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Development and testing of mid-infrared sensors for in-line process monitoring in biotechnology

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ABSTRACT

Three prototypes of mid-infrared (MIR) spectrometric sensor systems for simultaneous monitoring of ethanol and carbohydrates (in the present case glucose and fructose) in the course of biotechnological processes have been constructed based on recent developments in pyroelectric detection and fiber photonics. The sensors utilized were a grating spectrometer or a Fabry-Pérot interferometer adjusted for the detection of analytes' characteristic absorbance bands in the spectral region of "fingerprints" between 1050 and 950 cm^{-1}. The measurements were performed with an attenuated total reflection (ATR) probe connected to the spectrometer by a polycrystalline infrared fiber (PIR). Two probes with different ATR elements were tested: with a diamond crystal (for both spectrometers) and with a detachable PIR loop head (for grating spectrometer). The sensor performances were assessed and compared using partial least-squares (PLS) regression modeling and prediction statistics for two designed sample sets of binary ethanol–glucose and glucose–fructose aqueous solutions. The models based on the FT-IR spectroscopic analysis of the same designed samples using a diamond ATR probe (a "gold standard" method) were used as a benchmark. The system based on a grating spectrometer connected to an ATR probe with a PIR loop head was additionally tested under the process conditions of *Saccharomyces cerevisiae* fermentation. The resulting root mean-square errors of prediction were 4.74 and 13.33 g/L, for ethanol and glucose models, respectively. Simultaneously, NIR spectroscopy in the range 1100–2100 nm was used both for the analysis of designed samples and for the fermentation process monitoring. In the latter case a biomass content prediction model has been built along with those for ethanol and glucose. All tested full-spectroscopic and sensor-based methods of analysis have been compared and their practical applications discussed.

1. Introduction

Most biotechnological processes commonly applied worldwide are poorly regulated in terms of their chemical analysis, even those established in the industrial sphere. Typically, only pH, redox activity, dissolved oxygen, biomass and temperature are monitored using well-established in-line probes and sensors. In order to determine the concentrations of nutrient components of the medium, such as glucose and other sugars, as well as the most desired organic products, it is necessary to retrieve samples from the bioreactor for a conventional, e.g. chromatographic analysis. Due to the samples' complexity, inevitable periodicity and slowness of the laboratory analysis as well as high process speeds, common analytical methods are often insufficient to assure the required product quality and production efficiency [1]. Furthermore, close monitoring of various process parameters in the course of fermentation may be critical for fast counter-steering in case of a deviation from the normal process conditions.

The regulatory initiative of process analytical technology (PAT) issued about ten years ago [2] has started to change the process control strategies, specifically in the food and pharmaceutical sectors,

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stimulating the development of highly informative real-time (in-line) or near real-time (on-line) monitoring methods and analytical devices.

A leading role in the development of in-/on-line analysis in biotechnological process monitoring belongs to optical spectroscopy in near infrared (NIR) range up to 2500 nm (4000 cm^{-1}) [3–8] and fluorimetry [9,10]. Recently, some biotechnological applications of Raman spectroscopy have been reported [11–13]. Perfect adaptability of the optical techniques to in-line process analysis is related to the application of fiber optics and flexible probes on its basis. The spectral measurements are non-invasive and the resulting multivariate data contains a wealth of process information that can be used to analyze several parameters at once [10].

In actual fact, the data evaluation, particularly of NIR spectra, still remains a challenge that slows down the industrial distribution of spectroscopic systems. The process spectra usually have complex shapes with overlapping signals of individual compounds. The fermentation broth analysis is also complicated by the presence of various substances with unknown spectral properties and concentrations that affect the measured spectra. The spectra can also be strongly altered by process turbulence and by the light scattering by cells or gas bubbles in the optical pathway [14]. Collectively, these influences have to be taken into account, in order to obtain accurate and robust predictive models for quantitative determination of the monitored parameters. Recent advances of multivariate data analysis (also known as chemometrics) made an essential contribution to the development of instrumental analytical chemistry in general. Multivariate modeling is an important science-intensive part of the analytical method development for process monitoring [1]. It includes several methodologies: design of experiment (DoE) [15], exploratory factor analysis, multivariate regression algorithms, such as partial least-squares (PLS) [16] in addition to others.

The spectroscopy of mid-infrared region (MIR) 4000–400 cm^{-1} (2.5–25 μm) is rarely used for in-line analysis. Its application examples in biotechnology can be found in the following references [13,17–19]. This reluctance to use the highly sensitive and chemically specific MIR region in process analysis can be accounted for by high costs of general-purpose MIR spectrometers themselves and sometimes by a demanding handling under running process conditions that could not be avoided even in commercial systems. In-line MIR measurements may require liquid nitrogen for detector cooling or nitrogen gas atmosphere to avoid undesired absorption in free beams. Advantages and drawbacks of MIR compared to NIR spectroscopy are considered in [20]. Until recently, the price and limited availability of low-absorption fiber materials could be prohibitive for using convenient fiber-based MIR analyzers and probes.

Novel IR-fiber materials available nowadays cover practically the whole mid-infrared range. Chalcogenide IR (CIR) glasses based on arsenic trisulphide (As_2S_3) transmit light in the wavelength range 1.5–6 μm and have the highest transmittance among the known glass fiber materials [21]. Oxygen-free polycrystalline infrared (PIR) fibers manufactured of silver halides' solid solutions $AgCl_{1-x}Br_x$ ($0<x<1$) [22] possess necessary transparency from 3 μm up to 18 μm. Therefore, they are indispensable for MIR fiber-optics spectroscopy in the whole "fingerprint" region that is rich in chemical information due to the fundamental absorption frequencies of various functional groups.

Attenuated total reflection (ATR) spectroscopy is a measurement technique that is perfectly suited for MIR analysis of different materials. The total optical path of ATR analysis is formed by a typical penetration depth of ATR crystal's evanescent field (0.5–2 μm) times the number of internal reflections defined by the ATR element geometry. Short pathways are often advantageous considering that IR absorption coefficients of most substances are much higher than e.g. in the NIR range. The most common ATR crystals are made of ZrO_2, ZnSe, Ge, Si and diamond. The crystal shape (prism, cone, multi-bounce plate, etc.) can be varied depending on the probe type and sample nature [23]. Over the last few decades a number of ATR probes for MIR analysis have been developed and manufactured based on CIR and PIR fiber technology. These probes, typically coupled with Fourier-transform infrared (FT-IR) spectrometers, were successfully tested in various analytical spectroscopy applications, such as in-line process monitoring and in-vivo tissue analysis [23–29]. Current advancements of optical technologies, in particular, budget techniques for high-resolution IR detection [30–32] create prerequisites for the broadening of the application range of mid-infrared spectroscopy and for its increasing use in industrial and field applications.

