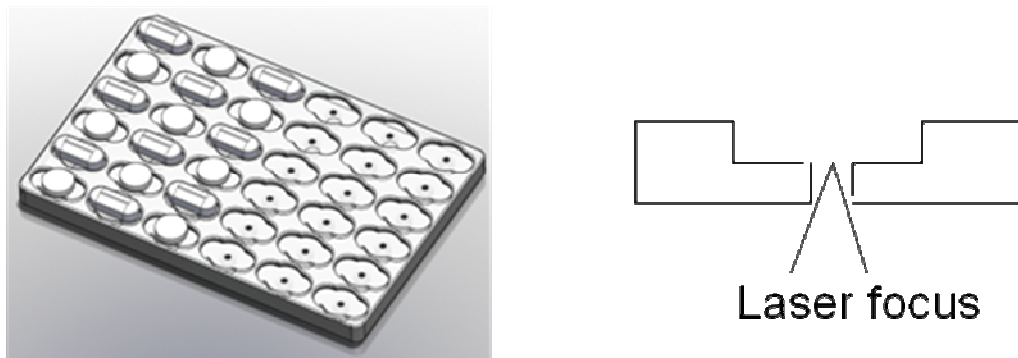


Figure 6. Tablet holder (left) partially filled with circular and oblong tablets, and cross-section of one well (right) showing the focus of the laser onto the bottom surface of the tablet without passing through any plate material.

Representative spectra of over-the-counter pharmaceutical tablets are displayed in Figure 7 using the tablet holder in the Identity with 785 nm laser excitation.

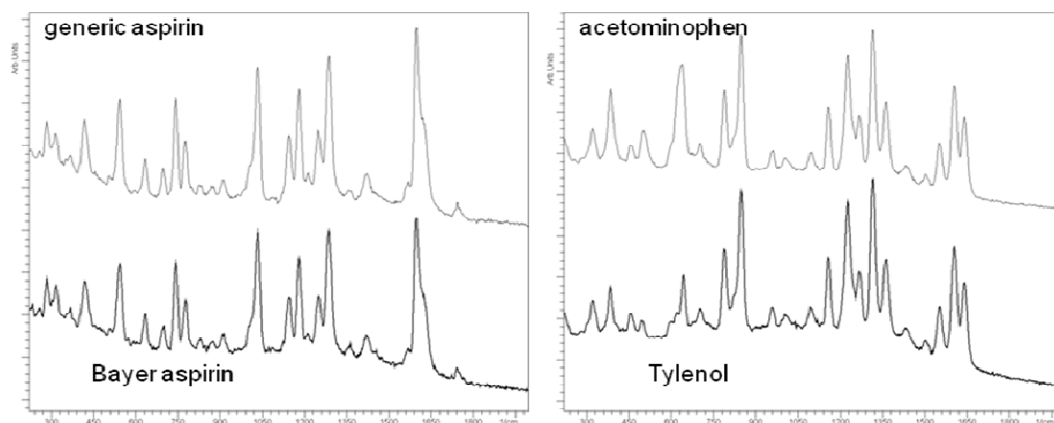


Figure 7. Spectra of over-the-counter pharmaceutical tablets collected with the tablet holder in the Identity plate reader. Spectra of Bayer aspirin (bottom) and generic aspirin (top) are displayed in the left panel, and spectra of Tylenol (Bottom) and generic acetaminophen (top) are displayed in the right panel. All spectra were collected using the Identity with 785 nm laser excitation.

Other Solids

Other solids, such as plastic sheets or films, can be measured using the solids sample plate. The solids sample plate is similar in concept to the tablet plate, with the exception that the plate is flat with 96 holes drilled through it to allow placement of solid samples over the small holes. As in the tablet plate, the bottom surface of the sample will be at the focal point of the laser for ideal sampling geometry, as shown in Figure 8.

