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Measurement of aqueous glucose in a model anterior chamber using Raman spectroscopy

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Many laboratories have been developing techniques that might be applied toward a non-invasive device that could optically measure aqueous humor glucose in the eye as a surrogate for blood glucose. We have configured a confocal Raman microscope that is capable of acquiring the Raman spectra of the aqueous humor *in vivo*. However, because of the potential for optical toxicity, a major design consideration is evaluating the tradeoff between the energy of exposure and measurement accuracy. Toward this end, we have developed a physical model of the anterior chamber in the eye for studying the feasibility of using a confocal Raman microscope for determining the concentration of glucose and other metabolites. The Raman spectrum of aqueous humor closely resembles the spectrum of its four primary Raman scatterers, (glucose, urea, lactate and ascorbate) mixed in normal saline. Aqueous mixtures of these analytes were placed under a contact lens resting on a quartz plate to form a physical model of the anterior chamber filled with aqueous humor. Calibration standards were prepared by non-collinearly varying the measured concentrations of the analytes over a range of 0–13 times the mean physiological levels found in aqueous humor. A confocal microscope was used to acquire spectra using an excitation wavelength of 785 nm. The accuracy to which the concentration with analytes could be optically measured was evaluated using the partial least-squares (PLS) algorithm and spectral datasets collected using exposure energies of 75, 150, 300, 900, and 1800 mJ. The optimum standard error of prediction (SEP) obtained for glucose was 34.3 mg dl^{-1} (2.72% of full range) using a 12-factor PLS model calculated using intensity-normalized spectra acquired over a free spectral range of 400–1800 cm^{-1} with 900 mJ excitation energy. Clinically acceptable predictability (4.6%) was obtained with 300 mJ total excitation energy. Similar results were obtained for urea (SEP = 8.84 mg dl^{-1}, 1.88%), ascorbate (SEP = 17.1 mg dl^{-1}, 8.22%) and lactate (SEP = 82.9 mg dl^{-1}, 7.59%) using 900 mJ exposure. Our results suggest that Raman spectroscopy may provide a feasible method of non-invasive glucose measurement if ocular toxicity can be avoided. Copyright © 2002 John Wiley & Sons, Ltd.

INTRODUCTION

It has been suggested that various optical techniques may be used to measure the glucose concentration within the aqueous humor of the eye.[1–4] The clear cornea provides direct optical access to the anterior chamber, which contains aqueous humor, a clear fluid that contains no blood cells and only small molecules such as glucose and dissolved oxygen. The glucose concentration within the aqueous humor has been shown to correlate with plasma glucose in animals and humans.[5–7] It has also been demonstrated that equilibration of glucose within the aqueous humor probably occurs within minutes.[2,8,9]

Among other optical techniques, Raman spectroscopy has been suggested as a means of non-invasively measuring aqueous humor (AH) glucose. Tarr and Steffes proposed the use of the stimulated Raman technique, but this would require probe lasers to traverse the anterior chamber for each wavelength sampled.[10,11] A number of investigators have utilized multivariate statistical methods in conjunction with near-infrared Raman spectroscopy to show that glucose and other constituents could be quantified with reasonable accuracy *in vitro* in AH, serum and whole blood.[12–15] We have previously demonstrated that the four Raman scatterers within aqueous humor (glucose, urea, ascorbate and lactate) can be measured *in vitro* in the physiological range using partial least-squares (PLS) and nonlinear methods (e.g. neural networks).[2,16]

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The purpose of this study was to determine the accuracy with which physiological levels of glucose and other Raman scattering constituents could be measured in a model system mimicking the anterior chamber of the eye. A secondary purpose was to investigate the relative benefit of normalizing the spectral information used to train a PLS algorithm on spectroscopic measurements of glucose. Two normalization techniques were evaluated, one using the 1640 cm^{-1} water band and the other based on normalizing training spectra against the band edge of the Rayleigh notch filter at 170 cm^{-1}. An additional purpose was to determine how the energy of exposure affected the accuracy of measuring glucose levels with Raman spectroscopy.