Here we report on the results of a collaborative project aimed at the development of a MIR sensor analyzer for in-line determination of ethanol, glucose and fructose in mixtures using the ATR probe technology. Three sensor prototypes have been developed using different up-to-date infrared detection units, probes and fibers. They were tested and validated on a designed sample set as well as under the conditions of a running *Saccharomyces cerevisiae* fermentation process. Simultaneously, the measurements were performed with NIR spectroscopy using a transflectance probe. Multivariate prediction models built on spectra of the designed samples measured with a diamond ATR probe coupled with a high-end laboratory FT-IR spectrometer were used as a benchmark. The prediction performances of different techniques have been compared and discussed.

2. Materials and methods

2.1. Designed sample sets

Two sample sets containing binary aqueous solutions ethanol–glucose (EG) and glucose–fructose (FG) were prepared by dissolving water-free pure ethanol (containing 1% of methyl ethyl ketone), D-glucose and D-fructose both obtained from Merck KGaA (Darmstadt, Germany) in de-ionized water (Fig. 1). Each set was composed in accordance with the "diagonal design" scheme [15]. This design provides independent (i.e. uncorrelated) variability of both component concentrations and their uniform distribution over 25 levels of the chosen concentration ranges. The diagonal experimental design has been developed for the multivariate calibration of multi-component mixtures and includes an integrated validation subset.

In the following text the samples are referred to by a prefix of the sample set (EG or FG) followed by the sample number indicated in the corresponding cell of the chart in Fig. 1. For instance, EG09 points at the sample containing 42 g/L of ethanol and 204 g/L of glucose. Similarly, FG20 states for 10 g/L of fructose and 24 g/L of glucose in the respective sample.

The solution samples were stored in tightly closed falcon tubes in a refrigerator at 4 ± 1 °C.

2.2. Fermentation process

An anaerobic *Saccharomyces cerevisiae* fermentation was chosen for the testing of developed analyzers under process conditions. Two processes were carried out in a bench-scale bioreactor system with an amateur control unit provided by Ulm University of Applied Sciences.

Total durations of first and second fermentations were 5 h and 32 min and 5 h and 52 min, respectively (Table S-1). Samples were periodically retrieved from the bioreactor and glucose and ethanol concentrations were determined by enzymatic UV tests [33,34] by R-BIOPHARM AG (Darmstadt, Germany) at 340 nm using a Büchi 900 photometer (Büchi Labortechnik GmbH, Essen, Germany).

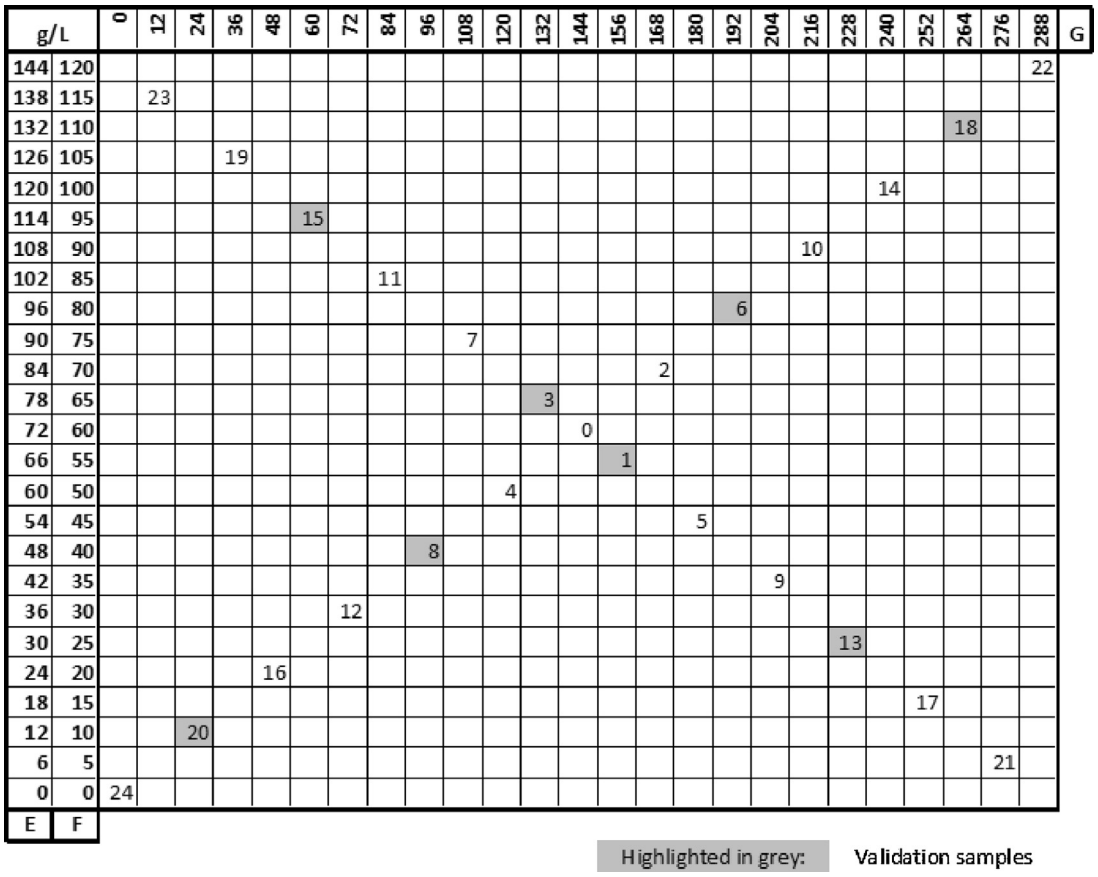

Fig. 1. Diagonal experimental design of ethanol–glucose (EG) and fructose–glucose (FG) sample sets.

After the fermentation process completion the resulting bioreactor broth was modified by adding of calculated quantities of glucose solution and ethanol in order to extend the content regions for a more robust calibration. The quantities of added glucose and ethanol where adjusted to minimize the correlation between the component concentrations in the whole data set. The resulting compositions of 41 process and post-process samples (Table S-1 in SM) were used as reference values in subsequent multivariate calibrations on spectral data.

Spectroscopic measurements were performed with a prototype system consisting of a grating spectrometer and a PIR-fiber ATR probe with a loop head described in the following sections. For more detail on the fermentation process see section S1.1 in supplementary materials (SM).