APPLICATION NOTE

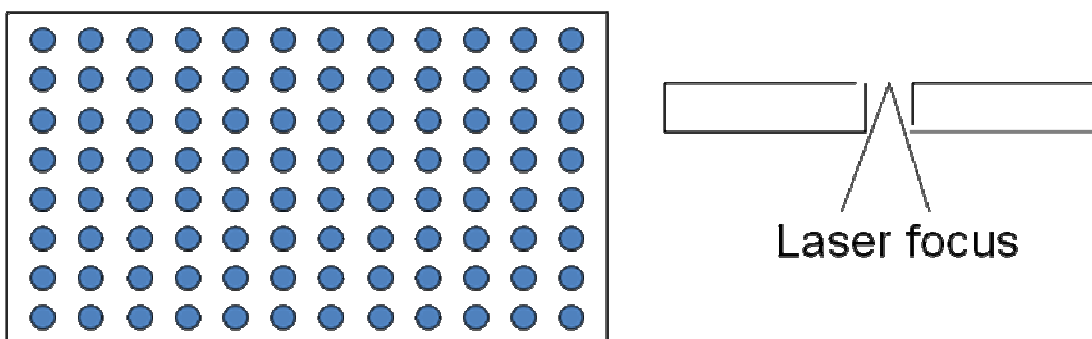


Figure 8. 96-hole Solids sample plate (left) and cross-section of one hole position (right) showing the focus of the laser onto the bottom surface of the solid sample without passing through any plate material.

Spectra of three plastic sheets collected on a 532 nm Identity system using the solids sample plate is displayed in Figure 9.

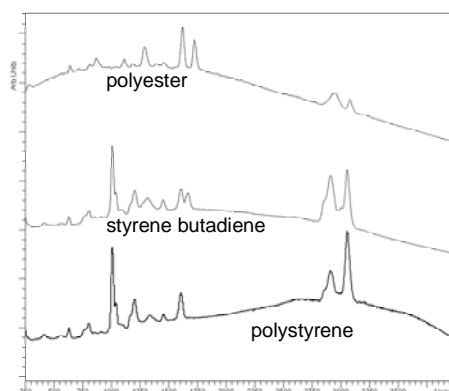


Figure 9. Spectra of polystyrene, styrene-butadiene, and polyester (bottom to top) collected using the solids sample plate in the Identity reader with 532 nm laser excitation.

Conclusion

The Identity Raman plate reader permits easy, high throughput sampling of a variety of sample forms, whether they be liquid, powdered, tablet or solid, with easy changeover using specially designed adaptor plates in the plate holder of the Identity. No adjustments, such as the focus of the laser, of the spectrometer are necessary to accomplish this task, so spectrometer performance is never compromised. High quality Raman spectra can be collected by users from a wide range of backgrounds, from the skilled spectroscopist to the laboratory technician.

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High Sensitivity Trace Detection using SERS-Active Microtiter Plates with the Identity Raman Plate Reader

Introduction

Raman spectroscopy is useful for the analysis of bulk liquids and solids due to its ability to produce chemical “fingerprints” of materials with unique spectral signatures. Such spectra can be used to identify chemical unknowns, to act as a quality control check of known compounds, or to determine the crystalline structure of solids, to name a few applications. The ability of Raman spectroscopy to perform trace analysis, however, is limited because the spontaneous Raman process is inherently weak, with typical detection limits in the pph or ppt range unless enhancement techniques are utilized. One method of increasing Raman sensitivity is surface-enhanced Raman spectroscopy (SERS), which can enhance Raman scattering by six orders of magnitude or more, improving detection limits to the ppb range. A detailed description of the SERS process is beyond the scope of this article, but suffice it to say that in SERS the Raman spectra of compounds attached to metal nano-particles are greatly enhanced by the plasmonic field generated by the metal particles. The reader is directed to a select group of references (1-9) for a more thorough description on the SERS process and theory.

A few previous reports have described SERS substrate preparation techniques for use with microplates (10-13), but all of these reported measurements employed unique SERS-active substrates prepared by the researchers, as well as custom built Raman spectrometer systems or Raman microscope systems. Many laboratories that have the need to measure trace components in high throughput, however, may not have the expertise to synthesize the SERS substrates with high reproducibility, nor the budget to acquire the high performance Raman instrumentation described in the previous research. Here we describe the use of commercially available SERS-active microtiter plates with the Identity, a novel low cost Raman microtiter plate reader, for high throughput trace analysis measurements, and show results which demonstrate the enhancement in the Raman spectrum that can be achieved in this format.

Experimental and Instrumentation

Benzenethiol and benzoic acid were obtained from Sigma Aldrich (Allentown, PA), and were used as is or diluted in HPLC grade methanol for normal Raman and SERS measurements.

All measurements were performed with a Digilab Identity Raman plate reader. The Identity, shown in Figure 1, is configurable with either a 532 nm or 785 nm laser and spectrometer with a Peltier-cooled CCD array detector capable of $<10\text{ cm}^{-1}$ spectral resolution. The Identity supports standard clear, flat bottom 96 and 384 well microtiter plates, as well as custom plate formats. The laser is focused through the bottom of the plate into the well for the analysis of liquids. Powders or coatings on the bottom of the well can be analyzed by placing the plate on a plate shim to raise the bottom of the well to the laser focal point. Raman scattering is collected in an 180° backscatter configuration. Glass bottom plates are typically recommended for measurement of solids or coatings to reduce the interference in the spectrum from the plate material. The Identity includes an x-y motorized stage for automatic analysis of the well positions defined in the control software plate map, a screenshot for which is shown in Figure 2.

