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Clinical chemistry and near infrared spectroscopy: technology for non-invasive glucose monitoring

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Non-invasive assays for blood glucose can be based on near infrared spectrometry of skin tissue using the diffuse reflectance technique. Using a straightforward spectral variable selection based on choices from the optimum partial least-squares (PLS) regression vector yields better results than using PLS calibration models with full spectrum evaluation previously reported. The pairs of variables are selected from the maxima and minima of the regression weights, respectively, in decreasing order. Substantial improvements in the prediction performance of such calibration models, compared to previous calibrations based on full spectrum evaluation, are obtained. Another aspect is the reduced number of spectral variables needed for robust calibration modeling. In addition, evidence is provided for the physical effect, as manifested by the spectral glucose absorptivities, underlying the individual single-person calibration models. Their regression vector structure shows very similar features as calculated for a glucose calibration experiment based on random human plasma samples. Novel techniques are presented for probing the intravascular fluid space using time-resolved near infrared spectroscopy of oral mucosa. The pulsatile blood spectrum can be derived from these diffuse reflectance lip spectra by Fourier analysis. Future applications and prospects for non-invasive blood analysis are discussed.

Keywords: near infrared spectroscopy, non-invasive glucose assay, multivariate calibration, multiple linear regression, pulsatile spectroscopy.

Introduction

Demands for versatile medical diagnostic methods have caused a tremendous increase in the application of near infrared (NIR) spectroscopy. Research into non-invasive instrumentation for metabolites based on such technology is currently carried out by many researchers; an overview to this field can be found in References 1–4. There are other spectroscopic techniques proposed for non-invasive monitoring. Recent publications cover assays based on luminescence decay of a long lifetime metal–ligand complex,[5] on optical activity[6] or by using Raman spectroscopy.[7,8] However, only feasibility studies have been presented so far using the latter techniques.

The monitoring of glucose, lactate, urea and others is of special medical interest as a guide to the metabolic state. In particular, glucose is a substance of high metabolic importance, since it can be considered as the main energy carrier for our organism. An endocrine regulation for this compound exists,

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maintaining the normal fasting level of glucose in peripheral venous blood of 70–110 mg dL^{-1} (3.9–6.1 mmol L^{-1}). The level in arterial blood is usually slightly raised compared to the venous level which depends on nutritional and metabolic conditions (between 15 and 30 mg dL^{-1} higher).

A glucose sensing device is an important application for patients with disorders in their carbohydrate metabolism, predominantly due to diabetes mellitus. About 4% of the population in developed countries are affected by this metabolic disease. In type I diabetes, a severe insulin deficiency exists due to the destruction of the beta cells producing this hormone. In type II diabetes, enough insulin may be available, but due to an insulin resistance in the target organs, glucose utilisation is perturbed. Therapy aims at regulating glucose levels comparable with metabolically healthy individuals, so that several daily determinations of blood glucose are essential for patients without adequate glycaemic control. Critical care also calls for frequent measurements, when bedside monitoring of severely ill patients is needed.

Due to its metabolic importance, determinations of glucose are the most frequently performed in the clinical laboratory. Its concentration is usually determined for either whole blood or derived fluids. Blood sampling can be done by vascular puncture using syringes; however, capillary blood is more easily obtained by lancing finger tips or other appropriate body parts. For routine analysis, the established methodology uses different enzymatic assays in combination with photometric or electrochemical detection, but new trends strive for reagentless assays using biosensor technology.[9]

Research activities are concentrated on the development of continuous methodology, either invasively by subcutaneously implanted devices (for reviews covering this field, see References 10 and 11) or non-invasively by spectroscopy. With current technology only inadequate glycemic control can be achieved, even for diabetic patients undergoing intensive insulin therapy. With the possibility of frequent measurements, such patients would gain advantages from a non-invasive glucose sensor. As a further development, a continuous readout of glucose levels could be used for a feedback-controlled insulin delivery system (artificial pancreas).