2.3. Probes

Two types of ATR fiber probes by art photonics GmbH (Berlin, Germany) were used. Both probes were based on PIR fibers as light guides, but used different ATR elements: a diamond crystal (hereinafter this probe is referred to as D-ATR) and detachable PIR loop (L-ATR). The L-ATR probe avoids using any expensive materials and technologies and thus is much cheaper than the D-ATR probe.

For the in-line NIR measurements in the fermentation broth an Avantes transflectance dip probe (Apeldoorn, The Netherlands) with an adjustable gap was used. The optical path length was set to the minimal value of 0.5 mm to compensate for high NIR absorption of water.

Further technical description of the probes is provided in section S1.2 of SM.

2.4. Full-range MIR and NIR spectroscopic analysis

To provide benchmark models for the subsequent performance testing of the developed sensor systems full-range MIR and NIR spectra of the designed EG- and FG-sample sets have been measured. MIR spectra were acquired with a Matrix-MF FT-IR spectrophotometer (Bruker AG, Ettlingen, Germany) with a D-ATR probe. NIR spectra in the wavelength range 1100–2100 nm were obtained using TIDAS S-1000 MS-T50/16 by J&M Analytik AG (Essingen, Germany). Deionized water at 30 °C was used as a reference in all spectroscopic measurements. In MIR analysis of designed sample sets the reference spectrum was renewed before each sample.

During the fermentation process NIR measurements were repeated at 5-min intervals in a time scan mode. Each time-scan measurement consisted of single spectra taken with 1-second intervals over three minutes. This acquisition technique was chosen to apply the "5%-quantile" preprocessing method, as described in section 2.6 below. Dark current and reference spectra were measured once right before the fermentation start.

Section S1.3 in SM provides more detail on full-spectroscopic MIR and NIR measurements.

2.5. Custom MIR detection systems

In the developed sensor systems two different MIR spectrometric technologies with pyroelectric detectors were tested. Their detailed technical specifications are given in section S1.4 of SM.

The first system was a grating spectrometer (GrS) built on a PY128LA line sensor by PYREOS (Edinburgh, Scotland, UK) with 128 sensing elements. The spectrometer setup is shown in Fig.

S-1. The spectral region of the sensor was tuned to cover two intensive ethanol peaks between 9 and 10 μm (approximately 1100–1000 cm^{-1}). Another one was based on a tunable Fabry-Pérot interferometer (FPI) sensor (LFP-80105-337) [35] from InfraTec GmbH (Dresden, Germany) [36] optimized for measurement in the range 8–10 μm (1250–1000 cm^{-1}).

Spectrum acquisition conditions with both custom MIR systems were the same as in the full-spectrum case (section 2.4).

2.6. Data analysis

Partial least-squares (PLS) regression used for quantitative modeling is a multivariate calibration method described elsewhere [37].

In-line NIR data from the fermentation process were pre-filtered using the so-called 5%-quantile method [14,38] eliminating the spectra affected by process-related disturbances.

Prior to the ethanol, glucose and fructose calibration NIR spectra were converted into their first or second derivatives using Savitzky–Golay's [39] algorithm with second-order polynomial and a window width individually adjusted to the modeled data. This pre-processing method was found to be advantageous for the modeling of spectral data of both designed sample sets and fermentation process.

The model performance was characterized by the root mean-square error of calibration (RMSEC), prediction (RMSEP, for a validation subset) and full cross-validation (RMSECV) as well as by the respective coefficients of determination R^2. Further detail on data analysis can be found in SM (section S1.5).

3. Results and discussion

3.1. Mid-infrared sensor development

In contrast to customary (e.g. Fourier-transform) infrared spectrophotometers, analyzers developed in this work are called sensors, despite their multivariate and thus spectrometric nature. Nevertheless, the term "sensor" seems suitable considering the reduction of traditional infrared range (4000–400 cm^{-1}) to a narrow target region of wavelength and significant miniaturization of devices. This technological advancement was possible due to the application of recently developed pyroelectric sensing technologies: PYREOS and FPI (section 2.5). Two analytical devices built here on their basis are new compared to state of the art. To our best knowledge, analyzers of this type are not available commercially, and their application to process analysis has not been published in scientific periodicals before. The expensiveness of modern full-range IR spectrophotometers often presents a prohibitive limitation for their industrial application. Five- to ten-fold price reduction attained by the developed sensors will promote the dissemination of process analytical technologies.

MIR sensor development can be considered as a technical downgrading. The most critical step is the replacement of "high-end" laboratory FT-IR spectrometer with a less expensive detection system customized for a specific analytical purpose. Modern FT-IR detection systems are based on a Michelson interferometer with mechanically varied arm lengths. The interference signal detected by a single detector as a function of time is then Fourier-transformed giving out an IR spectrum in the frequency domain. Although FT-IR spectrometers offer high resolution and cover a broad spectral range, they have some drawbacks, specifically, in respect of process control and production monitoring applications. In high-precision FT-IR analyzers detector cooling is usually required to reduce thermal noise. The presence of fine moving parts makes these devices expensive and too fragile for industrial applications. At the same time, the full spectral range is rarely necessary for industrial process monitoring tasks. Here, it is suggested that determination of the main nutrients and products in the course of fermentation or another biotechnological process may be based on a relatively narrow spectral range of "fingerprints". For such a narrow spectral range, simpler grating spectrometers free of any moving parts are much more efficient and robust than FT-IR devices. However, a detector array is required to register the IR spectrum generated by the optical grating. For practical reasons the setup must be operated at room temperature. Pyroelectric sensors with an operating temperature of 300 K and high spectral sensitivity around 10 μm present a plausible alternative to quantum-type sensor materials like HgCdTe that must be cooled and to thermal-type detectors, such as Golay cells that are not available as sensor arrays of required sensitivity and compactness.

Because of the small size of the pyroelectric PYREOS sensor array pixels no additional cooling is necessary. As a consequence, essential customization and optimization of the optical layout of the grating spectrometer (Fig. S-1) was necessary to get a well-focused spectrum in the required range onto the line detector with minimal losses. The modulated high-brightness MIR source has also been developed in this project, as the radiation source unit with necessary properties could not be commercially acquired. Its high brightness, which was necessary to get enough MIR radiation into the PIR fiber, has been achieved by using high-aperture aspheric mirror optics to image the MIR radiating ceramic source (Nernst source) onto the PIR fiber entrance. A mechanical shutter synchronized with the pyroelectric sensor array guarantees the modulation of the MIR source necessary for the pyroelectric sensor performance.

