Blood glucose assays based on infrared spectroscopy: Alternatives for medical diagnostics

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ABSTRACT

Infrared spectroscopy is nowadays frequently employed for applications in clinical chemistry. Since blood serves as the primary metabolic transport system in the body, its composition is the preferred indicator with respect to the pathophysiological condition of the patient. An important class of substances are the metabolites, including glucose, which are accessible by direct spectroscopic measurement without sample treatment. Multicomponent assays based on such technology are reagentless, fast and readily automated. Different *in-vitro* assays using mid- or near-infrared spectral data are presented including results from ex-vivo measurements using microdialysis and ATR spectroscopy for continuous blood glucose monitoring. Non-invasive sensing systems are under development for the determination of blood glucose, especially for diabetic patients or for monitoring in intensive care and surgery. Near-infrared spectrometry of skin tissue has been proposed, which allows a certain tissue volume to be integrally probed. On the other hand, fast measurements, such as used in pulse oximetry, can enable intravascular probing, i.e. collecting information on the arterial part of the vascular system (near-infrared plethysmography). Results and prospects for applications in non-invasive blood component assays are discussed.

Keywords: Fourier-transform infrared spectroscopy, clinical chemistry, glucose, *in-vitro* and *in-vivo* assays, multivariate calibration

1. INTRODUCTION

The applicability of infrared spectroscopy in clinical chemistry has been greatly increased by improvements in instrumentation and sample processing.[1] Blood serves as the primary metabolic transport system in the body, so its composition is the preferred indicator of the pathophysiological condition of a patient, although a variety of other biofluids is also accessible and can be analyzed. Consequently, the major application field for the clinical analyst consists of a combination of discrete sampling with subsequent analysis of many clinically relevant parameters. An important class are the metabolites such as glucose that are present within a concentration range allowing direct spectroscopic measurement without sample treatment. It must be emphasized that multicomponent assay methodology for blood substrates in biofluids carries several advantages, since it is reagentless, fast and readily automated. In addition, ex-vivo measurements using microdialysis and ATR mid-infrared spectroscopy allow for continuous blood glucose monitoring.

Besides improvements in the measurement technique, an essential prerequisite for reliable quantitative analysis is the application of statistical calibrations which afford a sufficient large number of calibration standards due to the complexity of the natural blood specimens seen in the clinical laboratory. Classical least-squares with curve-fitting of several component reference spectra is not applicable, because not all components contributing to the IR-spectrum of whole blood specimens are known. In the category of statistical calibrations, partial least-squares (PLS) or principal component regression (PCR)

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have been very successful algorithms employing full spectrum data. On the other hand, spectral variable selection as applied in multiple linear regression (MLR) can lead to improved and more robust calibration models than obtained by the traditional full spectrum approach which often relied on preselected, significant spectral intervals. The analytical performance of different *in-vitro* assays using mid- or near-infrared spectroscopy is compared here and possibilities for improvement are discussed.

Different non-invasive *in-vivo* sensing systems are under development for the determination of blood glucose, especially for diabetic patients or for direct monitoring in intensive care and surgery.[2,3] Measurements using near-infrared spectrometry of integral skin tissue have been proposed which possess the disadvantage that a great number of different factors, due to the complexity of tissue under spectrometric study, have to be considered for calibration modeling.[4] Fast measurements, such as used in pulse oximetry, can deliver intravascular probing by exploiting the blood volume changes caused by the rhythmic cardiac blood pressure alterations. The derived difference signals can be analyzed for metabolite concentrations, and the prospects for near-infrared spectroscopic plethysmography as a future application of such a technique in non-invasive blood component assays are discussed.

2. *IN-VITRO* ANALYSIS OF BIOFLUIDS

A Mid-Infrared *in-Vitro* Investigations of Human Plasma

The quantitative analysis of blood substrates in whole blood or blood plasma became possible through the attenuated total reflection (ATR) technique and access to water compatible IR-materials. In addition, as the aqueous phase in biofluids shows high absorption even at short sample pathlength, a major advantage is that this technique can deliver absorbance signals for quantitative analysis which are equivalent to a sample thickness of only a few micrometers in a transmission experiment. The filling of conventional cells of such pathlength with highly viscous biofluids is extremely difficult; interference fringes from multiple reflections within the cell additionally disturb the quantitative analysis of these spectra. A high spectral signal-to-noise ratio is absolutely essential to reach the concentration ranges envisaged for blood substrate analysis. Since the penetration depth of the infrared radiation field is in the order of the wavelength of the probing radiation, problems with this measurement technique arise from protein adsorption onto the ATR-crystal, which had to be overcome by special steps, i.e. cleaning and washing cycles to allow reproducible long-term measurements.