EXPERIMENTAL

An $f/1.8$ Kaiser Holospec Raman spectrometer was fiber optically coupled to a holographic probe head (Model HFPH-785) and used in conjunction with a Model BX60 Olympus microscope. An SDL (Model 8530) external cavity wavelength-stabilized laser diode operating at 785 nm was used as the excitation source. Near-infrared (NIR) radiation was selected to minimize the degree of fluorescence induced in *in vitro* or *in vivo* investigations. The instrument was designed to provide a Stokes-shifted free spectral range of 3455 cm^{-1} by imaging the diffracted output from a dual channel holographic grating onto a Princeton Instruments camera incorporating a 1024 × 256 pixel CCD (Model CCD-1024HRB) manufactured by EEV. The back-illuminated, deep-depletion, thick epitaxial silicon CCD was designed to provide high quantum efficiency in the NIR region without étaloning. However, because this chip cannot be operated in the multi-pinned phase (MPP) mode, it has a relatively high dark current, requiring that it be operated at low temperature (−80 °C).

Excitation light was routed to the microscope using a single-mode fiber with an ~5 μm core. The holographic probehead on the microscope was used to collimate the excitation light. Internal holographic filters were also used to remove Raman scattered light emanating from within the delivery fiber. The collimated light was focused through a microscope objective and onto the sample.

Raman scattered light from the sample was pre-filtered within the probehead to remove Rayleigh scattered light. The collected light was focused into a graded-index collection fiber, which guided the light to the spectrometer. Point-to-point imaging from the focal point of the objective to a metal aperture that was evaporated on the tip of the collection fiber provided the instrument with a degree of confocality inversely related to the aperture size.

Objectives with different numerical apertures and collection fibers with different aperture sizes were tested using the Raman microscope with a silicon wafer. The translation distance along the optical axis necessary to obtain the full width at half-maximum (FWHM) power of the 517 cm^{-1} silicon Raman peak was determined. The intensity of the 517 cm^{-1} peak at the focal point was also measured. The ultimate goal of this test was to determine which combination of objective and fiber could provide enough confocality to reject the Raman scattered light from the cornea and the lens of the eye while providing an adequate collection volume in the aqueous humor for high sensitivity. This test also allowed us to choose from among several objective lens–fiber aperture combinations for further testing in an anterior chamber model.

An acceptable degree of confocality (FWHM = 130 μm) with good signal strength was obtained using a 20× Olympus LCPlanFl objective and a 50 μm fiber collection aperture. This also provided a working distance of 6 mm, a comfortable distance for *in vivo* testing.

A physical model of the anterior chamber of the eye was constructed using a poly(methyl methacrylate) (PMMA) contact lens and an underlying quartz slide. This model anterior chamber held 50 μl of solution with a height of 1.6 mm from the quartz slide to the superficial surface of the contact lens (Fig. 1). Good rejection of the Raman signature from tissues should be achievable *in vivo* since the distance from the lens to the corneal surface in humans is more than twice that of our model anterior chamber.

One hundred solutions were prepared with various concentrations of glucose, lactate, urea, and ascorbic acid in normal saline to mimic aqueous humor. The concentration of these four principle constituents ranged incrementally from 0 to 13 times the normal concentrations of natural aqueous humor (the normal concentrations are glucose 97, lactate 84, ascorbate 16 and urea 36 mg dl^{-1}). The constituents were combined randomly to avoid collinear relationships. The intensity of the SDL laser was adjusted to provide an output power of 15 mW at the sample. Spectra from artificial aqueous humor (AAH) were collected 1 mm below the surface of the contact lens in the model anterior chamber. Five spectra for each of the samples were collected using integration times of 5, 10, 20, 60, and 120 s.