APPLICATION NOTE

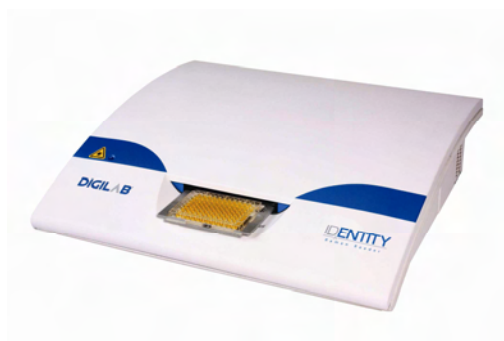


Figure 1. Digilab Identity Raman Microplate Reader

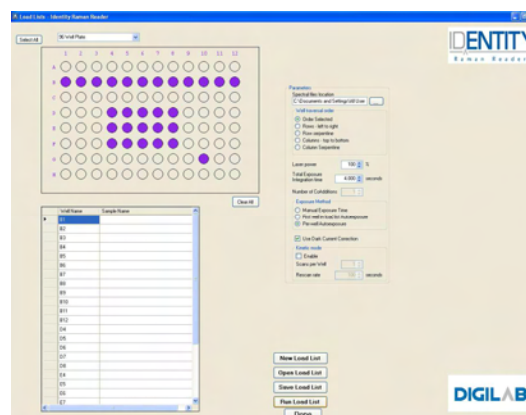


Figure 2. Identity load list view for creation of scan methods.

Well locations are selected by either clicking on individual wells, by clicking and dragging a rectangle over the desired wells with the mouse, or by clicking on Select All to scan all well locations. Laser power is set as a percentage of total laser power, and integration time can be set manually for all well locations, or automatically with the autoexposure selection. With autoexposure, the integration time is set by collecting a short pre-scan of the sample, from which the strongest peak in the Raman spectrum is determined to estimate the integration time necessary to nearly fill the dynamic range of the digitizer. If the estimated integration time is less than the entered exposure time, Raman scans are coadded to give the total exposure time.

The 96-well SERS-active microtiter plates consisted of ~ 1 mm thick coating of a patented (10) silver-doped sol-gel chemistry on glass bottom microtiter plates. The height of the microplate was adjusted to bring the SERS coating into the focal point of the laser, as shown schematically in Figure 3. Approximately 100-200 μL of each analyte in solution was added to individual SERS-active well for analysis. The analytes were allowed to passively diffuse into the porous sol-gel and to the immobilized silver particles.

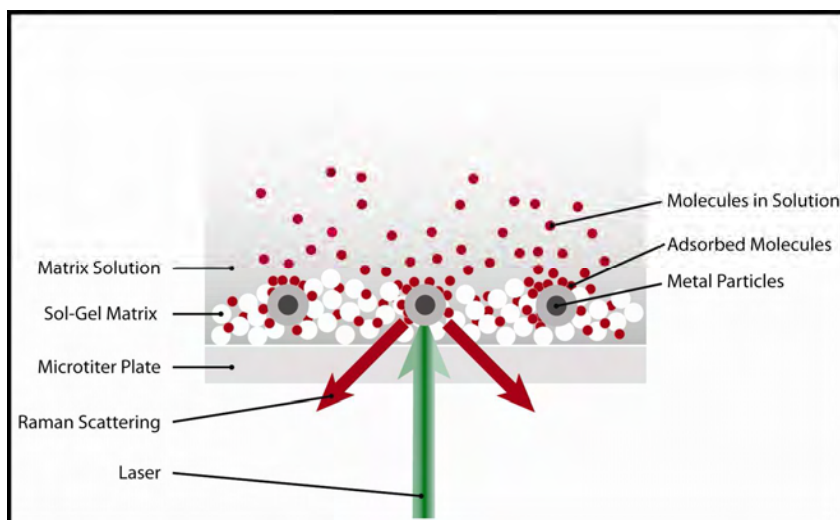


Figure 3. Schematic diagram of the Ag-colloid SERS coating on the well surface of a clear, flat bottom 96 well microtiter plate, showing the laser focus into the coating to obtain the SERS spectrum of analytes in solution that diffuse into the coating.