In the past, the micro-Circle cell has been successfully employed by us and others for mid-infrared investigations[5-8], as a flow-through cell of low inner volume (about 50 µL) is available which can be thermostatted and provides high optical throughput. On the other hand, IR-fiber based evanescent wave spectroscopy is showing a great potential for quantitative analysis of biofluids (see Fig. 1), because similar spectroscopic performance can be achieved by using much simpler and

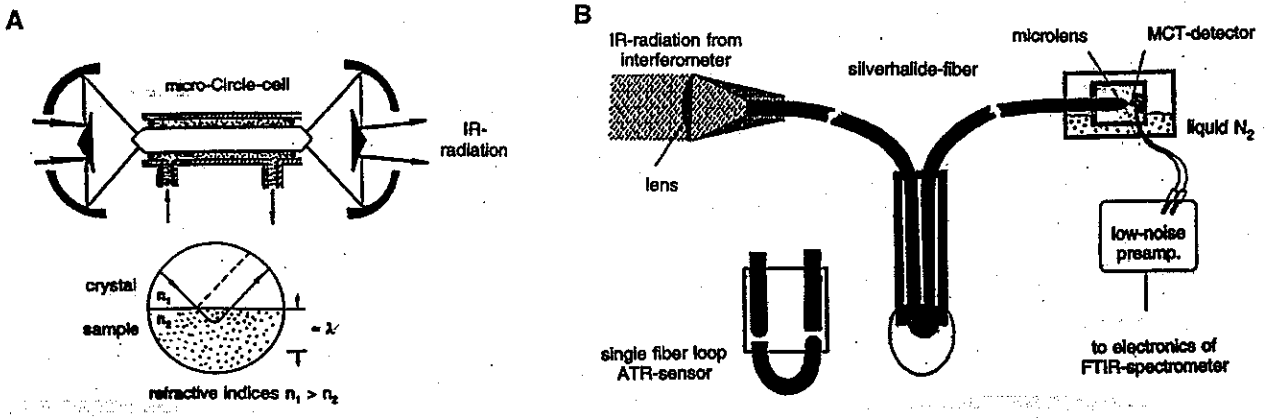

Fig. 1 Experiments using the ATR-technique: **A** micro-Circle cell accessory (reproduced by permission[9]), **B** simple and inexpensive setup for evanescent wave spectroscopy using fiber optics realized for silver halide material.

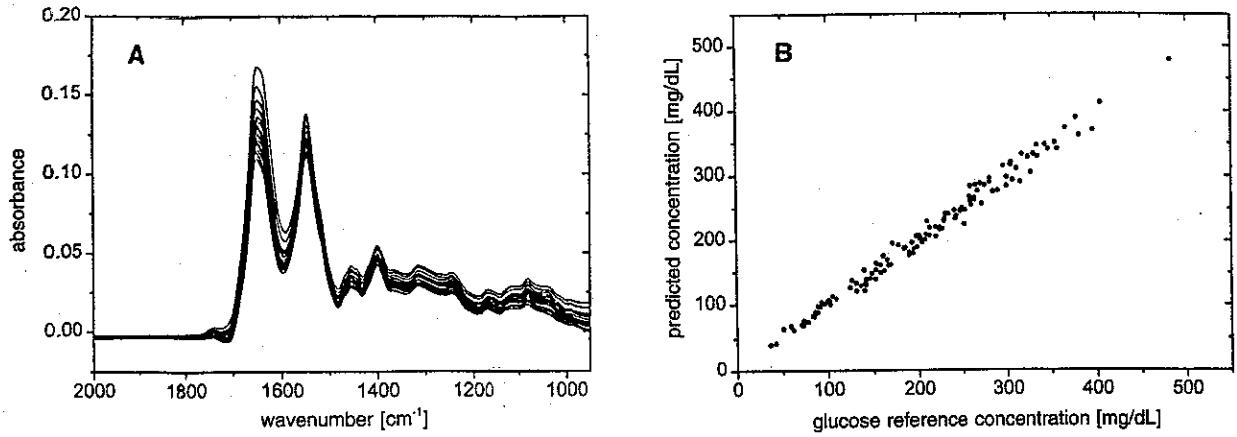

Fig. 2 Calibration experiment using ATR mid-infrared spectra of blood plasma: **A** absorbance spectra as measured versus a water filled micro-Circle cell, **B** prediction results for glucose using a PLS-calibration model based on absorbance values at 10 specially selected spectral variables within the interval of 1200 - 950 cm^{-1} vs. clinical reference values.

less expensive accessories than based on conventional ATR-techniques.[10] In addition, a tremendous miniaturization of the equipment can be realized using silver-halide fibers.

The results from a glucose assay are presented for human plasma using mid-infrared ATR spectra recorded from a hospital population of samples from 126 different patients. Partial least-squares was used for multivariate calibration based on spectral intervals in the fingerprint region selected for optimum prediction modeling. Different data, either plasma absorbance spectra with compensation of the water signal or logarithmized single beam spectra, were considered (see Fig. 2). The second option for building statistical models, using single beam spectra, provides a lower noise level and less systematic variations due to cell filling compared to the two step measurement needed for absorbance spectra. However, some variations in the spectra due to spectrometer instability over the measurement time of the spectral calibration and prediction data exist. The prediction performance of calibration models based on a set of spectral variables selected from the extreme values of the optimum full spectrum PLS regression vector[11] is also given for comparison in Table 1.