Non-invasive instrumentation for patient self-monitoring can be based on NIR spectroscopy. This has been recognised through many *in vitro* studies, e.g. see References 12 and 13 and the literature cited there. The main research emphasis is on the development of reliable glucose assays, for which the selectivity can only be achieved using multivariate calibration, i.e. taking into account the NIR fingerprint spectrum of a compound. This conclusion can also be drawn from recent Monte Carlo simulations when studying the optical effects from glucose and other physiological factors or analytes for *in vivo* measurements.[14] This latter study was devoted to the short-wave NIR range, but is equally applicable to longer wavelengths. In a different approach, the main emphasis was on the scattering coefficient influenced by a varying glucose concentration.[15–17] Photoacoustic NIR techniques, less affected by tissue scattering as experienced for diffuse reflectance, have also been proposed.[18,19] Further contributions were presented by Danzer *et al.* for improving non-invasive glucose assays using NIR spectrometry by means of significant outlier removal.[20,21] Our approach here is based on a spectral variable selection to achieve a better prediction performance of statistical calibration models than usually obtained with full spectrum evaluation.[22] This strategy uses the extreme values in the optimum PLS regression vector which is different from a method employed recently by McShane *et al.*[23] for a simple analytical task using information from spectral regions with largest spectral variance. Another strategy was followed using a genetic algorithm-based method for wavelength selection.[24] For both *in vitro* applications spectral data from the transparency window between 4700 and 4200 cm^{-1}, as experienced for transmission measurements of aqueous solutions, were used which exclude, however, non-invasive glucose monitoring due to the limited penetration depth of NIR radiation of such wavelengths into skin tissue.

The quality of the statistical calibration models based on oral glucose tolerance testing has been questioned recently.[4,25] It is well known that pickup of spurious drift effects can significantly influence the results from multivariate statistical calibrations, especially with regard to continuous monitoring. An appropriate experimental design with randomised sampling is necessary for such critical applications

when the analytical signals are comparable to the prevailing noise level or signal drifting.[26] Our strategy in the past has been to employ complex experiments avoiding temporal chance correlations. We present additional data providing evidence for the physical effects (i.e. glucose absorptivity) underlying the non-invasive assay using the diffuse reflectance technique, which is applied to the measurement of skin tissue. Further development of non-invasive technology for glucose determination is presented, which is based on time-resolved NIR spectroscopy allowing the probing of the intravascular fluid space modulated by the heart beat.

Experimental

Experiments were aimed at single person monitoring using the diffuse reflectance NIR spectra of the lower inner lip. The spectral measurements were carried out using a Fourier transform IR spectrometer model IFS-66 from Bruker equipped with tungsten lamp, CaF_2 beamsplitter and a liquid nitrogen cooled InSb detector from Infrared Associates (Suffolk, UK). A total of 1200 interferograms providing a spectral resolution of 32 cm^{-1} were averaged, and the resulting measurement time was about 1 minute. More details about the specially optimised diffuse reflectance accessory have been published elsewhere.[27]

Capillary blood samples for the glucose determination in the laboratory were taken by puncture of the fingertip using capillary pipettes. The blood glucose concentration was determined by the enzymatic hexokinase/G6P-DH method. Details of the calibration design have been presented earlier.[28,29] One experiment was performed with extensive, non-standardised oral glucose tolerance testing (OGTT) during a two-day campaign. To support the results from this calibration experiment, a second test was performed with the same diabetic patient who actively followed computer-generated random glucose time profiles over two weeks.

For the time-resolved measurements the spectrometer software for gas chromatography/FT-IR coupling was employed. A total of nine interferograms with the same resolution as given above were averaged within 0.5 s. The diffuse reflectance lip spectra were recorded for a measurement time of at least two minutes. Subsequent background spectra could be recorded using Spectralon reflectance standard material from Labsphere (North Sutton, NH, USA). The spectra were transferred to a personal computer for further processing using MATLAB (The Mathworks, South Natick, MA, USA).

Multivariate calibration using partial least-squares (PLS) has been very successful for the quantitative analysis of several parameters in many clinical blood assays. The PLS algorithm was applied for a linear regression of the spectral data between 9000 and 5500 cm^{-1} (λ = 1.1–1.8 µm) against the probands' blood glucose concentration values. Root mean squared errors of prediction ($RMSEP = (\Sigma (c_{ref,I} - c_{pred,i})^2/M)^{1/2}$ with M samples) were estimated by cross-validation using the "leave one out" strategy, but also packages of five standards were omitted to test the robustness of the calibration models. Programs for the input of spectral data, PLS calibration and cross-validation were written in MATLAB. Use was also made of the signal processing toolbox. Further details of the calibration strategy including detection of outliers are described in References 30 and 31.