The novelty of the second approach using a micromechanical Fabry-Pérot interferometer with a pyroelectric sensor is based on the novel combination of the above described customized high-brightness modulated MIR source with the compact FPI system. Both approaches in said specific configuration have not been described previously.

ATR probe with a PIR fiber loop as a measurement unit is an important component of the whole sensor system. The previous publication on the probe development [23] is mainly devoted to a discussion of signal strength and sensitivity of differently shaped loops. In that work, test spectra of a fermentation reaction were shown, but no data analysis was provided to prove the feasibility of quantitative analysis. The new ATR probe design described in the present work is of particular interest for modern biotechnology. Its detachable head allows using the ATR element in disposable bioreactors as a small flange mounted in the reactor wall. By itself, the present combination of a fiber-based ATR probe with an infrared sensor is a new type of the process analytical device. The probe with a PIR-loop element is three to five times cheaper than a diamond probe at the same or similar signal performance.

3.2. Comparison of different spectroscopic and sensor techniques using designed sample sets

Fig. S-2 presents MIR spectra of EG- and FG-sets of the design samples (Fig. 1) in the range 2999–700 cm^{-1} obtained with the "gold standard" method including the high-resolution FT-IR spectrometer and a diamond ATR element that is the most preferred material for precise analysis. PLS regression modeling and validation statistics for full-spectrum MIR data is presented in Table 1.

Generally, the spectral similarity of EG- and FG-samples is accounted for by their structural similarity in terms of functional groups composing the molecules of ethanol, glucose and fructose. Thus, similar broad signals located between 2800 and 3000 cm^{-1} in Fig. S-2a and b are basically assigned to different C–H stretching vibrations of aliphatic groups that are present in all three components of the studied mixtures. Because of the low signal intensity and selectivity, this spectral region can hardly be useful for the

Table 1
PLS regression statistics for designed EG- and FG-sets.

Method and Dataset	Range	nLV[a]	Calibration		Cross-validation		Prediction[b]	
			RMSE	R^2	RMSE	R^2	RMSE	R^2
Ethanol (EG-set)								
FT-IR&D-ATR	2999–700 cm^{-1}	2	5.52	0.984	7.16	0.975	5.41	0.980
FT-IR&D-ATR	1049–951 cm^{-1}	2	4.55	0.989	5.79	0.983	5.10	0.983
GrS&D-ATR	1082–1006 cm^{-1}	2	5.18	0.986	6.67	0.978	5.63	0.979
GrS&L-ATR	1093–1015 cm^{-1}	2	7.76	0.968	10.58	0.945	8.34	0.953
FPI&L-ATR	1250–990 cm^{-1}	2	7.48	0.970	9.74	0.953	5.75	0.978
NIR_SG2D13.2[c]	1127–2090 nm	3	3.93	0.992	5.09	0.987	3.96	0.990
Glucose (EG-set)								
FT-IR&D-ATR	2999–700 cm^{-1}	2	3.71	0.998	4.38	0.998	3.58	0.998
FT-IR&D-ATR	1049–951 cm^{-1}	2	5.47	0.996	6.66	0.995	5.53	0.995
GrS&D-ATR	1082–1006 cm^{-1}	2	9.77	0.987	11.99	0.982	10.87	0.980
GrS&L-ATR	1093–1015 cm^{-1}	2	9.12	0.989	11.95	0.982	11.62	0.977
FPI&L-ATR	1250–990 cm^{-1}	2	8.30	0.991	9.87	0.988	5.96	0.994
NIR_SG2D13.2	1127–2090 nm	3	6.29	0.995	8.06	0.992	5.79	0.994
Fructose (FG-set)								
FT-IR&D-ATR	2999–700 cm^{-1}	3	0.79	1.000	1.81	0.998	1.41	0.998
FT-IR&D-ATR	1049–951 cm^{-1}	3	0.96	0.999	1.14	0.999	1.11	0.999
GrS&D-ATR	1082–1006 cm^{-1}	3	2.21	0.996	3.82	0.990	3.71	0.987
GrS&L-ATR	1093–1015 cm^{-1}	3	11.07	0.906	17.10	0.793	16.45	0.739
FPI&L-ATR	1250–990 cm^{-1}	3	7.23	0.960	13.54	0.870	8.43	0.931
FPI&L-ATR_o2o21[d]	1127–2090 nm	3	2.92	0.993	6.64	0.968	1.58	0.998
NIR_SG1D15.2[e]	1132–2086 nm	4	11.99	0.886	19.47	0.725	14.57	0.797
Glucose (FG-set)								
FT-IR&D-ATR	2999–700 cm^{-1}	3	2.17	0.999	3.50	0.998	1.78	0.999
FT-IR&D-ATR	1049–951 cm^{-1}	3	2.35	0.999	2.92	0.999	1.82	0.999
GrS&D-ATR	1082–1006 cm^{-1}	3	3.76	0.998	6.76	0.994	4.03	0.997
GrS&L-ATR	1093–1015 cm^{-1}	3	7.78	0.992	14.11	0.975	9.30	0.986
FPI&L-ATR	1250–990 cm^{-1}	2	26.26	0.908	29.77	0.891	14.43	0.965
FPI&L-ATR_o2o21	1127–2090 nm	2	8.03	0.991	9.65	0.988	10.45	0.982
NIR_SG1D15.2	1132–2086 nm	4	12.83	0.977	20.80	0.946	15.12	0.962

[a] The number of latent variables used in the PLS model.
[b] Validation dataset by design (section 2.1).
[c] NIR spectra transformed by Savitzky–Golay 2nd derivative algorithm with 2nd order polynomial and window of 13 points.
[d] FPI&L-ATR data without outlying samples FG02 and FG21.
[e] NIR spectra transformed by Savitzky–Golay 1st derivative algorithm with 2nd order polynomial and window of 15 points.

present analytical purpose. The same conclusion can be made for the adjacent range from 1800 cm^{-1} toward higher wavenumbers also containing minor and poorly characteristic absorption bands. Negative signals at 1570 cm^{-1} are related to the water content reduction compared to the reference.

Above 1700 cm^{-1} the spectra are generally characterized by lower signal-to-noise ratio. Worse spectral quality in this range comes from the essentially (2–3 times) worse transmittance of the PIR fiber [22]. Besides, spectral quality at 2000–2300 cm^{-1} is strongly affected by a wide diamond absorption gap. This is the main disadvantage of diamond as an ATR element that limits its application, for example, for the analysis of some catalysts and petrochemical products.