B Coupling of Microdialysis and Mid-Infrared ATR Spectroscopy

Worldwide extensive effort is being put into the development of glucose sensors for *in vivo* monitoring, which will improve glycemic control in patients with disorders of their carbohydrate metabolism, because the continuous measurement of

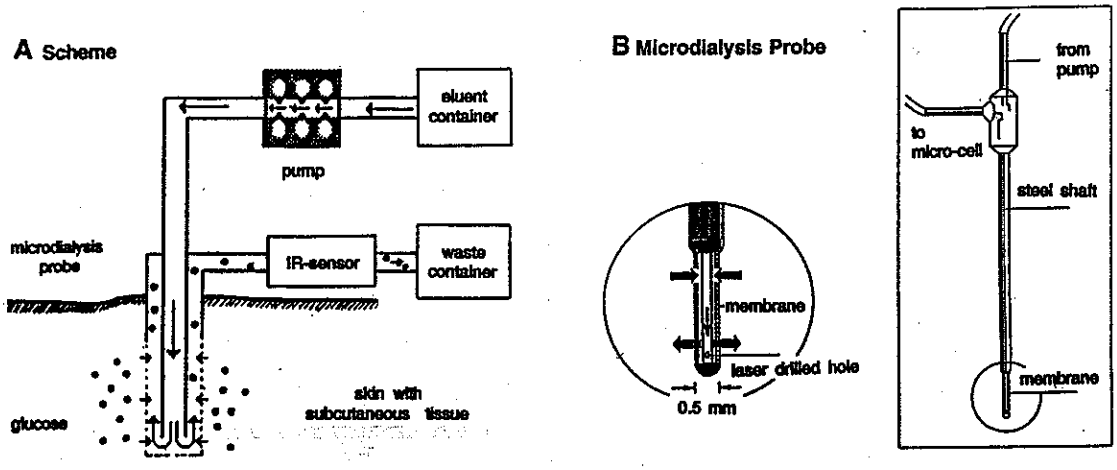

Fig. 3 Experimental scheme for the coupling of a microdialysis probe with an IR-spectroscopic glucose sensing device: **A** Scheme for continuous monitoring, **B** microdialysis probe (length of the membrane covered shaft was 16 mm).

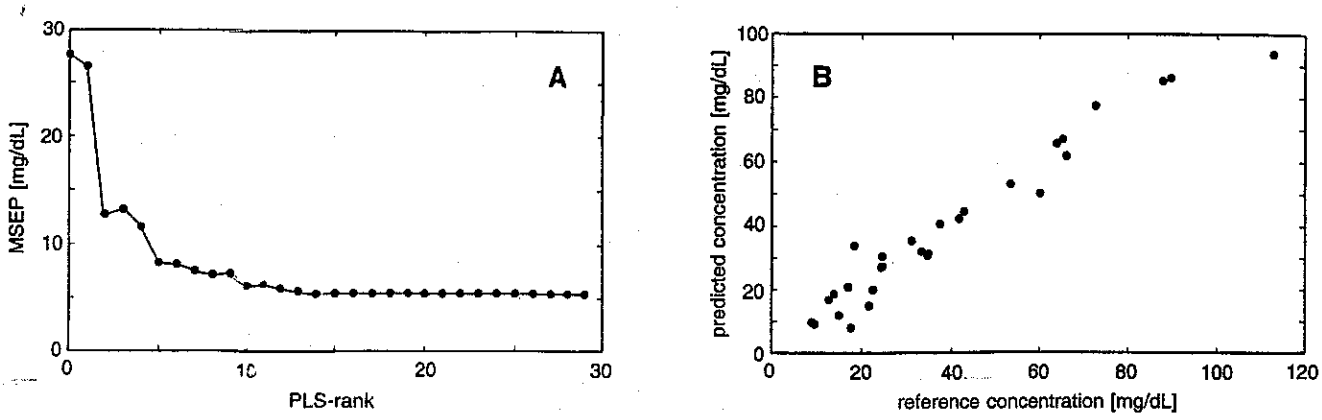

Fig. 4 Calibration results for glucose in microdialysates from seven different patients: **A** MSEP statistics obtained from leave-one-out cross-validation vs. PLS rank, **B** predicted concentrations vs. measured clinical reference values.

glucose is essential for individuals without adequate blood sugar regulation. Such a device is particularly desirable in an artificial pancreas for patients with diabetes mellitus to achieve optimal metabolic control. There are currently two invasive approaches proposed and evaluated: the use of electrochemical enzyme biosensors implanted directly into the subcutaneous tissue or in combination with a microdialysis probe (see also Fig. 3).[12] As the lifetime of the enzyme sensors is usually

Table 1. *In-vitro* glucose calibration results for different sample populations. Glucose reference concentrations were determined by a hexokinase/glucose-6-phosphate dehydrogenase assay.[13] Mid-infrared measurements were done by using the ATR-technique; for the near-infrared spectra a transmission cell of 1 mm pathlength was employed. The mean-square prediction errors (MSEP) are given for full spectrum evaluation and with spectral variable selection (c_{av} is the average concentration and σ_{pop} the population standard deviation).

data	no. of samples	c_{av} (mg/dL)	σ_{pop} (mg/dL)	no. of variables	MSEP (mg/dL)
Glucose in blood plasma (spectral calibration range 1200 - 950 cm^{-1})					
-log (single beam)	126	207.9	91.2	49	9.8
				18	9.5
absorbance	126	207.9	91.2	49	10.4
				14	10.3
Glucose in dialysates from blood plasma (spectral calibration range 1200 - 950 cm^{-1})					
absorbance	29	39.9	27.1	49	6.1
Glucose in water (spectral calibration range 1200 - 950 cm^{-1})					
absorbance	17	39.7	22.1	49	1.3
Glucose in blood plasma (spectral calibration range 6800 - 5450 and 4750 - 4200 cm^{-1})					
-log (single beam)	124	206.8	91.5	62	16.2
				24	16.4
absorbance	124	206.8	91.5	62	18.0
				26	16.3