A novel strategy for spectral variable selection based on PLS regression vector choices has recently been described by us which has been very successful for our clinical applications.[22] It is based on the pairwise selection of extrema of the optimum PLS regression vector calculated for pre-selected spectral intervals. These were chosen by spectral *a priori* information for the component to be studied.

Multivariate calibration based on spectral variable selection

An overview of the spectral data obtained during the single person calibration experiments is provided by Figure 1. The primary information are the single beam inner lip spectra which were calculated from the apodised interferograms including phase correction, see Figure 1(c). Usually, absorbance spectra as shown in Figure 1(b) are considered as in-

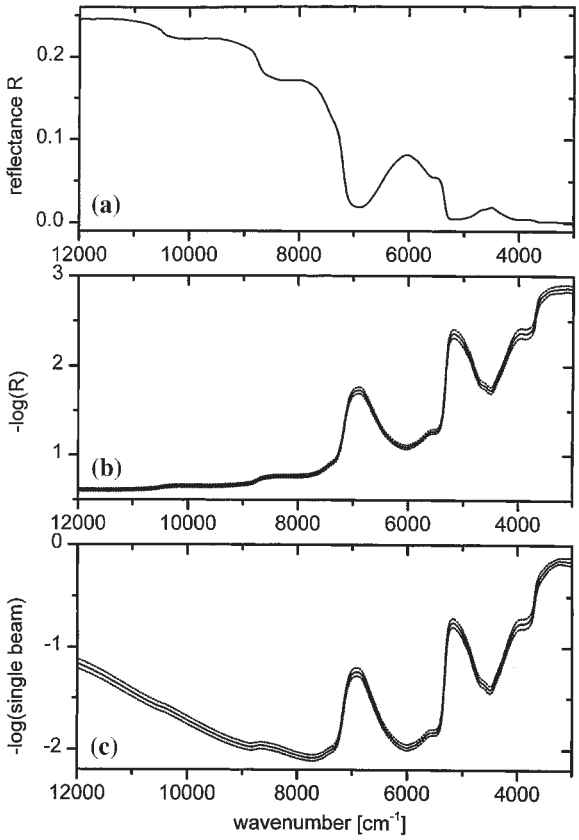

Figure 1. Near infrared diffuse reflectance spectra of oral mucosa: (a) mean reflectance spectrum as measured from 136 single person lip spectra, (b) spectrum transformed into absorbance analogue units, (c) logarithmised mean single beam spectrum (the latter two spectra are with population standard deviation).

put data for quantitative assays. However, as we could show for statistical calibrations,[12,32] logarithmised single beam spectra of human plasma samples can also be considered for calibration modelling. These have the advantage that the prediction performance is better due to a reduced noise level compared to calibration models based on absorbance data, because for the latter a noise-containing background spectrum is required. The background in the NIR domain is usually without significant spectral features, as opposed to the mid-infrared spectral range, so modelling of the background can be achieved with only a few additional factors compared to the optimum calibration model obtained with absorbance spectra.

The blood glucose profiles resulting from the intake of several glucose potions and the administration of insulin are well described in previous publications.[2,29] Sampling was done during a time period of about 15 hours and different up-and-down features in the temporal glucose concentration profiles were chosen to reduce the potential pickup of chance correlations. The glucose values at the time of spectrum recording were obtained by spline approximation using data with concentrations obtained by capillary blood testing. Furthermore, an impulse invariant designed Butterworth filter of first order with a time constants of 10 min was applied to the time dependent blood glucose profiles to estimate the average lip tissue glucose concentrations at the time when the spectra were taken. For support of the results obtained by using oral glucose tolerance tests, a randomised one-person experiment was performed over the duration of two weeks. Different daily glucose levels were approached with the sequence selected at random by the computer. The optimum PLS calibration models with full spectrum evaluation between 9000 and 5475 cm^{-1} have been previously discussed in detail.[28,29]

The improvements we achieved with this data using the novel strategy with spectral variable selection[22] are significant, because we find reduced standard errors of prediction of about 15% relative to the *RMSEP* from the full spectrum PLS models. The computational workload is small compared to a model selection considered recently and based on genetic algorithms.[24,33] Interestingly, it could be demonstrated in a recent publication[34] that, with a suitable variable selection, multiple linear regression models can be more efficient than PLS or PCR calibration models. The results of our non-invasive glucose assays are summarised in Table 1.