The most intense absorbance in both datasets is observed in the "fingerprint" region. Ethanol exhibits here two distinct peaks at about 1090 cm^{-1} and 1050 cm^{-1} related to C–O and C–C stretching vibrations that are supported by a less intensive C–H bend signal at 880 cm^{-1}. Glucose and fructose in this region are represented by a complex combination of poorly resolved (even in FT-IR spectra!) and hardly interpretable bands (Figs. 2a and 3a, and S-2). Two most intensive distinguishing peaks observed at about 1060 and 1030 cm^{-1} (1057 and 1025 cm^{-1} in [40]) presumably belong to C–O stretching vibrations of fructose and glucose, respectively. This assignment follows from the experimental and calculated data reported in the literature [40,41]. Further interpretation of D-glucose and D-fructose absorption bands can be found in [40]. In terms of quantitative analysis, it is very important that despite general similarity, ethanol, glucose and fructose have distinguishable spectral signatures clearly observed in the fingerprint region by FT-IR spectroscopy with the D-ATR probe (Figs. 2a and 3a). The region 1149–951 cm^{-1} (taking data spacing into account) seems to be the most suited for quantitative analysis performed in the present study.

Indeed, limiting the modeling to the chosen range does not lead to any essential loss in the model performance compared to the full-range data and in some cases even results in a reduction of the validation errors (Table 1). The "gold standard" models for this region are important to evaluate the performances of developed sensor systems basically operating in the same interval of wavenumbers. In general, the prediction accuracy achieved by this analytical method is to be taken as the ultimate case. RMSE validation accuracies of 5–6 g/L and 1–2 g/L for EG- and FG-sets, respectively can be characterized as very high and suitable for a wide range of practical analytical needs. Somewhat lower accuracy in the case of EG-set, in particular for the ethanol model (RMSECV = 5.79) is accounted for by the presence of an outlying sample EG22 clearly observed in Fig. 2b. Also in the sensor data models (Fig. 2e, h and k) the predicted ethanol content in EG22 tends to be higher than its reference value, thus evidencing a sample preparation issue rather than spectral outlier. On the other hand, all PLS models for EG-set in the chosen range require two latent variables (LVs), which is a sign of robustness for the two-component system. In contrast to it, the FG-models make use of 3 LVs. An additional factor may be necessary to compensate for a non-linearity present in the data due to a saturation of spectral responses of the two hydrocarbons at their high concentrations in the solution.

The replacement of Matrix-MF FT-IR spectrometer with the developed grating-based sensor (GrS) is accompanied by an

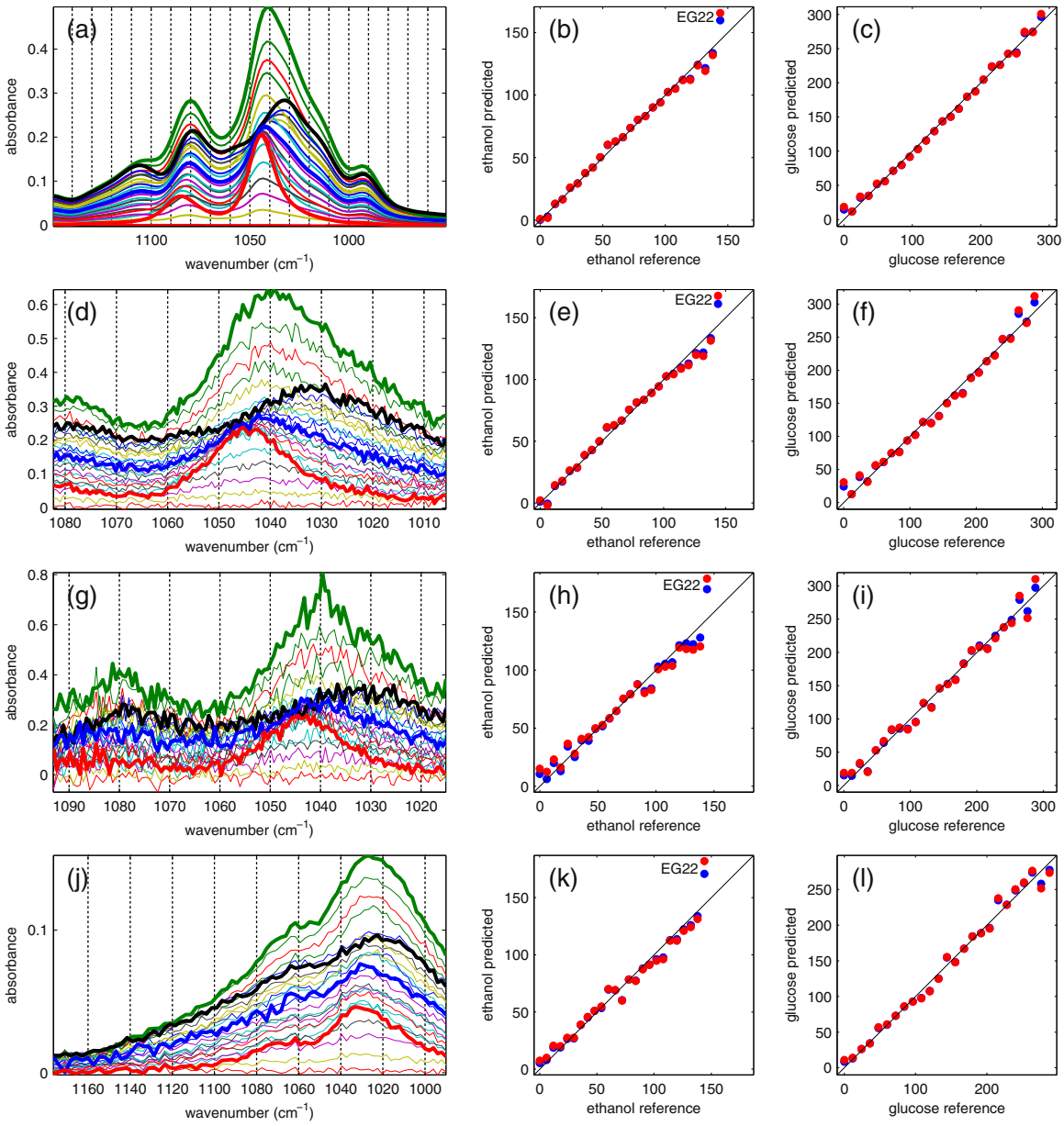

Fig. 2. Spectra of EG-set of samples (left column) acquired with the following techniques: (a) FT-IR with D-ATR probe (the following demonstrative samples are highlighted with a bold line and color: green—22; black—21; blue—0 and red—23; see Fig. 1), (d) GrS with D-ATR probe, (g) GrS with L-ATR probe and (j) FPI with L-ATR probe; middle (b, e, h, k) and right (c, f, i, l) columns show respective PLS-predicted versus reference plots for ethanol and glucose (blue and red markers correspond to RMSEC and RMSECV statistics, respectively) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

Table 2
PLS calibration and validation statistics for ethanol, glucose and biomass based on samples of two fermentation processes and their post-process modifications.

