Broadband laser-based mid-IR spectroscopy for analysis of proteins and monitoring of enzyme activity

Andreas Schwaighofer *, Christopher K. Akhgar, Bernhard Lendl *

Research Division of Environmental Analytics, Process Analytics and Sensors, Institute of Chemical Technologies and Analytics, Technische Universität Wien, Getreidemarkt 9, 1060 Vienna, Austria

HIGHLIGHTS

- Broadband mid-infrared (IR) external cavity-quantum cascade laser (EC-QCL) spectroscopy.
- Measurement and analysis of the protein amide I and amide II region.
- IR spectra of proteins measured at concentrations as low as 0.25 mg mL^{-1}.
- Monitoring of Lipase-catalyzed saponification of triacetin.
- Laser-based IR spectroscopy poses a fast, sensitive and rugged analysis technique.

GRAPHICAL ABSTRACT
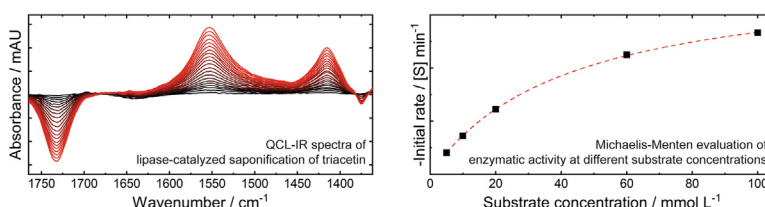
Broadband EC-QCL based mid-IR spectroscopy
✓ Spectral coverage: 400 cm^{-1} ✓ High SNR
✓ Protein amide I+II region ✓ Fast, compact, rugged

ARTICLE INFO

ABSTRACT

Laser-based infrared (IR) spectroscopy is an emerging key technology for the analysis of solutes and for real-time reaction monitoring in liquids. Larger applicable pathlengths compared to the traditional gold standard Fourier transform IR (FTIR) spectroscopy enable robust measurements of analytes in a strongly absorbing matrix such as water. Recent advancements in laser development also provide large accessible spectral coverage thus overcoming an inherent drawback of laser-based IR spectroscopy.

In this work, we benchmark a commercial room temperature operated broadband external cavity-quantum cascade laser (EC-QCL)-IR spectrometer with a spectral coverage of 400 cm^{-1} against FTIR spectroscopy and showcase its application for measuring the secondary structure of proteins in water, and for monitoring the lipase-catalyzed saponification of triacetin. Regarding the obtained limit of detection (LOD), the laser-based spectrometer compared well to a research-grade FTIR spectrometer employing a liquid nitrogen cooled detector. With respect to a routine FTIR spectrometer equipped with a room temperature operated pyroelectric detector, a 15-fold increase in LOD was obtained in the spectral range of 1600–1700 cm^{-1}. Characteristic spectral features in the amide I and amide II region of three representative proteins with different secondary structures could be measured at concentrations as low as 0.25 mg mL^{-1}. Enzymatic hydrolysis of triacetin by lipase was monitored, demonstrating the advantage of a broad spectral coverage for following complex chemical reactions. The obtained results in combination with the portability and small footprint of the employed spectrometer opens a wide range of future applications in protein analysis and industrial process control, which cannot be readily met by FTIR spectroscopy without recurring to liquid nitrogen cooled detectors.

* Corresponding authors.
E-mail addresses: andreas.schwaighofer@tuwien.ac.at (A. Schwaighofer), bernhard.lendl@tuwien.ac.at (B. Lendl).

1. Introduction

Mid-infrared (mid-IR) spectroscopy is a powerful and versatile technique for the analysis of structure and dynamics of polypeptides and proteins [1–3]. Vibrations of the polypeptide repeat unit result in nine characteristic group frequencies in the mid-IR region that are referred to as amide bands. Among those, the amide I band (1700–1600 cm^{-1}) and amide II band (1600–1500 cm^{-1}) were shown to be most sensitive to protein structure. The sensitivity to individual secondary structure elements originates in differing patterns of hydrogen bonding, dipole–dipole interactions, and geometric orientations in the α-helices, β-sheets, turns, and random coil structures that induce different frequencies of the C=O and N-H vibrations. The resulting characteristic band shapes and positions can be then correlated with the specific secondary structure folding [4].

Fourier-transform IR (FTIR) spectroscopy is the established and most widespread instrumentation in this spectral region. FTIR spectrometers are commonly equipped with thermal light sources such as Globars that emit broadband and constant but rather low power radiation across the mid-IR range. The low emission intensity can lead to limitations regarding measurements of analytes present in a highly absorbing matrix such as water. For mid-IR spectroscopy of proteins, there is the pronounced challenge of the HOH-bending band of water near 1643 cm^{-1} that overlaps with the protein amide I band. As a consequence, the optical path is restricted to < 10 μm for FTIR transmission measurements in order to avoid total IR absorption [2,3]. These short path lengths lead to impaired sensitivity because of the lower band absorbances and limited robustness due to higher probability of cell clogging.