An example of the improved calibrations, based on the data set with time delayed concentration data, is given in Figure 2. Part (a) shows the root mean squared error of prediction (*RMSEP*) dependent on the number of orthogonal PLS factors which is equivalent to the rank of the PLS calibration matrix to be inverted for solving the linear equation system. Besides the results from the leave-one-out strategy, also those from leave-five-out cross-validation is provided for testing calibration robustness. The regression vector calculated from the optimum PLS

Table 1. Root mean squared glucose prediction errors (*RMSEP*) obtained for various single person calibration experiments with diffuse reflectance lip spectra (full spectrum evaluation was done with 115 spectral variables within the interval 9000–5475 cm^{-1}, wavenumber spacing 30.8 cm^{-1}, see also text).

(A) calibration data	Root mean squared prediction errors 132 spectra with OGTT	
	full spectrum evaluation	variable selection
–log S_{lip}, interpolated conc.	45.6 mg dL^{-1}	38.9 mg dL^{-1} (30 variables)
delayed glucose profile	43.0 mg dL^{-1}	36.4 mg dL^{-1} (26 variables)
(B) calibration data	216 spectra with random tests	
	full spectrum evaluation	variable selection
–log S_{lip}, interpolated conc.	51.9 mg dL^{-1}	46.8 mg dL^{-1} (32 variables)

calibration model is the basis for the spectral variable selection. The *RMSEP* values dependent on the number of spectral data points is shown in the subplot. A minimum is found for 26 variables (the optimum PLS model using these variables can be calculated from 16 PLS factors). It is interesting to see that the least-squares solution with full rank inversion provides nearly the same prediction errors, and the difference in the prediction errors obtained from cross-validation with one or five standards left out is only 1.8 mg dL^{-1} for the optimum PLS models.

The number of PLS factors necessary for calibration with full spectrum employment has been questioned recently by Arnold.[4] However, the results shown here give clear evidence about the optical effects upon which our calibrations are based on. This is well supported by a comparison of the glucose regression vector sections obtained for the human plasma population[12] with that evolved for the single person OGTT-experiment covering the same interval, see Figure 3. The inversion of minima and maxima, as obvious for the spectral variables around 5500 cm^{-1}, has also been observed in other cases (see Reference 22 with respect to a total protein calibration). The regression vectors for the other experiments show impressively similar structures. In the past, the structure of the full-spectrum PLS regression vectors, including studies into how they evolve during the process of optimisation, was investigated by us;[35] these show significant similarity for both experiments, either with OGTT or random sampling. Such data must be seen in connection with the aqueous glucose absorptivity data which is displayed in Figure 4. In addition to the difference spectra calculated from absorbance data of the glucose solutions (with slight compensation of water absorbance), the spectrum of glass-like glucose is also presented for comparison which is very similar to the spectral features obtained from aqueous solutions.[36]

NIR pulse spectrometry

Integral tissue probing suffers many limitations as described in detail.[1] As most clinical parameters are obtained by the analysis of blood, it is highly desirable to have access to similar information by non-invasive spectrometric means. However, since the blood volume represents only a small fraction of the total skin tissue probed, the signal changes due to the pulsatile blood flow are minimal when compared to the total tissue water. Such a measurement principle has been applied in pulse oximetry for many years and has recently been reviewed.[37] However, this technique has not yet been applied for metabolite measurements due to limitations in spectral signal-to-noise ratio, so far observed for *in vivo* NIR measurements.