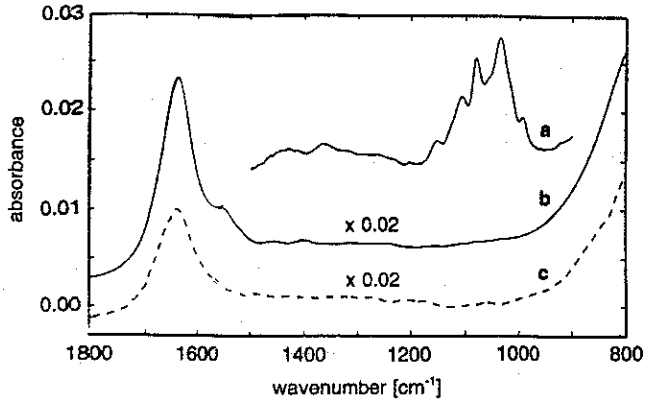

Fig. 5 Absorbance spectra recorded by using different ATR accessories: a) glucose in aqueous solution (0.5 %) with water absorbance compensation measured with the micro-Circle cell; b) serum as measured using a fiber loop sensor (see also text) calculated vs. a spectral background with air exposed fiber; c) water as measured using a flow-through cell with fiber centered inside a teflon hose (inner volume 4.5 µL, fiber length for the two latter devices was 18 mm, respectively).

limited, detection by infrared spectrometry is a promising alternative. The microdialysis simplifies the biotic matrix considerably, because compounds of low molecular weight can be separated from proteins, eliminating the problems in continuous measurements arising from protein adsorption onto the ATR crystal surface. However, a dilution of the original solution concentration is observed depending on the flow-rate of the perfusion fluid. Micro flow-through cells with internal volumes of only a few microliters are needed to follow the dynamics of the *in vivo* blood glucose concentration profiles in diabetic patients.

Microdialysates were obtained from blood plasma from seven patients; for more experimental details see[14]. Multivariate calibration with spectral data from 1200 to 950 cm^{-1} was carried out based on the partial least-squares algorithm. The average prediction error for glucose evaluated for a calibration model from 10 PLS factors by cross-validation was 6.1 mg/dL. The performance can be considerably improved when a constant matrix such as obtained in continuous single-person sampling is provided. Simulation with aqueous glucose solutions yielded a mean squared prediction error of 1.3 mg/dL which is excellent for monitoring of low glucose concentrations down to 15 mg/dL (see also Fig. 4 and Table 1).

With improved optical and mechanical properties of crystalline silver halide fibers the development of novel accessories, in particular of flow-through cells with an inner volume of a few microliters or disposable sensor parts for sample measurement, is possible. These features enable process monitoring, for example, when only minute sample volumes at low flow rates are available such as in the case of *in-vivo* blood glucose sensing using microdialysis as discussed above. The performance of some fiber-optic sensors based on evanescent field absorption has been compared with conventional ATR

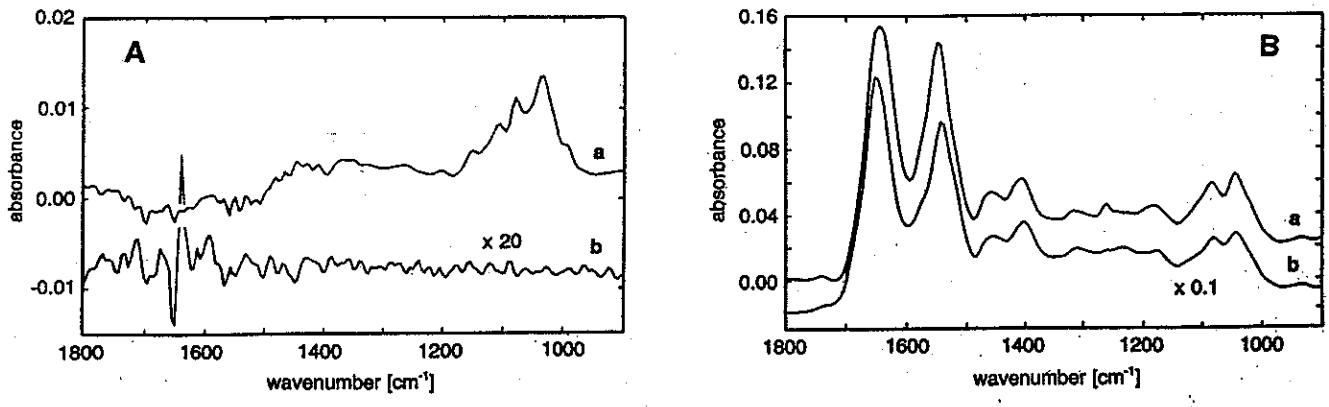

Fig. 6 Absorbance spectra as measured with a fiber loop sensor (spectral resolution 8 cm^{-1}, 600 interferograms accumulated, Blackman-Harris three-term apodization): **A** Spectrum of aqueous glucose (0.5 %) with water absorbance compensation and noise level estimated by ratioing two water spectra, **B** Spectra from a liquid serum sample with water absorbance subtraction (a) and from a dry serum film on the fiber loop (see also text).