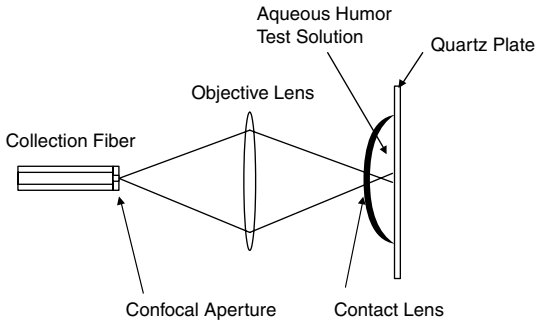

Figure 1. Method of confocal collection of Raman spectra from a model anterior chamber consisting of aqueous solution between a PMMA contact lens and quartz plate.

A variety of chemometric techniques are available to determine the concentration of one or more constituents within a sample, based on its Raman spectrum. These methods include partial least-squares, complete least-squares, principal component regression and neural network techniques.[17–20]

We used PLS because, unlike many techniques, it requires a knowledge of only the concentration of the constituent of interest. Complete knowledge of the other constituents within the training samples is not required, but it assumes that constituents within the training set are representative of those expected in the test set.

The commercial software PLSplus/IQ (Galactic Industries) was used to implement the PLS analysis. Two types of normalization techniques were employed prior to entering data into the PLS regression model. Rayleigh normalization of the aqueous humor spectra was performed by dividing each collected spectra by its maximum value. The maximum occurs roughly at 170 cm^{-1} and corresponds to leak-through from the holographic notch filters in the probehead and spectrometer. The use of this technique may compensate for variations in laser intensity across the training set. However, this technique may not be ideal for developing a PLS model designed for use in a clinical instrument because of unit-to-unit variations in the excitation wavelength and the band-edge of the holographic notch filters. The second normalization technique tested utilized the 1640 cm^{-1} water band. This technique is believed to be more robust, since slight variations in wavelength should not significantly effect normalization of this relatively wide spectral band. Non-normalized spectra were also processed to determine the relative benefit of the two normalization techniques.

After the normalization step, the average intensity of the training data at each wavelength collected was subtracted from the intensity of each training spectrum. This procedure is known as mean centering and is often used in developing PLS regression models. For each PLS model, every training sample was included, i.e. no potential outliers were eliminated.

RESULTS

Raman spectra from the anterior chamber model using a contact lens are shown in Fig. 2. The spectra of the contact lens, aqueous solution and quartz plate can be clearly distinguished, demonstrating the effectiveness of the confocal collection system. Similarly, the spectra of the cornea, aqueous humor, and lens can be clearly distinguished when the system is applied to an anesthetized rabbit (Fig. 3).

Two types of PLS models were developed using a common set of Raman spectra acquired from 100 AAH samples collected within the model anterior chamber at various exposure energies. The first type of PLS model was developed using spectral data that spanned the entire free spectral range (400–3255 cm^{-1}) of the instrument. The

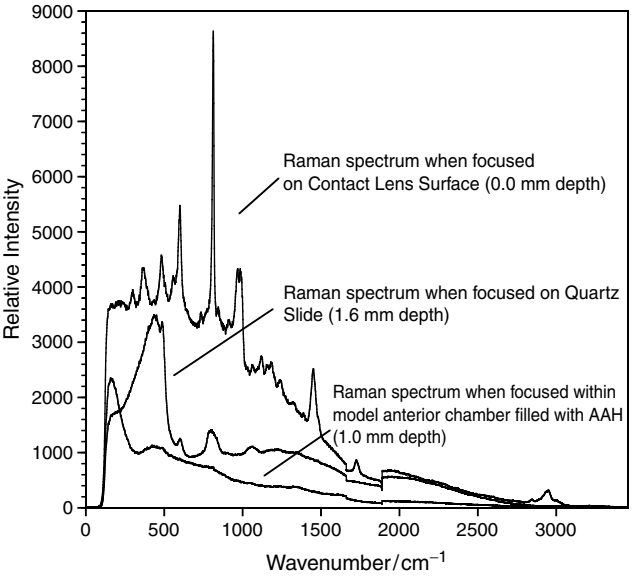

Figure 2. The Raman spectra of aqueous solution confocally collected from a depth of 1.0 mm beneath the surface of the contact lens can be clearly distinguished from the spectra of the PMMA contact lens and the quartz plate.