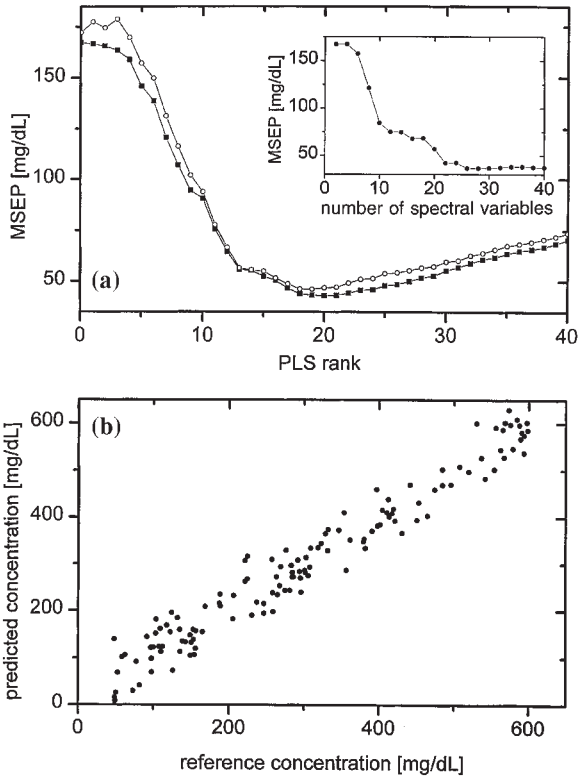

Figure 2. Calibration results for blood glucose using 132 diffuse reflectance lip spectra obtained from a single-person experiment with non-standard oral glucose tolerance testing and delayed glucose concentration profiles (see text): (a) root mean squared error of prediction (*RMSEP*) with full spectrum evaluation versus PLS model rank (for the spectral interval see Table 1; the leave-one-out cross-validation results are given by squares, results from leave-five-out are illustrated by open circles); the subplot provides the results from optimum PLS models as calculated with an increasing number of especially selected spectral variables. (b) predicted concentrations versus reference values (least-squares fit: $c_{pred} = 11.5 + 0.962\ c_{ref}$; $R^2 = 0.952$; calibration model based on 26 variables with an optimum PLS-rank of 18).

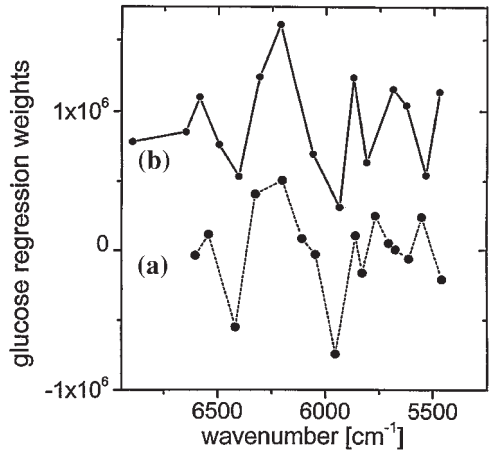

Figure 3. PLS regression vector sections for glucose concentration prediction with common spectral variables: (a) weights obtained for calibration with random human plasma samples from 124 different patients;[12] (b) weights obtained for calibration using a single-person experiment with 132 lip spectra recorded during non-standard oral glucose tolerance testing (offset and scaled; for calibration results see also Figure 2).

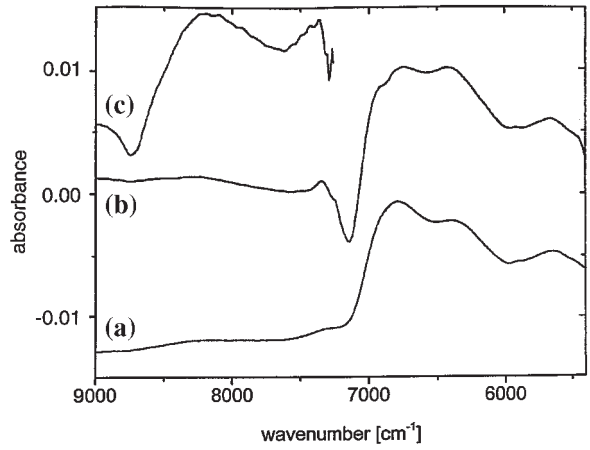

Figure 4. Glucose absorbance spectra within the spectral range considered for a non-invasive spectrometric assay: (a) spectrum from a glass-like glucose sample prepared from syrup (arbitrarily scaled); (b) difference spectrum of a 5.0% glucose solution as measured in a 0.5 mm cell, and (c) difference spectrum of a 2.5% glucose solution as measured in a 10 mm cell, both at a temperature of 30°C and with water absorbance compensation (offset for clarity).