equipment, especially for studying microsamples of biofluids.[10] The following Figures 5 and 6 illustrate the potential of fiber based accessories. In a fiber sensor, consisting of a fiber bent perpendicularly to a nearly full turn (total fiber length 18 mm), special fiber modes can be excited, so that about the same absorbance signals in the finger print region were obtained for aqueous solutions as by using a micro-Circle cell. A special flow-through cell with an inner volume of 4.5 µL delivered smaller absorbance signals, e.g. for water (see Fig. 5), than for the latter devices. The spectral signal-to-noise ratio achievable for quantifying glucose is satisfactory under the experimental conditions chosen in Fig. 6A. A detection limit below 1 mg/dL can be reached based on the univariate analysis at the glucose peak maximum and the rms-noise level. Multivariate data evaluation is expected to lead to an even lower detection limit. Figure 6B illustrates the performance of a fiber-based ATR device based on dry film measurement affording only a microliter sample volume. The serum film was created just by dipping the fiber loop into the biofluid and subsequent rapid drying by exposing it to the atmosphere. Further investigations are necessary to evaluate the full potential of these unpretentious measurement techniques for glucose assays applicable for medical diagnostics.

C Near-Infrared *in-Vitro* Investigations of Human Plasma

Near-infrared spectroscopy also offers splendid opportunities for quantitative assays in clinical chemistry, although the information content of the spectra in that spectral range is lower compared to the prevailing information contained in mid-infrared spectra with plentiful bands from fundamental and combination vibrations. In the near-infrared range, molecules absorb radiation for excitation of overtone and combination bands assigned to vibrations of molecular moieties with hydrogen atoms involved, such as O-H, C-H and N-H. The short-wave near infrared carries higher overtone bands with even smaller absorptivities, so that transmission is usually increased, and aqueous solutions can be measured with cuvettes up to a centimeter pathlength. We were interested in a comparison of glucose assays based on different spectral ranges to decide on which technique would be most appropriate for the *in-vitro* blood analysis. Therefore, the blood plasma sample population from a hospital was studied by using a transmission cell of 1 mm pathlength. An example of the resulting spectra is given in Fig. 7A which shows the absorbance spectrum of a plasma sample as measured versus the empty and water filled cell, respectively. An alternative is to measure dry films from biofluids using the diffuse reflectance technique. This offers the potential of extending the analytical range of quantitation by eliminating the masking and disturbing water absorptions. Further investigations have to be carried out into this challenging dimension of clinical infrared spectroscopy.[15]

Multivariate calibrations were calculated based on water compensated plasma absorbance spectra, as well as on logarithmized single beam spectra. In addition to the full spectrum evaluation, calibration models with spectral variable

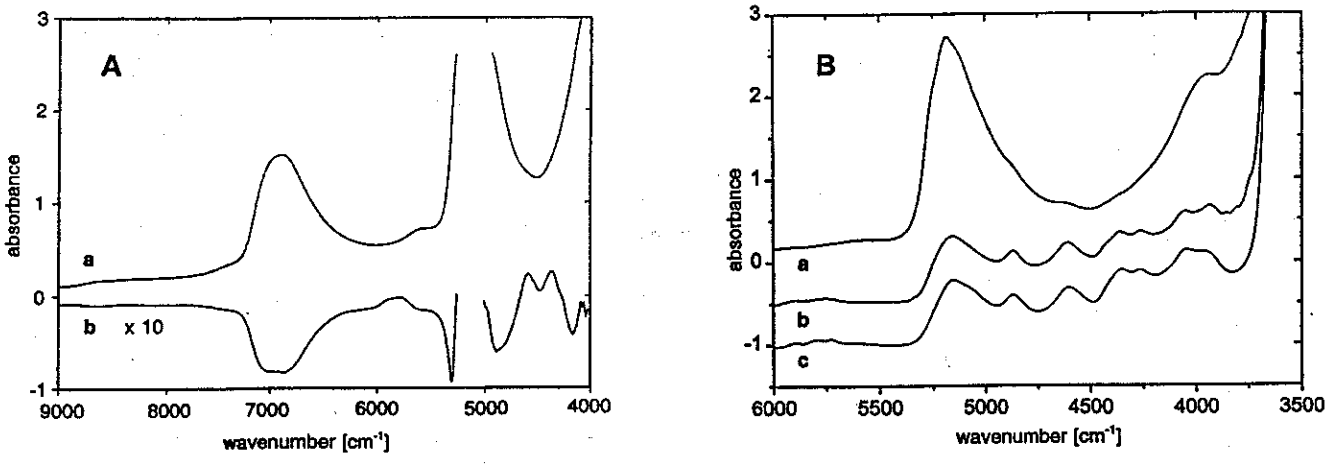

Fig. 7 Near infrared spectra of biotic fluids: **A** Absorbance spectra of a plasma sample obtained with a 1 mm pathlength quartz cell as calculated vs. the empty (a) and water filled cell (b); **B** Diffuse reflectance spectra of whole blood (a) and of dried films from blood (b) and plasma (c) as measured on a gold coated rough substrate of sand paper (the latter two traces are enlarged for clarity).