Spectra of benzenethiol, benzoic acid and 2,4-dinitrotoluene were collected using a 532 nm laser at 30 mW laser power and a 10 second total exposure time using the autoexposure setting. Each

APPLICATION NOTE

spectrum was corrected using a dark current collected with the same integration time and number of coadditions as the sample spectrum. For these measurements total per well measurement time is thus approximately 20 seconds for collection of the sample spectrum and dark current spectrum, and the total time to scan an entire 96 well plate would be approximately 34 minutes. Sample diffusion occurs from the time the sample is added to the well to the time the plate is mounted for scanning, and does not add to the overall analysis time.

Results and Discussion

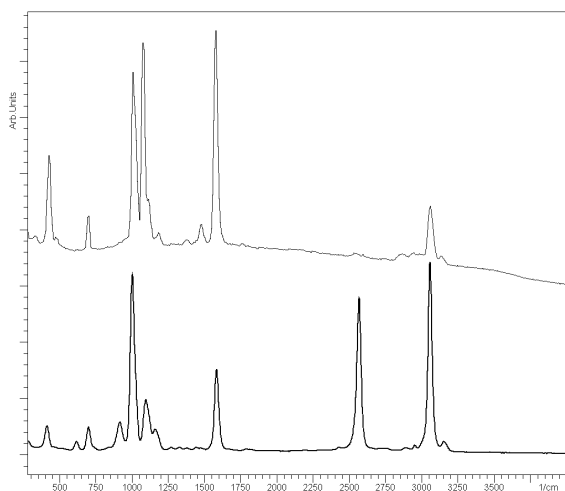


Figure 4. SERS (top) and Raman (bottom) spectra of 10 ppm in water and neat benzenethiol respectively. Both spectra were collected with a 532 nm laser at 30 mW laser power and 10 second measurement time.

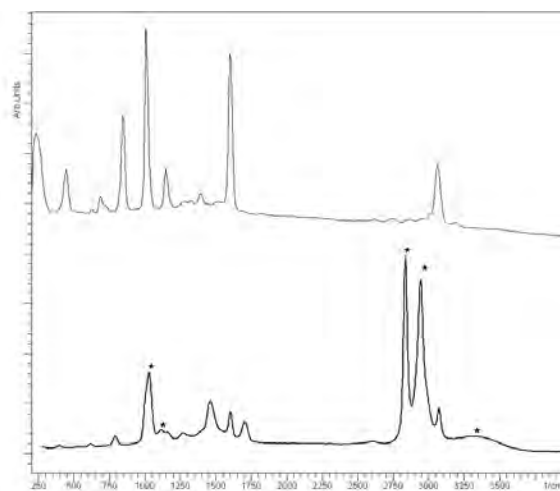


Figure 5. SERS (top) and Raman (bottom) spectra of 10 ppm and 10% benzoic acid in methanol, respectively. Methanol bands are indicated with (*) in the bulk Raman spectrum. Both spectra were collected with a 532 nm laser at 30 mW and 10 second measurement time

The SERS spectrum of a solution of 10 ppm benzenethiol in methanol is compared to the Raman spectrum of neat benzenethiol in Figure 4. Benzenethiol is highly SERS-active, and is often used to establish SERS-activity for various SERS substrates. A brief examination of the normal Raman spectrum compared to the SERS spectrum demonstrates the changes in relative peak intensity and position to the spectrum that can be induced by the SERS effect. In the case of benzenethiol, sulfur forms a chemical bond with silver as substantiated by the disappearance of the 2550 cm^{-1} peak due to thiol (S-H). Such a bond will also change the electron density and hence the bond strength of several vibrational modes and consequently their spectral peak positions, as well as intensities. Such bonding will also influence the proximity and orientation of the vibrational modes with respect to the metal surface and the plasmon field, again influencing peak intensity.

The goal of this preliminary study was to establish the ability of SERS-active microtiter plates and a Raman plate reader to perform high throughput analysis of trace chemicals, and only modest low concentrations were measured. Nevertheless, a comparison of the aromatic ring mode intensity at $\sim 1580\text{ cm}^{-1}$ for the SERS of the 10 ppm sample and the Raman of the pure sample was used to estimate a 3×10^4 enhancement factor. This modest enhancement factor underestimates the enhancements that can be expected at lower concentrations, as the SERS intensity is limited by the available silver surface area, which typically becomes saturated in the 1 to 10 ppm analyte concentration range. Furthermore, the signal-to-noise ratio suggests that

APPLICATION NOTE

significantly lower concentrations, such as ppb, could easily be measured (as is true for all the spectra presented).

Next, benzoic acid, which represents a functional group common to many drugs, was examined. A similar comparison of the Raman to SERS spectra for benzoic acid in methanol at 10 wt% (10^5 ppm) to 10 ppm, respectively, is displayed in Figure 5.