Results of time-resolved measurements on human oral mucosa are reported using diffuse reflectance spectroscopy, which is based on the same accessory as mentioned above for time integrating inner lip measurements. The first individual lip spectra obtained for an exemplary experiment with fast measurements are shown in Figure 5(a). The logarithmised single beam lip spectra were pre-processed by a Savitzky–Golay smoothing with a quadratic polynomial of 25 data points and calculated as differences against the first measured spec-

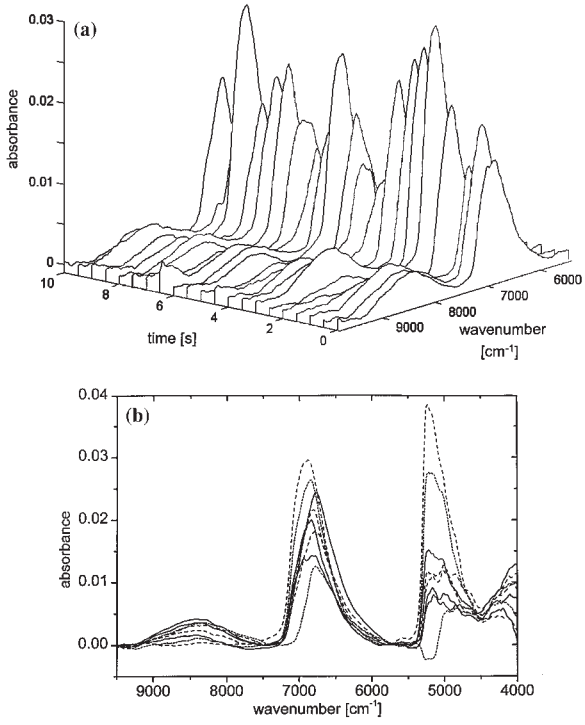

Figure 5. Some time-resolved inner lip spectra of a single person shown as differences versus the first measured single beam lip spectrum after Savitzky–Golay smoothing (see text) (a) and absorbance differences after application of a baseline correction using a quadratic polynomial and four base points, respectively (b).

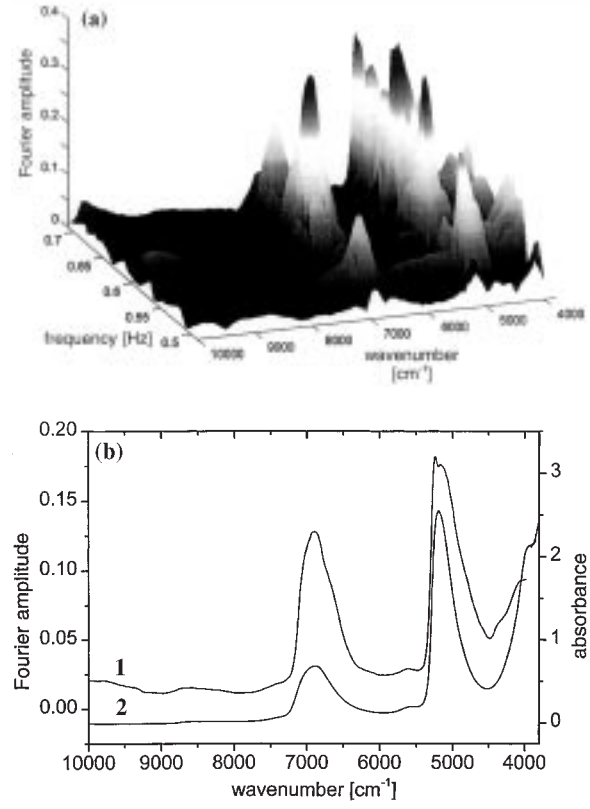

Figure 6. Three-dimensional plot of spectral Fourier amplitudes illustrating the pulsatile spectral components in the NIR diffuse reflectance spectra of human oral mucosa due to blood volume variations caused by the cardiac cycle (a); averaged spectral Fourier amplitude coefficients from frequency interval between 0.59 and 0.65 Hz (trace 1) and absorbance spectrum for water as measured in a 0.5 mm cell (trace 2, right ordinate scale) (b).

trum of the data set. The difference spectra, after application of a polynomial baseline fitted to four predefined spectral intervals which are located in the spectrum minima, are presented in Figure 5(b) displaying also a broader spectral range. In addition, it illustrates the intensity changes caused by changes in the arterial blood compartment associated with the cardiac cycle. Furthermore, a significant shifting of the absorption band maximum around 7000 cm^{-1} is obvious, which suggests a change in the water bonding structure. The reason for this could be dynamic effects arising from the blood pulsation, i.e. different shearing forces due to changing blood velocity. However, this must be investigated within future experiments.