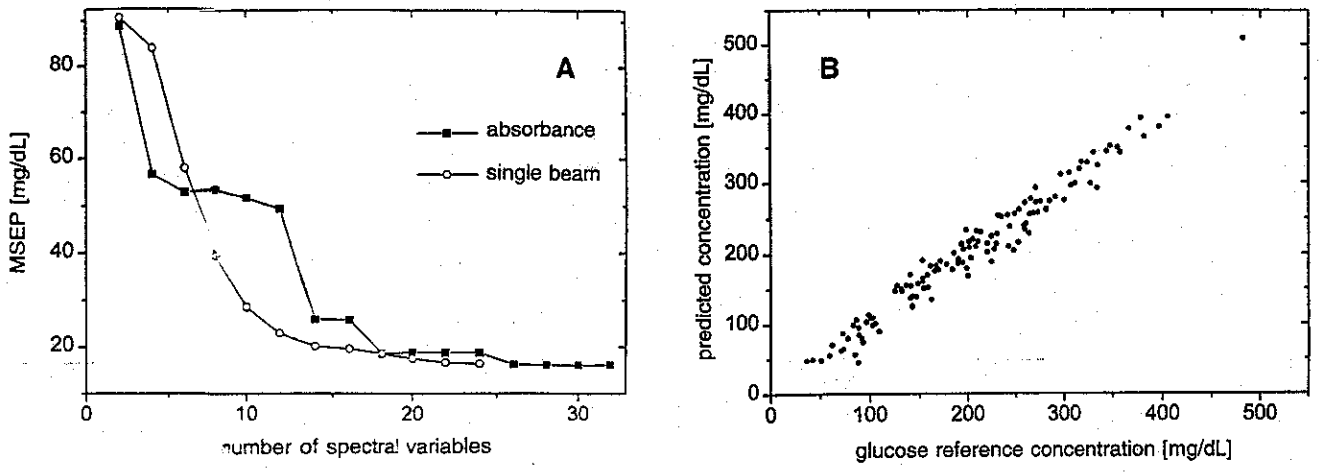

Fig. 8 PLS-calibration results for glucose in human plasma: **A** Optimum MSEP statistics obtained from leave-one-out cross-validation in dependence of the number of especially selected spectral variables for absorbance spectra and logarithmized single beam spectra, **B** predicted concentrations from a single beam calibration model (24 spectral variables) vs. clinical reference values.

selection were also performed, for which the results are also summarized in Table 1. In Figure 8 the optimum MSEP for models with a varying number of spectral variables is given illustrating that about 18 variables within the preselected spectral transmission windows are needed to have sufficient prediction capability for glucose concentrations. In Figure 7B the predicted concentrations obtained from a single-beam-calibration model using leave-one-out cross-validation are plotted versus the clinical reference values. The average performance of best mid-infrared and near-infrared calibration models lie apart by at least a factor of 1.7 when comparing MSEP-values and rating them against the overall best result. This can be explained by the lower data selectivity, as in particular overlap of substrate absorption bands with strong bands from water in the near-infrared range exists. The hydrogen bonding network in aqueous solutions, and therefore their absorption spectrum, is known to be rather sensitive to several experimental and intrinsic parameters, e.g. temperature.

3. IN-VIVO MEASUREMENTS

Owing to the large water absorptivities in the mid-infrared, penetration of IR-radiation is not sufficient to establish transcutaneous measurements of metabolites in tissue. A way out of this dilemma is to use near-infrared spectroscopy for which the water absorptivities are much smaller. Due to the optical constants of tissue, i.e. the spectral absorption and scattering

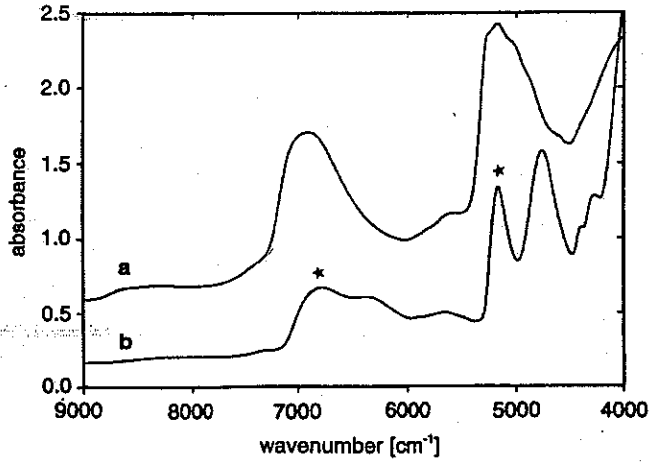

Fig. 9 Diffuse reflectance spectrum (in absorbance analogue units) of the inner lip of a test person as measured by a special accessory[16] (a) and absorbance spectrum of a glass-like glucose film showing the same spectral dependence as found for aqueous solutions (b); water residual absorption bands are marked by asterisks.

coefficients, a wavelength dependent penetration depth for such radiation exists. For wavelengths between 600 and 1300 nm (16700 - 7700 cm^{-1}, the so-called therapeutic window) opportunities are available for transmission measurements of body tissues. Another window exists from 1600 to 1850 nm (6250 - 5400 cm^{-1}) between two water absorption bands, which can be used for diffuse reflectance measurements. Such a measurement technique was favoured by us based on our experiences with *in-vitro* spectroscopic glucose assays.[1] Generally, the measurement conditions for the *in-vitro* analysis of biofluids can be better controlled with regard to temperature stability, sample homogeneity and optical pathlength than for *in-vivo* measurements with tissues being of greater complexity than found for biofluids.