Raman peaks for methanol are marked with an asterisk. Since methanol is not SERS-active, its Raman peaks do not appear in the SERS spectrum. This also illustrates the benefits of laser excitation through the bottom of the plate, as opposed to from the top and through any solvent, which would then contribute to the SERS spectrum.

Conclusion

The principle of high throughput trace analysis has been demonstrated with commercially available SERS-active 96 well microtiter plates in the Identity Raman plate reader. Quality spectra were obtained from samples that demonstrate the sensitivity achievable by SERS in a microplate format. Although only modest enhancement factors were estimated, this was attributed to the high concentration of the samples measured during this preliminary study, and sub ppm detection limits should be expected. This is also supported by the excellent signal-to-noise ratios for all of the SERS measurements. Finally, the speed of analysis, 10 seconds per well, suggests that the combination of SERS-active microtiter plates and a Raman reader has great potential for high-throughput trace analysis for many applications.

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Quantitative Analysis of Fructose in Water with the Identity Raman Plate Reader

INTRODUCTION

The Identity Raman plate reader is a Raman spectrometer system designed to quickly analyze samples in microtiter plates. This format is ideal for performing quantitative analysis of liquid solutions. The application of fructose in water is chosen as an example because a wide variety of food products and beverages, such as soda pop, incorporate high fructose sugar and water as the primary ingredients. In fact, most non-diet sodas typically contain thirty to forty grams of total sugar in a 355 mL (12 oz.) container, with the sugar content comprising approximately 10% by weight of the total. This application note will describe the preparation of standard fructose-water solutions, and the collection of their spectra, along with spectra of unknown solutions, all within a single plate scan on the Identity Raman plate reader.

Sample Preparation

A stock solution of 20% by weight fructose was prepared by dissolving 2 grams of fructose into 8 ml of Nano-pure water. Five 1 ml calibration standards were prepared by taking aliquots of the stock solution and diluting to obtain final concentrations of 20%, 15%, 10%, and 5% by weight, with pure water as the 0% standard. In addition, blind unknown standards were prepared from the stock solution to run as samples in the method

Procedure

Three hundred microliters of all standard and unknown solutions were pipetted into wells of clear, flat-bottom 96-well polystyrene microtiter plates. Three replicates of each standard, and 2 replicates of each unknown, were pipetted into separate wells, and Raman spectra of each sample were collected in triplicate in one plate scan method on the Identity. All spectra were acquired in the Identity Raman microplate reader, shown in Figure 1, using 70 mW of laser power at 532 nm, coadding 15 scans of 4 second integration time for a total measurement time of 60 seconds per sample. A screen shot of the method file from the Identity software is displayed in Figure 2, showing the selection of the wells, the laser and spectrometer control settings, along with the activation of the kinetics scan parameters to automatically collect three replicate scans of the samples. In total, 66 individual spectra were

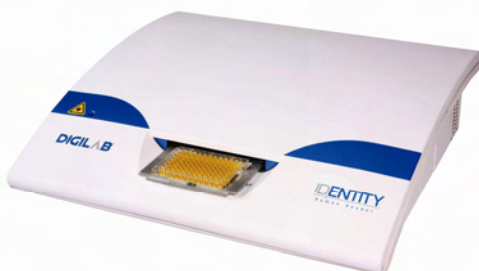


Figure 1. Identity Raman plate reader

automatically collected, with a total plate collection time of approximately 67 minutes.