Analyte	Preprocessing	Range	nLV[a]	Calibration		Cross-validation		Prediction[b]	
				RMSE	R^2	RMSE	R^2	RMSE	R^2
NIR spectrometer with transflectance probe									
Biomass	None	1100–1859 nm	2	0.43	0.952	0.47	0.946	0.48	0.928
Ethanol	SG2D2.15[c]	1132–1872 nm	4	1.65	0.992	2.05	0.988	2.10	0.989
Glucose	SG2D2.15	1132–1872 nm	4	3.08	0.995	3.71	0.993	3.96	0.993
Grating-based sensor with L-ATR probe									
Ethanol	None	1093–1015 cm^{-1}	3	3.86	0.956	4.87	0.934	4.74	0.945
Glucose	None	1093–1015 cm^{-1}	4	12.86	0.918	17.25	0.859	13.33	0.918

[a] The number of latent variables used in the PLS model.
[b] Custom validation dataset (Table S-1).
[c] Savitzky–Golay 2nd derivative algorithm with 2nd order polynomial and window of 15 points.

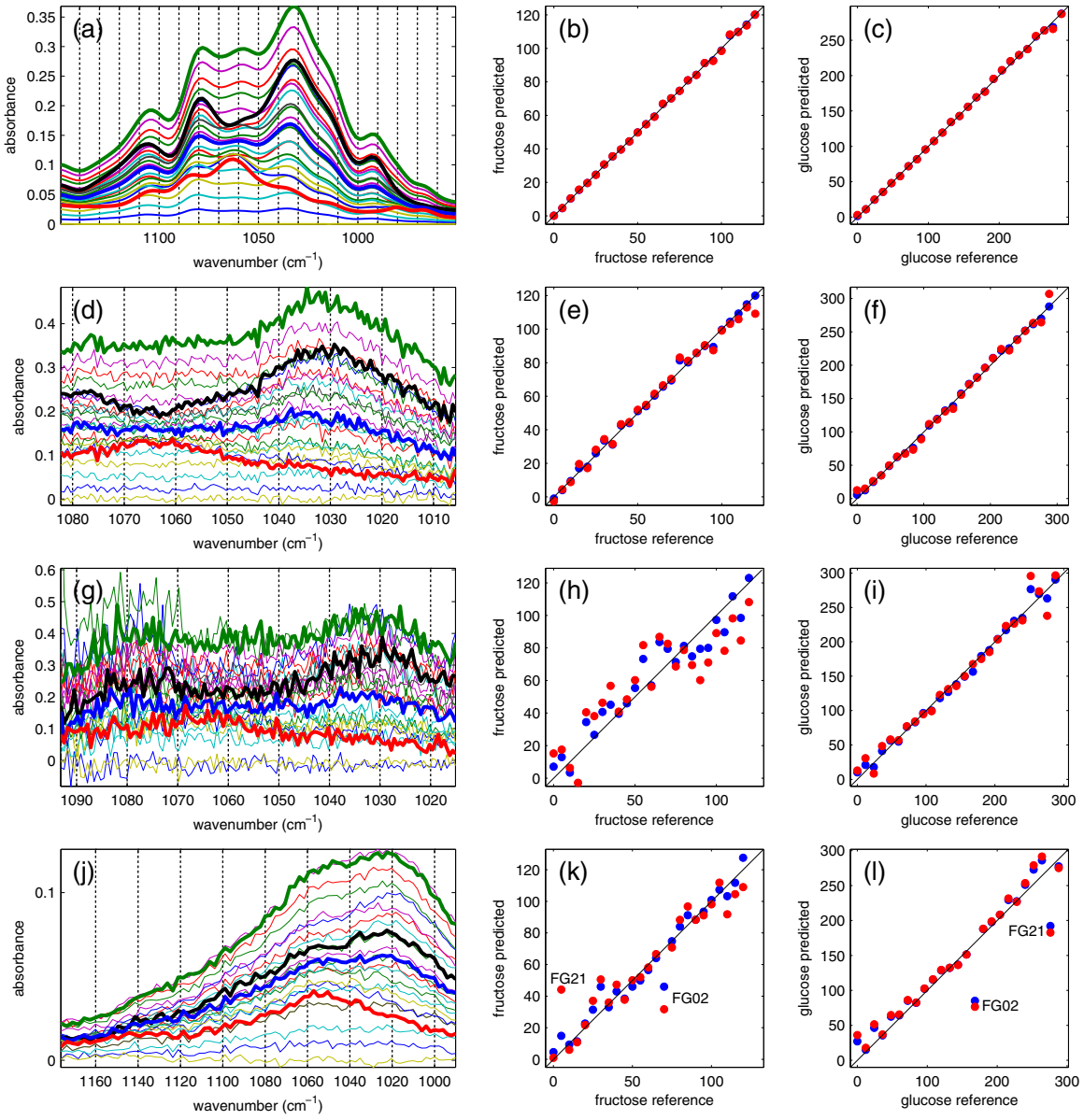

Fig. 3. Spectra of FG-set of samples (left column) acquired with the following techniques: (a) FT-IR with D-ATR probe (see color assignment in the capture of Fig. 2), (d) GrS with D-ATR probe, (g) GrS with L-ATR probe and (j) FPI with L-ATR probe; middle (b, e, h, k) and right (c, f, i, l) columns show respective PLS-predicted versus reference plots for fructose and glucose (blue and red markers correspond to RMSEC and RMSECV statistics, respectively) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

expected resolution loss that is clearly seen in spectra of designed samples (compare (a) and (d) graphs in Figs. 2 and 3). Another striking feature of the GrS&D-ATR sensor's data is the noisy character of spectra. However, a closer look at the spectra in Figs. 2d and 3d reveals that this effect is only partially explained by the measurement noise. Some spikes tend to repeat from spectrum to spectrum, and therefore, they reflect a roughness of detector's pixel structure, which is much less destructive than noise in terms of multivariate modeling. Observed reduction of spectral quality and selectivity had an inevitable effect on the prediction performance of all multivariate models. Thus, glucose RMSECV has increased to 12.0 g/L and 6.8 g/L in EG- and FG-sets, respectively, while the ethanol model suffered to the least extent (Table 1). Nevertheless, the R^2-values indicate general model healthiness that is also illustrated by predicted versus measured plots presented in Figs. 2e, f and 3e, f.