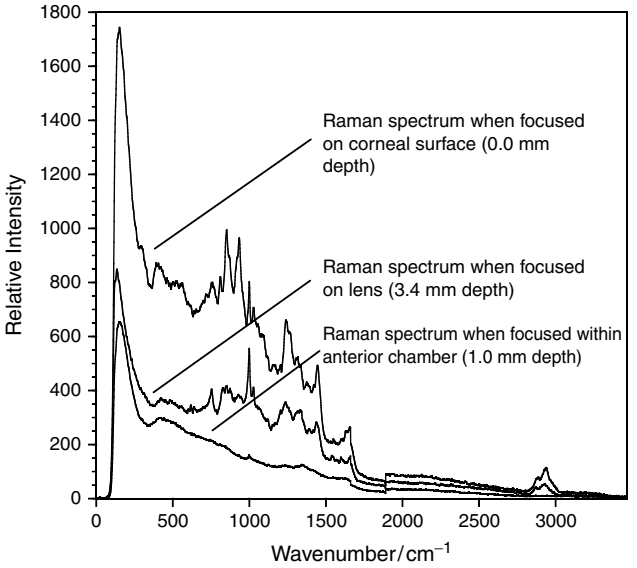

Figure 3. The Raman spectra of natural aqueous humor confocally collected from the anterior chamber of an anesthetized rabbit can be clearly distinguished from the spectra of the cornea and lens.

second type of PLS model was developed using spectra from a low-wavenumber subset (400–1800 cm^{-1}). Cross-validation testing of both types of PLS models revealed that, independent of the energy of exposure utilized, the longer wavelength band did not contribute to the accuracy with which PLS predicts glucose concentration. Consequently, all further results will be reported for spectra acquired from 400–1800 cm^{-1}.

Normalization, exposure time and the number of factors utilized in the PLS model influence the accuracy which glucose levels can be measured. These tradeoffs were evaluated by evaluating different PLS models in which these parameters were systematically varied and subsequently tested using cross-validation.

The accuracy to which an analyte could be optically measured given the various parameters used in this study was quantified by calculating the standard error of cross-validation (SECV) and the correlation coefficient (R^2). The SECV and R^2 values were calculated for PLS models incorporating (1) non-normalized spectra, (2) spectra which were normalized at the band-edge of the Rayleigh cut-off peak (170 cm^{-1}) and (3) spectra which were normalized on the Raman water peak (1640 cm^{-1}). This calculation was performed for normalized and non-normalized training sets acquired using exposure energies of 150, 300, 900 and 1800 mJ.

The effects that normalization and the number of factors have on the accuracy of the PLS calibration models are shown for glucose in Fig. 4. These spectra were obtained from samples of AAH measured within the model anterior chamber using an exposure of 900 mJ (15 mW × 60 s). SECVs for PLS models utilizing up to 24 factors were evaluated, but no improvement in accuracy of glucose measurement was seen in PLS using more than 12 factors. Based on the minimum SECV, the plots suggest that optimum results are obtained with 12 PLS factors and spectra that have been normalized with respect to the 170 cm^{-1} Rayleigh notch filter band-edge.