The analytical form of the spectral pulsatile signal, as known from pulse-oximetry, varies a bit, but is mainly of sinusoidal shape modified by higher harmonics.[38] The Fourier analysis of the time dependent, discretely sampled logarithmised single beam intensities assigned to the individual spectral variables provides us with the spectral Fourier amplitudes at different frequencies which is shown in Figure 6(a). Prominent features show up around a frequency component of 0.6 Hz, which reflects a pulse rate of 84 heart beats per minute. This arises from the fact that an inappropriate sampling of two spectra per second was chosen leading to folding at the Nyquist frequency limit which is known as aliasing. Also higher harmonics of the pulsatile signals are folded into the frequency band resulting

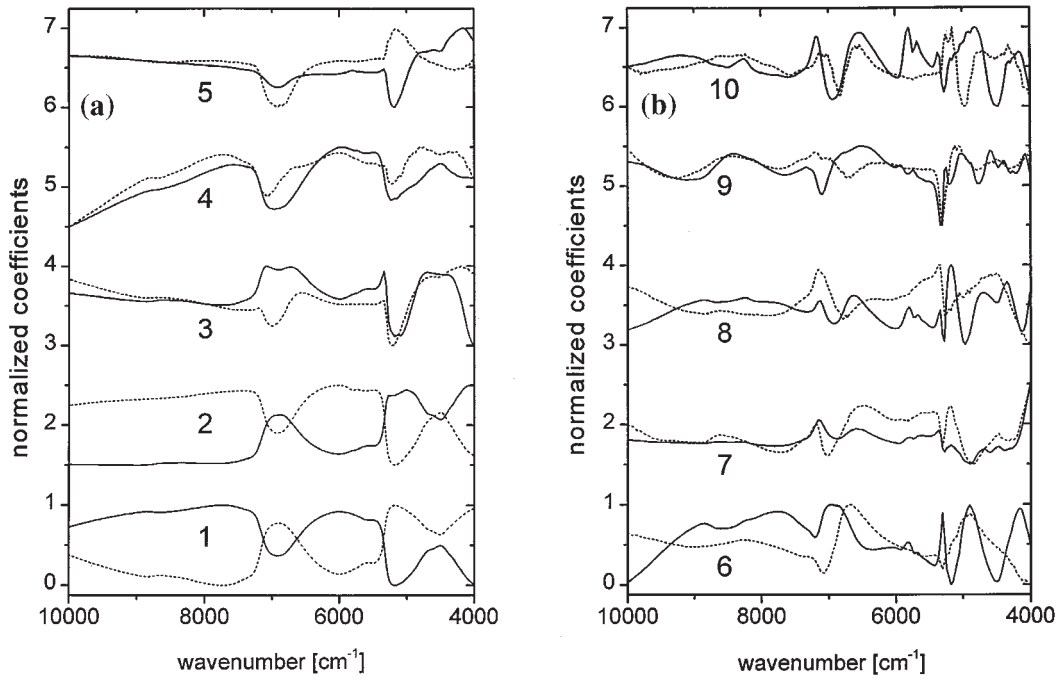

Figure 7. Factor spectra from a principle component analysis of the logarithmised inner lip spectra of a single person recorded during the calibration experiment with non-standard oral glucose tolerance testing (solid lines) and of the logarithmised inner lip spectra from a different person obtained during about one minute (sampling frequency: two spectra recorded per second; dashed lines): (a) provides the minimum-maximum normalised loading spectra of the first five principal components, and (b) gives additional five factor spectra related to further decreasing singular values of the original calibration spectra matrix.

from our chosen spectral sampling. Nevertheless, a unique and corrected assignment is still possible with the *a priori* information about the heart beat frequency. Investigations were carried out to determine to what extent the frequency folding effects the Fourier coefficients of the fundamental frequency of the time-dependent signal induced by the beating heart. No interference was observed for the fundamental and the second intense overtone Fourier coefficients which appear at a frequency window around 0.8 Hz for the situation studied. Further simulations with signals from pulse oximetry also showed that the fundamental component can be resolved from higher harmonics.[13]