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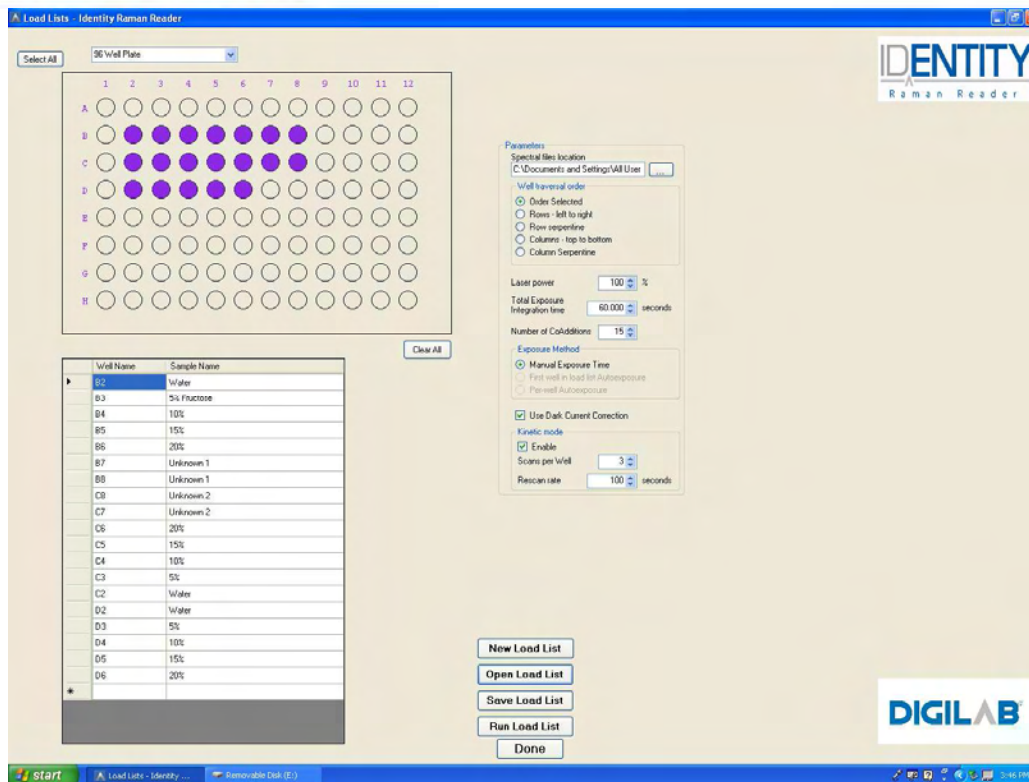


Figure 2. Load List page of the Identity software for the scan method described here. Wells are selected by clicking on individual wells or drawing a rectangular box around a group of wells with the mouse. When wells are selected a data table opens up for the operator to input textual information about the individual samples in the wells. Laser power is set as a total of total laser power (70 mw for the 532 nm laser), and integration time and coadditions can either be set manually or automatically with the autoexposure selection. Lastly, triplicate scans were collected using the kinetic mode.

Representative spectra from one row of calibration samples are displayed in Figure 3, showing the variation in the spectrum as the fructose concentration is increased from 0 to 20 weight percent fructose in water.

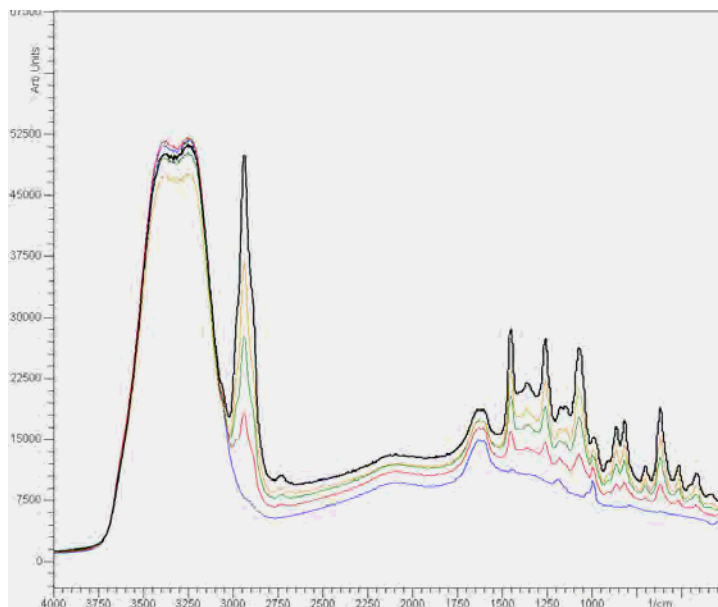


Figure 3. Calibration spectrum from Row B of the plate scan, with concentrations of 0, 5, 10, 15 and 20 weight % of fructose in water (bottom to top). Only water and weak polystyrene bands are observed in the 0% standard spectrum. The polystyrene bands from the microplate have constant intensity in all spectra and are not accounted for in the calibration methods.

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Quantitative Calibration and Results

All calibration spectra were imported into Labcognition's (Cologne, Germany) Panorama software, and both univariate and multivariate (Partial Least Squares, PLS) calibration methods were generated. In the univariate method the integrated area for the vibration centered at 619 cm^{-1} , from 551.5 to 666 cm^{-1} , was selected for the triplicate readings of the 15 calibration spectra as shown in Figure 4. This spectral region was used for analysis because of the strength