Several papers about non-invasive glucose measurements have been published by our group[17-19]. Near-infrared diffuse reflectance spectra of the human inner lip were evaluated for PLS-calibration with spectral data between 9000 and 5500 cm^{-1}. Non-standard oral glucose tolerance testing and random testing were carried out within single-person calibration experiments. A time lag of about 10 min. in the glucose concentration profile within the spectroscopically probed tissue volume, compared to capillary concentrations, could be estimated from the quasi-continuously obtained calibration data. In a multi-person calibration experiment, the venous reference data provided better prediction modeling than the capillary glucose concentration values. The average mean squared prediction errors for calibration models based on single-person data were about 2.5 mmol/L (45 mg/dL), and prediction errors for multi-person calibrations were 10 mg/dL higher. Further improvements were recently achieved using our spectral variable selection for PLS-calibration models.[11] By this approach a 15 % reduction of the average prediction error could be achieved. The results of the *in-vivo* calibration experiments are summarized in Table 2.

Integral tissue probing suffers many limitations as described in detail in Reference.[4] 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

Table 2. *In-vivo* glucose calibration results for different experiments. The near-infrared spectra of the inner lip were recorded using a special diffuse reflectance accessory. The mean-square prediction errors are given for full spectrum evaluation and with spectral variable selection.

data	no. of samples	c_{av} (mg/dL)	σ_{pop} (mg/dL)	no. of variables	MSEP (mg/dL)
Single person with oral glucose tolerance testing (spectral calibration range 9000 - 5475 cm^{-1})					
-log (single beam)/ capillary blood	132	300.4	167.9	115	45.6
				30	38.9
-log (single beam)/ delayed glucose profile	132	299.1	166.5	115	43.0
				26	36.4
Single person with random glucose testing (spectral calibration range 9000 - 5475 cm^{-1})					
-log (single beam)/ capillary blood	216	268.2	162.3	115	51.9
				32	46.8
Multi-person experiment with random glucose testing (spectral calibration range 9000 - 7420 and 7000 - 5920 cm^{-1})					
absorbance/ venous blood	381	148.7	75.3	88	55.4
				22	54.1

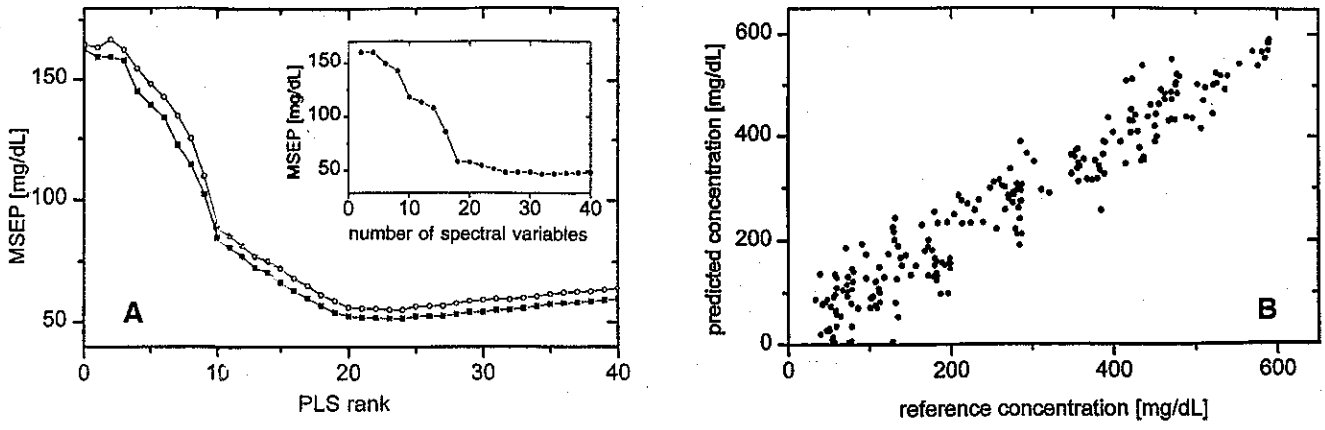

Fig. 10 Calibration results for blood glucose using diffuse reflectance lip spectra obtained from a single-person experiment with random testing: **A** mean square error of prediction with full spectrum evaluation (for the spectral interval see Table 2; the leave-one-out cross-validation results are 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 for the optimum PLS model based on 32 spectral variables.

applied in pulse oximetry for many years and has recently been reviewed.[20] A Fourier analysis of pulse oximetry data provides us with the necessary sampling frequency to resolve the fundamental amplitudes of the heart beat modulated periodic blood volume variations (see Fig. 11). However, this technique has not yet been applied for metabolite measurements due to limitations in spectral signal-to-noise ratio, so far achieved for *in-vivo* near-infrared measurements.

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 with two spectra per second are shown in Fig. 12A. The difference spectra are calculated as differences against the first measured spectrum of the data set, which illustrates the intensity variations caused by changes in the arterial blood compartment associated with the cardiac cycle. The logarithmized single beam lip spectra were preprocessed by a Savitzky-Golay smoothing with a quadratic polynomial of 25 data points, before the Fourier transform of the time dependent absorbance signals from each spectral variable was calculated.