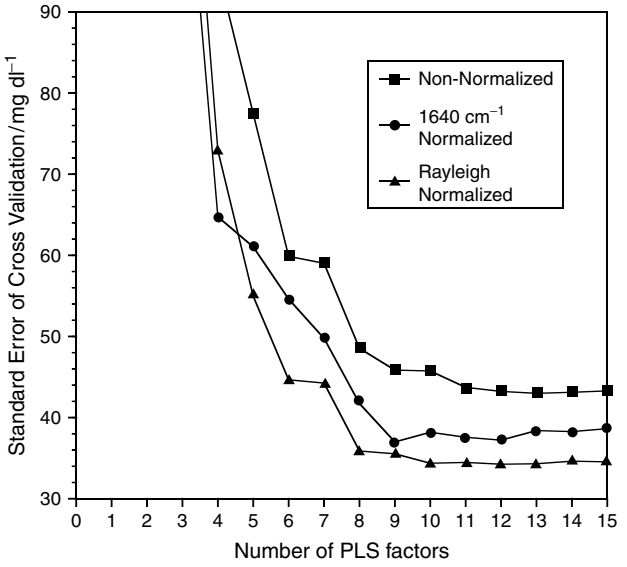

Figure 4. The Standard Error of Cross-Validation (SECV) is shown as a function of the normalization. The Raman spectra of 100 training samples were each collected with an exposure of 900 mJ at 785 nm utilizing a contact lens model of the anterior chamber.

SECV and R^2 are plotted as a function of energy of exposure in Figs 5 and 6, respectively. These statistics were calculated for glucose using PLS models derived with either normalized or non-normalized sets of training spectra. The performance of the PLS calibration models is shown for both 9 and 12 factors [the optimum number of factors determined by using the *F*-ratio in PRESS (Prediction Residual Error Sum of Squares calculations) was nine; the optimum number of factors for minimizing SECV was 12]. Both the SECV and R^2 improved with energy of exposure up to 900 mJ (60 s × 15 mW). Spectral normalization improved the accuracy to which glucose levels could be predicted, but there was no significant difference in the SECV and R^2 obtained using either normalization technique regardless of the energy of exposure.

The true performance of a PLS model may be overestimated when cross-validation methods are used in situations where the number of training samples does not greatly exceed the number of factors. To validate the sample size used to develop the PLS models in this study, we divided our a set of 100 spectra acquired with 900 mJ exposures into two groups, even and odd sets of 50 spectra. A 12-factor PLS model for glucose was developed using the set of 50 odd AAH spectra. The accuracy to which this calibration model could predict the concentration of the even samples was measured in terms of standard error of prediction (SEP) and R^2. In Table 1, these results are compared with the cross-validation

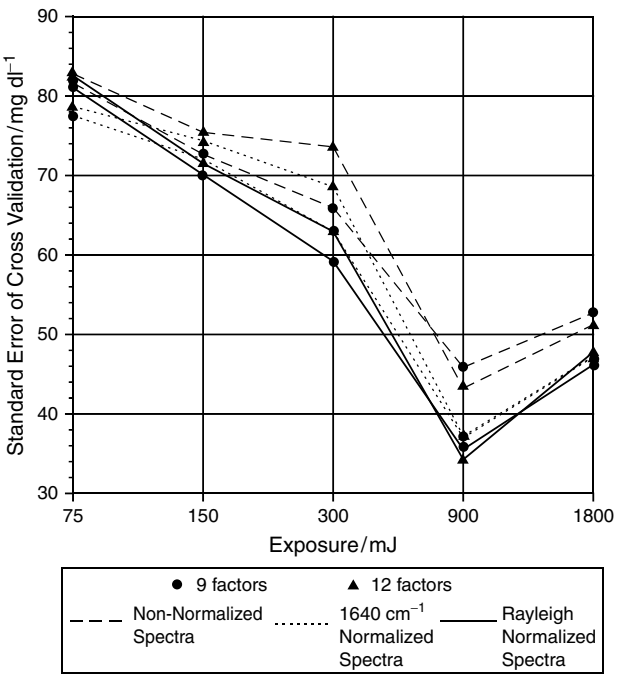

Figure 5. SECV values are shown as a function of energy of exposure and are used to describe the relative accuracy that physiological levels of glucose can be quantified in 100 samples with or without normalization of the spectra prior to applying PLS.