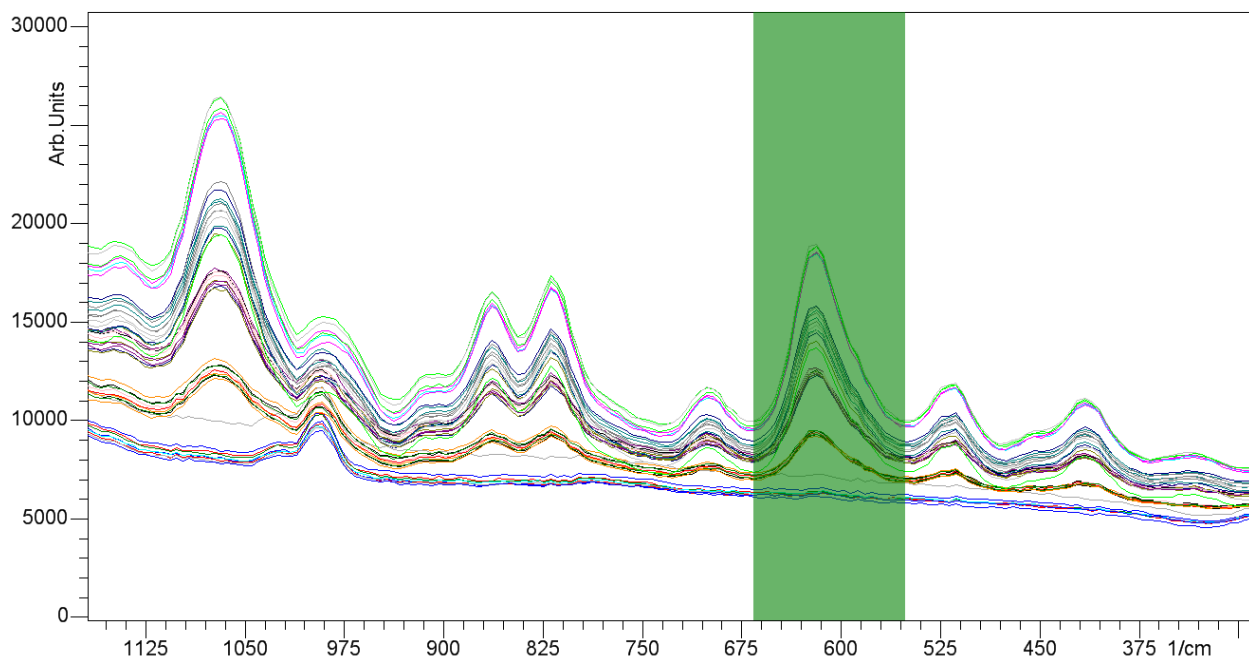


Figure 4. Integrated area region selection for univariate calibration of fructose in water calibration

of the band and it is free from overlap with water or polystyrene. Predicted results for the triplicate readings of the duplicate unknown samples are tabulated in Table 1, which were prepared as 4.0 % and 8.0% solutions. It should be noted that pipetting uncertainty can lead to an error of 0.2 wt % in the prepared concentrations of the standards and “unknown” samples (the standard deviation in the table below is related more to the precision of the measurement than the accuracy). Thus the two “unknown” samples have fructose concentrations of $4.0 \pm 0.2\%$, and $8.0 \pm 0.2\%$, and the predicted concentrations

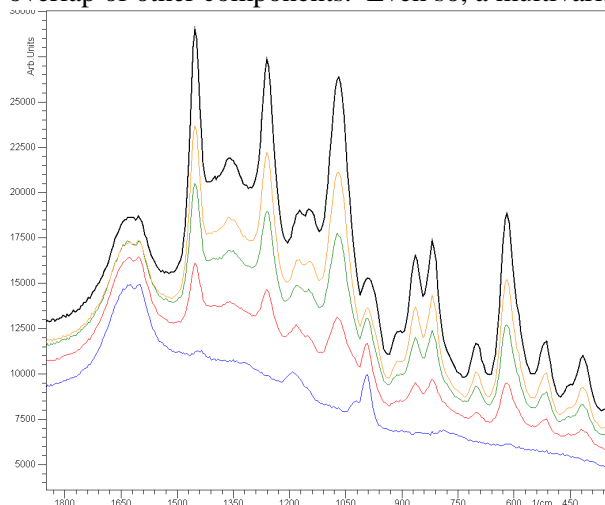
	Unknown 1 (B7)	Unknown 1 (C7)	Unknown 2 (B8)	Unknown 2 (C8)
	4.1	4.0	8.1	7.9
	4.0	3.9	8.0	7.9
	4.0	4.2	8.4	8.0
Ave.	4.0		8.1	
St. Dev.	0.1		0.2	

Table 1. Predicted fructose weight % concentrations for the triplicate readings of the two duplicate unknown samples using the univariate calibration. Unknown 1 was prepared as a 4.0 % solution, and Unknown 2 as an 8.0% solution.

agree with the prepared values within the error of the measurement.