In Figure 6(b) the pulsatile spectrum as obtained from the Fourier transformation of the time-resolved diffuse reflectance lip spectra is compared with the water absorbance spectrum as recorded with a transmission cell of 0.5 mm pathlength. The water absorbance alterations due to the cardiac blood pressure changes are about 20 mAU for the water band at 6900 cm^{-1}. This is equivalent to a water layer of 15 µm thickness, which is about a factor of 50 smaller than obtained for the integrative measurements discussed above. In comparison to the human plasma study[12] for which a cell of 1 mm pathlength was applied, there is even a factor of 70 between the corresponding water absorbance values. It is noteworthy, that the ratio of the maximum amplitudes of the water combination band at 5200 cm^{-1} and of the overtone band at 6900 cm^{-1} is much smaller for the pulsatile spectrum than for the water absorbance spectrum recorded in transmission. This can be explained by the significantly different penetration depths for the NIR radiation realised for those wavelengths. There are effects on the spectral water band shape depending on the cardiac phase which has been illustrated in Figure 5(b), with the result that

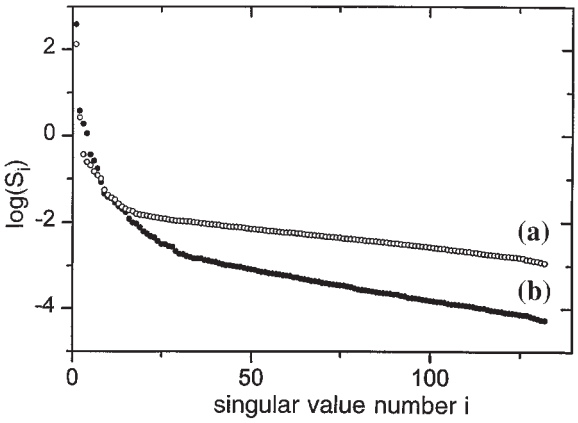

Figure 8. Logarithmic plot of the singular values from a singular value decomposition of two lip tissue spectra matrices (logarithmised single beam data between 10000 and 4000 cm^{-1}) versus their numbers: (a) spectra obtained from fast measurements resolving the pulsatile components from cardiac modulation (each spectrum is the average from 9 interferograms), (b) spectra recorded during a two-day OGTT experiment (each is the average from 1200 interferograms).

some water band broadening will be noticed for the pulsatile spectrum.

The different complexity of the spectral data obtained during the OGTT-experiment with the diabetic patient, compared with the spectral set recorded during time-resolved measurements, can be shown by a principal component analysis of both sets of spectral data. The first 10 factor spectra, normalised to the same min–max distance of their vector components, are presented in Figure 7. As the first five vectors show great similarity due to the dominating variance contributions from tissue water, the other factor spectra from the integrative inner lip measurements possibly demonstrate that different sampling tissue sites increase the complexity of the data. As a consequence, calibration modelling with a greater number of factors is required for efficient glucose assays than would be necessary using pulse spectrometry.

In Figure 8 the values from the singular value decomposition of the original logarithmised spectrum matrices are plotted in a logarithmic scale versus the number of the corresponding principle components, and one notices the steepest decline for the first nine singular values for both data sets. The slope becomes smaller for the next nine singular values for the time-resolved spectra, whereas about 20 principal components are needed to change the slope again in the plot of the singular values calculated for the time integrated inner lip spectra. The residual components can be related to random noise. This illustrates the different complexity of the spectral data compared here.

Conclusions

The performance of non-invasive blood glucose assays using diffuse reflectance lip spectra is still not acceptable for the normal and hypoglycaemic concentration ranges, even with PLS calibrations based on spectral variable selection. Significant improvements could, however, be achieved compared to previous results based on full spectrum evaluation of pre-selected intervals. In addition, evidence is provided for the physical effect, i.e. radiation absorption by glucose, underlying the individual calibration models.

NIR pulse spectrometry of skin tissue using diffuse reflectance measurements is a very promising technique. However, further investigations have to be carried out into which improvements can be gained from dedicated signal processing. Finally, pulsatile spectra of adequate signal-to-noise ratio have to be evaluated for calibrations for metabolites and other blood substrates. Improved time resolution measurements can better isolate the fundamental cardiac pulse frequencies or lock-in techniques might be applied for direct difference spectrum generation of the pulsatile blood component spectrum. Since such a technique allows the probing of a part of the vascular fluid, the perturbations from the tissue can be excluded, so that non-invasive blood analysis will be more reliable. In particular, this is important because most medical diagnostic expert systems are based on parameter data determined from blood samples.

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