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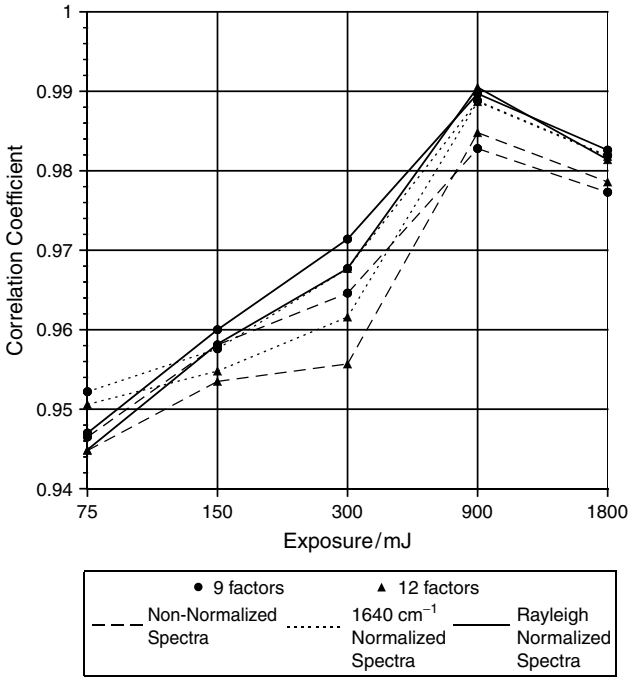

Figure 6. Regression correlation coefficients are shown as a function of energy of exposure and are used to describe the relative accuracy that physiological levels of glucose can be quantified using 100 samples processed with or without normalization of the spectra prior to applying PLS.

Table 1. Cross-validation and prediction statistics for PLS models trained on 50 AAH samples

	Cross-validation statistics		Prediction statistics	
Normalization method	SECV/mg dl^{-1}	R^2	SEP/mg dl^{-1}	R^2
None	56.59	0.9734	52.08	0.9895
1640 cm^{-1} normalized spectra	48.91	0.9801	44.39	0.9924
Rayleigh normalized spectra	47.26	0.9814	43.35	0.9927

statistics (SECV and R^2) obtained using the odd training samples alone. The prediction statistics compared favorably with those obtained using cross-validation. Therefore, the cross-validation method does not significantly overestimate the PLS predictive accuracy when at least 50 samples are used with 12 factors in our model, and even greater accuracy would be expected using cross-validation with 100 samples.

The Clark grid provides ranges in which errors in glucose measurement would impact treatment decisions or clinical outcomes.[21] The cross-validation results for PLS models tested using spectral samples with AAH glucose levels below 500 mM are plotted on a Clark grid in Fig. 7. PLS models were developed for exposures of 900 mJ (60 s) and 300 mJ