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The univariate calibration works quite well in this analysis because the sample set is composed of a simple bi-component mixture, and it is possible to select a band for the analyte that is free from overlap of other components. Even so, a multivariate method was also developed to demonstrate



its applicability for such mixtures. The spectral region from 350 to 1850 cm^{-1} was selected for a PLS calibration as this region contains spectral information about all components in the system (fructose, water and polystyrene), as shown for a subset of the calibration spectra in Figure 5.

Figure 5. Spectral region selected for PLS calibration for the entire set of calibration spectra, from which a subset are displayed here showing the variation in the spectra from 0% fructose (bottom) to 20% fructose (top).

The PLS calibration accounts for all variability in the data set, as reported in a plot of the Predicted Residual Error Sum of Squares (PRESS) versus factor number. The factors are the individual variables the calibration is fitting to the model, and an examination of the PRESS plot, displayed in Figure 6, indicates that three factors will account for all variability in this data set.

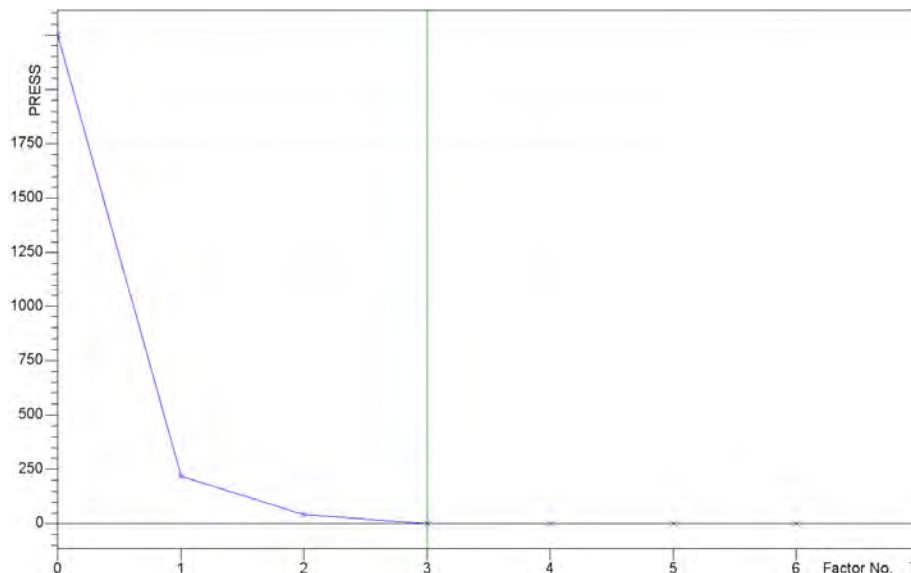


Figure 6. PRESS values versus factor number in the PLS calibration for the fructose in water data set. The PRESS plot indicates that three factors will adequately model this system.

The benefit of a multivariate calibration is that it will account for variability in the data set that may not be obvious from a casual inspection of the spectra, as long as that variability can be modeled in the calibration data. Such variability could be due to any number of effects that can cause variability in the spectra, such as concentration dependent chemical interactions between components, temperature changes, or variability in the measurement system. Thus the PLS method best models the calibration data set with three variables in this case when there is only one independent variable (% fructose) that was controlled by the analyst.

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Concentration predictions for the “unknown” samples using the multivariate calibration are shown in Table 2. Again we conclude that the analytical method predicts the concentrations of the “unknown” samples within the error of the measurement.

	Unknown 1 (B7)	Unknown 1 (C7)	Unknown 1 (B8)	Unknown 1 (C8)
	3.8	4.0	7.7	7.9
	3.8	3.9	7.7	7.9
	3.8	4.0	7.8	7.9
Ave.		3.9		7.8
St. Dev.		0.1		0.1

Table 21. Predicted fructose weight % concentrations for the triplicate readings of the two duplicate unknown samples using the multivariate calibration. Unknown 1 was prepared as a 4.0 +/- 0.2 % solution, and Unknown 2 as an 8.0 +/- 0.2 % solution.

Conclusions

The Identity was used to collect replicate standard spectra for generation of quantitative calibrations, and replicate “unknown” sample spectra, all within one plate scan method. In total, 66 spectra were collected automatically without any operator intervention once the plate scan method was started. The standard spectra were imported into Panorama, a third party software package in which both univariate and multivariate calibrations were generated, producing equivalently accurate results for the prediction of unknown concentrations of fructose in water at concentration levels typical for food and beverage products.

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