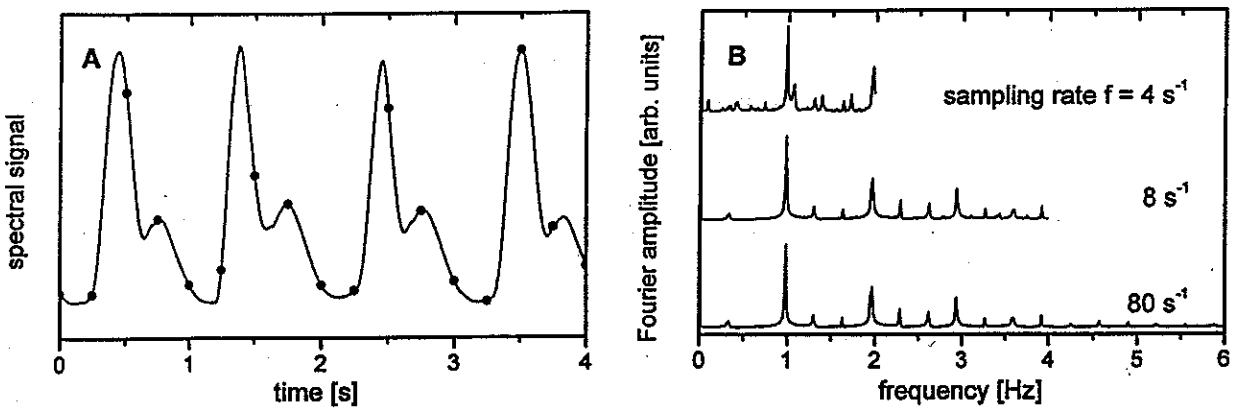

Fig. 11 Pulse oximetry traces, observed for example for hemoglobin absorption at 580 nm and sampled every 250 ms (**A**) and Fourier-transform data obtained for different sampling intervals resulting in different Nyquist frequency limits; for 4 and 8 Hz sampling traces aliasing is evident (**B**).

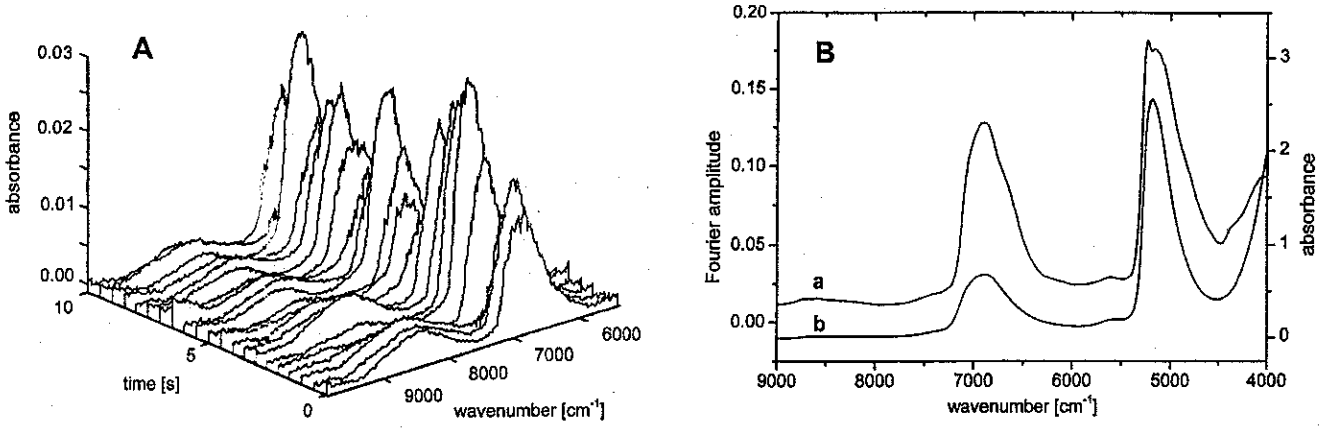

Fig. 12 Time resolved measurements of inner lip spectra from a single person shown as differences versus the first measured single beam lip spectrum (**A**); averaged spectral Fourier amplitude coefficients from frequency interval between 0.59 and 0.65 Hz (a) and absorbance spectrum for water as measured in a 0.5 mm cell (**b**, right ordinate scale) (**B**).

In Figure 12B 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 mA.U. 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 for which a cell of 1 mm pathlength was applied, there is 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 near infrared radiation realized for these two wavelengths.

CONCLUSIONS

The analytical performance of in-vitro assays can be considered as satisfactory, especially when mid-infrared spectral data, recorded by the ATR technique, are considered. The prediction quality is good enough to allow continuous glucose monitoring using a microdialysis probe which has to be subcutaneously implanted. By this technique concentrations down to 15 mg/dL due to dilution by low perfusion flow have to be quantified with a relative standard deviation of about 10 %. Future routine analysis in the clinical laboratory might rely on dry film measurements, carried out by transmission, attenuated total reflection or reflection-absorption. Such techniques need only blood sample volumes of less than 1 µL which can be obtained by less-invasive and painless methods contrary to those nowadays in use.

The problem with spectrometric non-invasive glucose assays is that a constant, concentration independent prediction error exists, as long as spectral data and modeling are still inadequate for practical use, which is an adverse and inacceptable condition for measurements especially in the hypoglycemic range. As a guideline, a relative concentration prediction error of 15 % or better over the clinically relevant range between 30 and 350 mg/dL could be considered sufficient, so that further improvements are essential compared to the results achieved here. Near infrared pulse spectrometry of skin tissue using diffuse reflectance measurements is a very promising technique. However, further investigations have to be carried out into obtaining improvements through dedicated signal processing. Finally, pulsatile spectra of adequate signal-to-noise ratio have to be evaluated for calibrations for metabolites and other blood substrates.

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