Refusal of diamond in the ATR element and its replacement with a PIR loop is associated with a dramatic price reduction of the whole sensor analyzer system. This downgrading has an immediate effect on the spectral quality (Figs. 2g and 3g) and model performances (Figs. 2h, i and 3h, i). Spectral roughness becomes even stronger, and the noise share becomes dominating over the pixel structure. Although there are two internal reflections both in the PIR-fiber probe and in the diamond ATR probe, a stronger absorbance and related signal reduction in the PIR fiber material can be a straightforward explanation of the observed spectrum decay. The fructose model has experienced the worst damage: its RMSECV fell down to 17.1 g/L (compare to 1.1 g/L in the ultimate FT-IR&D-ATR case). Perhaps, much closer spectral similarity with glucose, compared to another component pair, makes fructose calibration more susceptible to the spectral quality. Hence, although the spectral resolution in GrS&D-ATR and GrS&L-ATR is the same, further deterioration introduced by the loop element worked out to be critical in the latter case. In spite of an essential loss of performance compared to the "gold standard" of analysis, component prediction accuracies in the EG- and FG-sets achieved by the GrS&L-ATR sensor (RMSECV of 10–15 g/L, except for fructose) stay practicable for a number of

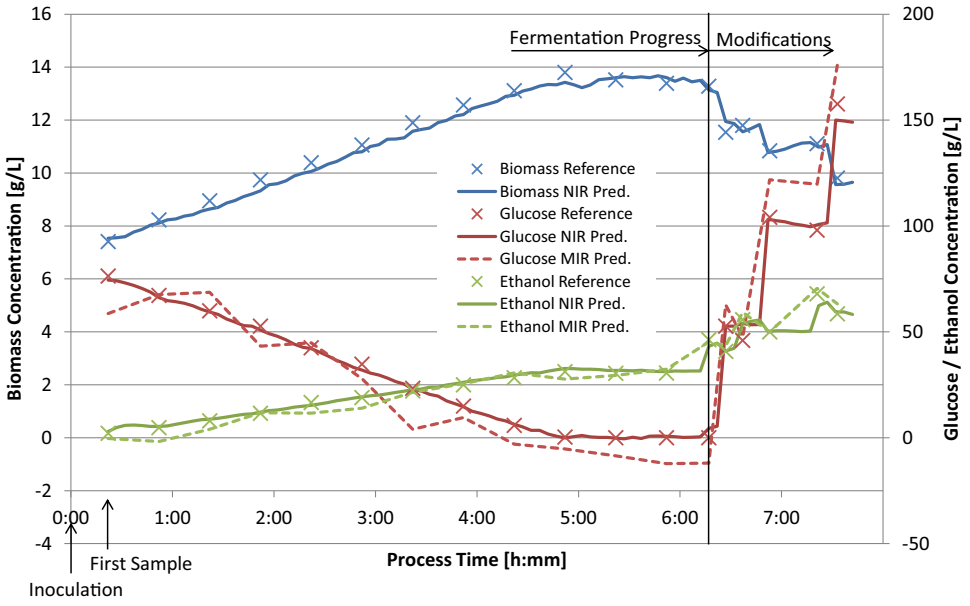

Fig. 4. Biomass (blue), glucose (red) and ethanol (green) concentration profiles during the second fermentation process (Table S-1) and post-process modifications. Crosses: concentrations determined by reference analysis; straight lines: prediction from NIR spectra taken every 5 min (as corresponds to 41 calibration samples); dashed lines: prediction from MIR spectra for each of the 41 calibration samples (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

process monitoring applications, such as in the food industry. The suggested sensor system is capable of separate analysis of different hydrocarbons in a mixture, which is a big advantage compared to the existing low-budget, e.g. refractometric, analyzers. Therefore, it can be concluded that multivariate regression is capable of handling the reduction of spectral selectivity introduced by the downgrading of sensor parts. The main error source in the developed grating-based sensors is the experimental and measurement accuracy rather than insufficient spectral selectivity.

It was also important to compare two MIR spectrometer technologies extensively developed nowadays, Fabry-Pérot interferometer (FPI) and PYREOS-based GrS, to each other using the two available designed sample sets. Spectra obtained with an L-ATR probe coupled with the FPI sensor (FPI&L-ATR) are presented in Figs. 2j and 3j. Although the noisiness of spectra is essentially lower than in the grating-based sensors, it is also evident that spectral selectivity of FPI is the worst among the studied MIR spectrometers. This effect is partially explained by about twice lower nominal spectral resolution. The component peaks seem much less resolved and absorbance intensity ratios of the "marker" samples indicated by the thick lines in Figs. 2j and 3j look different from those in other spectroscopic methods: see (a), (d), (g) graphs in Figs. 2 and 3. An evident advantage of FPI is a broader wavenumber interval covered by this detector compared to the studied PYREOS model. In the present application, however, this advantage does not bring any noticeable gain, as the sample spectra do not have any informative signals above 1170 cm^{-1}, and thus, this region was excluded from analysis. Prediction accuracies offered by the FPI&L-ATR system are generally comparable to the GrS&L-ATR results. They are even slightly better, except for glucose in FG-set that gives out RMSECV of about 30 g/L due to the presence of two bad outliers: FG02 and FG21 (Fig. 3l). In contrast to the ethanol outlier in Fig. 2b, e, h and k, caused by a sample preparation error, those glucose model outliers have only appeared in the FPI case and are thus related to the spectral deviations. These deviations can be accounted for by the well-known sensitivity of the FPI measurement to any external mechanical impact being one of the main known technical problems of this technology. After the removal of above outliers, the FPI&L-ATR models seem somewhat better than GrS&L-ATR ones (Table 1). It should also be noted that the glucose model in FG-set is built with two LVs, and is therefore simpler than in the case of GrS.

The results obtained by MIR spectroscopic analysis of the same designed samples were also compared to the measurements by NIR spectroscopy. Although NIR spectroscopy does not belong to the most precise spectroscopic methods in aqueous media, its wide application to the analysis of biotechnological objects and processes [3–8,10] makes this comparison interesting and methodologically useful.