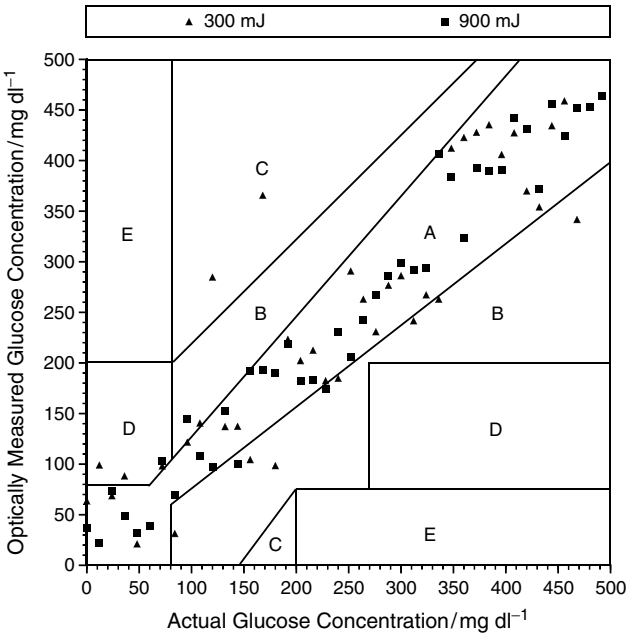

Figure 7. The Clark grid is designed to describe the clinical utility of blood glucose monitoring instruments. The points on the grid are from AAH samples tested with 75, 300 and 900 mJ exposure energies. Data in region A represent data that are 'clinically accurate.' Data in region B are less than 'clinically accurate,' but pose no danger to the patient. Data in region C represent measurements that could result in 'overcorrection.' Data within region D 'represent a dangerous failure of the instrument to detect and treat.' Data region E represents data that would lead to 'erroneous treatment,' opposite of that actually required.

(20 s). Each spectrum was pre-normalized with the 170 cm^{-1} Rayleigh cut-off peak. The data points in Fig. 7 lie primarily within the clinically acceptable range.

We also utilized cross-validation to predict accuracy of PLS for other Raman scattering constituents within AAH. Figure 8 shows the SECV that was obtained for each of the constituents using spectra that are normalized by the Rayleigh band edge using 900 mJ of exposure. The minimum SECV obtained for each analyte were glucose SEP = 34.3 mg dl^{-1} (2.72%), urea SEP = 8.84 mg dl^{-1} (1.88%), ascorbate SEP = 17.1 mg dl^{-1} (8.22%) and lactate SEP = 82.9 mg dl^{-1} (7.59%). The percentage accuracies indicated are with respect to 0–13 times the normal physiological range of each analyte.

CONCLUSIONS

We have demonstrated good predictability of confocal Raman spectroscopy over a physiological range of glucose concentrations using partial least-squares analysis in an anterior chamber model. The reliability of this

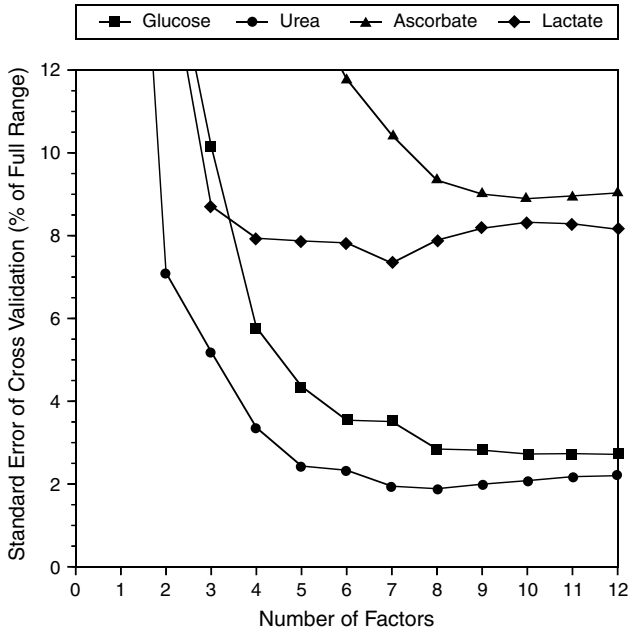

Figure 8. SECV is shown as a function of the number of PLS factors for the four Raman scatterers in aqueous humor. The Raman spectra of 100 training samples were each collected with an exposure of 900 mJ at 785 nm utilizing a contact lens model of the anterior chamber. Each spectrum was mean-centered and intensity normalized with respect to the Rayleigh notch filter cut-off peak at 170 cm^{-1} before being incorporated into the PLS model.

technique is within clinically acceptable parameters. Lactate, urea and ascorbate can also be measured with this technique.