From NIR-spectra of EG- and FG-sets of samples (Fig. S-3a, c) it can be seen that overwhelming absorption of water strongly complicates the analysis of other constituents. The main spectral variance here is due to the changing water content. This indirect correlation with dissolved component concentration is not helpful for their quantitative analysis, because their characteristic features are strongly masked with water bands making them hardly distinguishable from one another, and therefore, influenced by the dynamic range of spectral measurement. Application of spectral derivatives of second order in EG-data (Fig. S-3b) and of first order for FG-set (Fig. S-3d) serves to emphasize the chemical differences and hence to improve the model performance. An extensive pretreatment applied makes the models for EG-samples comparable to the FT-IR&D-ATR method, but at the expense of an additional LV, pointing at possible non-linearity and related robustness issues. Calibration models for the FG-set of samples exhibit impracticable performances (RMSECV about 20 g/L at four LVs) evidencing the failure of the NIR system to distinguish glucose and fructose from their fine spectral differences in the presence of dominating water.

The above observations evidence that ATR MIR spectroscopy is generally preferred to NIR transmittance (transflectance) spectroscopy in the analysis of ethanol, glucose and fructose at concentrations that typically occur in the fermentation processes.

3.3. *In-line monitoring of a fermentation process with NIR and MIR sensors*

One of the developed sensor systems, grating spectrometer with a PIR-loop ATR probe, was tested under the conditions of a

running *Saccharomyces cerevisiae* fermentation process. In parallel, NIR spectra were acquired using a transflectance probe. Two fermentation processes were analyzed. Along with the spectroscopic analysis, 41 samples were taken from the fermentation broth in the process course and during its post-process modifications (Table S-1). This data was used to build predictive models for ethanol, glucose and biomass content. PLS modeling statistics is summarized in Table 2.

Both traditional NIR analyzer and developed MIR sensor show satisfactory validation statistics. Generally, the results are somewhat worse than those in the analysis of designed EG-samples. (R^2 values of cross-validation should be compared because of the difference in the ethanol and glucose concentration ranges.) PLS models were validated using both full cross-validation and prediction on a subset of 15 process samples. The sequence of test samples was selected and slightly modified in order to span the component concentration ranges adequately but avoiding their minimum and maximum values (Table S-1). The analysis has shown that PLS successfully captured both in- and inter-process variability. The models for ethanol and glucose included an additional LV, compared to the designed EG-set and to the models based on a single fermentation process (Table S-2). This is an expected complication at the model transfer into real-life conditions. General consistency of different modeling and validation statistics presented in Table 2 to each other and to previously reported analysis of designed EG-mixtures (Table 1) supports our conclusion about feasibility of using the developed low-range MIR analyzer with a loop ATR probe for process monitoring in biotechnology as a viable alternative to relatively well established NIR spectroscopy (Fig. 4).

The spectra used for the biomass model building were not preprocessed, because the descending background is mainly related to the scatter by the yeast cells and thus, contains information on the biomass content. Therefore, scatter by yeast cells that complicate NIR transflectance analysis of ethanol and glucose turns into an advantage enabling the biomass prediction. The ATR spectra have no sensitivity to the biomass content because of IR-light's low penetration depth into the sample. The biomass prediction model is reasonably simple (only two LVs) and the accuracy it provides (RMSECV = 0.47 and R^2CV = 0.946) evidences the method's practical usability, e.g. as an additional option at in-line analysis of components dissolved in the fermentation broth.

In general, the budget combination of the grating-based PYREOS sensor with the ATR loop-probe still loses the accuracy competition to the more traditional wide-range NIR spectroscopy, especially in the case of glucose analysis. However, the much lower price of the MIR sensor makes the methods competitive in a practical sense, in particular, considering that the technical advancement of the MIR sensor technology is only beginning. The ability of budget ATR sensors to distinguish different hydrocarbons in a typical fermentation mixture is another significant advantage compared to traditional NIR-spectroscopy method.

4. Conclusions

Functioning prototypes of three mid-infrared sensor systems, consisting of a simplified spectrometer equipped with an ATR probe, for simultaneous determination of ethanol, glucose and fructose (and potentially, other carbohydrates) have been developed, and their prediction performances were critically assessed in a systematic fashion.

The technological simplification and hence price reduction of the developed sensor systems compared to the full-range laboratory MIR or NIR spectroscopic analysis was achieved at the expense of spectral resolution reduction and by limiting of the wavelength range to a narrow interval containing the most informative responses of the studied components. This modification has been possible due to the application of up-to-date pyroelectric detectors. Additionally, the application of PIR fibers and ATR probes on their basis enables the application of MIR sensors for in-line process monitoring in the biotechnology.

Technical downgrading applied here results in some inevitable loss in performance compared to FT-IR spectroscopy and a diamond ATR probe (taken for benchmark method). But this loss in developed MIR sensors is much lower than it could be anticipated, considering the dramatic reduction of detected signal information. The sensor prototypes developed in the present study can be transformed into a full-featured analytical system capable of solving a wide range of analytical problems in biotechnological process monitoring. The methodical scrutiny, with which this investigation was performed here, provides a proof of general feasibility of suggested approach to the MIR sensor development on the basis of up-to-date detection and light guiding technologies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2015.07.118

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Martin Hessling finished his diploma in physics at the University of Bonn (Germany) with a thesis on laser spectroscopy in 1993. Meanwhile he has more than 20 years experience in the development of optical systems for life science applications. After his PhD on fluorescence spectroscopy of atmospheric trace gases at the Juelich Research Center, he became postdoc at the Laser Laboratorium Goettingen, before he joined the Business Unit Biophotonics of Rodenstock Precision Optics/Linos Photonics. Since 2005 he is professor at the Ulm University of Applied Sciences (Germany) with research foci on spectral analysis of bioprocesses and disinfection by UV radiation.

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Thomas Hellmuth has a PhD degree in physics (University of Munich). He was at the research department of Carl Zeiss (Germany) from 1985 to 1993 and head of engineering of Humphrey Instruments (USA) between 1993 and 1995. Since 1995 he is professor for optics and laser technology at Aalen University of Applied Sciences. He has been responsible for several research projects and published a number of articles and patents in the field of optoelectronic devices for healthcare and sensor technologies.

Maria Jose Barraza Bernal has got her master of science degree in photonics from the University of Applied Sciences in Aalen (Germany, 2012) with a thesis on design and simulation of an attenuated total reflection probe for a MIR spectrometer. In 2013 she worked on Biosens project performing optical simulations of infrared sensors and scattering models. Since 2014 she is member of the Zeiss Vision Science Lab at the Institute for Ophthalmic Research, Eberhards Karls University in Tübingen and writes her PhD in the field of Psychophysics.

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Hans Meyer has a degree in fine mechanics (University of Applied Sciences, Aalen). He works at the research and development department of J&M Analytik AG (Germany) since 1997 and is head of this department since 2007. He has been responsible for several research projects and patents in the field of optoelectronic devices for spectroscopy applications and sensor technologies.