A training set of 50 samples was adequate for estimating glucose with PLS, with 12 as the optimum number of factors. Normalization of the spectra on independent spectral features such as the 1640 cm^{-1} water peak or the Rayleigh notch filter cut-off peak improves the performance of the PLS algorithm. It may be preferable to normalize on the 1640 cm^{-1}-water peak, as it should not vary among instruments, whereas the Rayleigh peak is dependent upon the specific notch filter.

A total energy exposure of 300 mJ provides acceptable accuracy of glucose measurement. This exposure level provides reasonable integration times at power levels which are likely to be above those recommended by the American National Standards Institute (ANSI) with our present numerical aperture,[22] but below levels believed to be toxic the eye.[23] The energy density that is safe for the eye at 785 nm, however, has yet to be empirically determined.

Because we can eliminate the 1800–3255 cm^{-1} band with no degradation in performance, the use of a dual-plex grating and an NIR optimized chip requiring non-MPP mode operation and very low temperature (−80 °C) operation may not be necessary. Low noise and relatively inexpensive MPP mode CCDs can be operated at higher temperatures than non-MPP devices. Quantum efficiency also improves at slightly higher operating temperatures. This makes the potential for development of a portable commercial device more feasible.

Acknowledgments

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REFERENCES

1. Coté GL. *J. Clin. Eng.* 1997; **22**: 253.
2. McNichols RJ, Cote GL. *J. Biomed. Opt.* 2000; **5**: 5.
3. Klonoff D. *Clin. Diabetes* 1998; **16**: 43.
4. Khalil O. *Clin. Chem.* 1999; **45**: 165.
5. Borchert MS, Storrie-Lombardi MC, Lambert JL. *Diabetes Technol. Ther.* 1999; **1**: 145.
6. Pohjola S. *Acta Ophthalmol., Suppl.* 1966; **88**: 1.
7. Schrader WF, Grajewski RS, Meuer P, Kiefer W. *Invest. Ophthalmol. Visual Sci. (Suppl.)* 2000; **41**: S77.
8. Steffes P. *Diabetes Technol. Ther.* 1999; **1**: 129.
9. Cameron BD, Baba JS, Cote GL. *Diabetes Technol. Ther.* 2001; **3**: 201.
10. Tarr RV, Steffes PG. US Patent 5 243 983, 1993.
11. Tarr RV, Steffes PG. *IEEE Lasers Electro-opt. Soc. Newsl.* 1998; **12**: 22.
12. Goetz MJ, Cote GL, Erckens R, March W, Motamedi M. *IEEE Trans. Biomed. Eng.* 1995; **42**: 728.
13. Berger A, Wang Y, Feld M. *Appl. Opt.* 1996; **35**: 209.
14. Berger J, Koo TW, Itzkan I, Horowitz G, Feld M. *Appl. Opt.* 1999; **38**: 2916.
15. Qu J, Wilson B, Suria D. *Appl. Opt.* 1999; **38**: 5491.
16. Lambert J, Storrie-Lombardi M, Borchert M. *IEEE Lasers Electro-opt. Soc. Newsl.* 1998; **12**: 19.
17. Geladi P, Kowalski B. *Anal. Chim. Acta* 1986; **185**: 1.
18. Haaland D, Thomas E. *Anal. Chem.* 1988; **60**: 1193.
19. Berger A, Koo T, Itzkan I, Feld M. *Anal. Chem.* 1998; **70**: 623.
20. Brown C. *Spectroscopy* 1986; **1**: 23.
21. Clark WL, Cox D, Gonder-Frederick LA, Carter W, Pohl SL. *Diabetes Care* 1987; **10**: 622.
22. *American National Standard for the Safe Use of Lasers*, ANSI Z-136.1-2000, American National Standards Institute, NY, NY, 2000.
23. Ediger MN, Landry RJ, Sliney DH. *Diabetes Technol. Ther.* 1999; **1**: 239.