More than two decades ago, quantum cascade lasers (QCLs) were introduced as a polarized, coherent and high power light source in the mid-IR region [5]. They allow stable operation at room temperature and provide spectral power densities higher than a factor of 10^4 compared with thermal light sources [6]. Since the commercial availability of external cavity-QCLs (EC-QCLs), which offer broadband spectral tuning in the range of several hundred wavenumbers, this type of light source has been increasingly used for studies of liquid samples [7]. It was shown that the high available emission power of QCLs enable mid-IR transmission measurements using an optical path 4–5 times larger than with conventional FTIR spectroscopy [8]. For analysis of proteins, academic setups employing EC-QCLs were reported for investigation of the amide I region [9,10] as well as amide I + II regions [11], finally surpassing the performance of FTIR spectroscopy in terms of limit of detection at similar measurement times [12]. These techniques were employed for analysis of protein structure after chemical [13], thermal [10] and pH-induced [14] denaturation. Recently there was also introduced a commercially available QCL-based IR spectroscopy system, (AQS3pro, RedShiftBio) that covers only the amide I spectral range and provides better performance than FTIR spectroscopy in terms of LOD at approximately 10-fold measurement time [15].

A drawback for laser-based IR setups so far was the limited accessible spectral range. Even though EC-QCLs with spectral coverages of several hundred wavenumbers are achievable [16,17], and even larger tuning ranges of>1000 cm^{-1} provided by a single device can be obtained by beam combination of multiple EC-QCL modules, uneven spectral tuning densities of QCLs pose a problem for implementation of broadband laser-based IR transmission setups [11,12]. For analysis of proteins, a broader spectral range allows not only to monitor protein structure changes in the amide I + II bands but also to reveal IR signatures of substrates and products of enzymatic reactions. Furthermore, particularly for chemometric analysis additional and more detailed information can be gained by analysis of a larger spectral range [14,18–20].

In this work we employ a commercial broadband laser-based IR spectrometer for protein secondary structure analysis. The performance of the instrument is benchmarked against FTIR spectroscopy. Furthermore, we utilize the large available spectral region (400 cm^{-1}) to follow the enzymatic hydrolysis of triacetin by lipase.

2. Materials and methods

2.1. Reagents and samples

Bovine serum albumin, lysozyme from chicken egg white, and γ-globulins from bovine blood (≥97%), lipase from Candida rugosa (Type VII, ≥700 unit/mg solid) were purchased from Sigma-Aldrich (Steinheim, Germany). Triacetin was purchased from Merck (Darmstadt, Germany). For protein secondary structure measurements, proper amounts of protein powder were dissolved in water. For enzymatic activity measurements, stock solutions of 5 mg mL^{-1} lipase and 250 mM triacetin were prepared. Ultrapure water (resistivity: 18 MΩ cm) was purified with a Milli-Q system from Millipore (Bedford, MA).

2.2. Laser-based IR spectroscopy

Laser-based IR spectra were recorded with a ChemDetect Analyzer (Daylight Solutions Inc., San Diego, USA), equipped with a 25 μm diamond transmission flow cell and an EC-QCL providing a spectral coverage between 1350 and 1770 cm^{-1}. External water cooling for the laser head was set to 17 °C. For protein secondary structure measurements, 91 scans were recorded and averaged at a measurement time of 45 s. For enzymatic activity measurements, the enzyme and substrate stock solutions were mixed to obtain the desired concentrations. Subsequently, the resulting solutions were thoroughly vortexed and injected into the sampling cell. At first, a reference spectrum was recorded and afterwards sample spectra were recorded for 20 min. at a time-interval of 20 s (60 scans). The spectrometer was flushed with dry air to decrease the influence of water vapor from the atmosphere. If necessary, absorption bands of water vapor in the atmosphere were subtracted during post-processing. The recorded spectra were treated by Savitzky − Golay smoothing (order: 2, window: 15 points), which resulted in a spectral resolution of 3.6 cm^{-1}, determined by comparison of the band width to FTIR spectra of water vapor. Spectra recording was performed with the ChemDetect software package. Data processing and analysis was conducted with in-house code developed in MatLab R2020b (MathWorks, Inc., Natick, MA, 2014).

2.3. FTIR spectroscopy

FTIR absorption measurements were performed using a Bruker Vertex 80v FTIR spectrometer (Ettlingen, Germany) equipped with a liquid nitrogen cooled HgCdTe (mercury cadmium telluride) detector (D* = 4 × 10^{10} cm $Hz^{0.5}$ W^{-1} at 9.2 μm) and a Bruker Tensor 37 FTIR spectrometer equipped with a DLaTGS (deuterated lanthanum α-alanine doped triglycine sulfate) detector (D* = 6 × 10^8 cm $Hz^{0.5}$ W^{-1} at 9.2 μm). The samples were manually injected into a flow cell, equipped with two CaF_2 windows and an 8 μm-thick spacer. During measurements, the spectrometer was constantly flushed with dry air for at least 10 min prior to data acquisition until water vapor absorption was sufficiently constant. Spectra were acquired with a spectral resolution of 3.6 cm^{-1} in double-sided acquisition mode. A total of 341 (Vertex operated at 80 kHz scanner velocity) and 48 (Tensor operated at 10 kHz scanner velocity) scans were averaged per spectrum (acquisition time: 45 s), which was calculated using a Blackman-Harris 3-term

apodization function and a zero-filling factor of 2. All spectra were acquired at 25 °C. Spectra were analyzed using the software package OPUS 7.2 (Bruker, Ettlingen, Germany). Water vapor absorption bands were subtracted, if required.

3. Results and discussion

3.1. Mid-IR spectra of proteins recorded with the laser-based IR spectrometer

Laser-based IR spectra of proteins with different secondary structures were recorded at varying concentrations. The broad tuning range of the employed spectrometer enabled recording IR spectra beyond the amide I and amide II regions and allowed to perform qualitative and quantitative evaluation. The examined proteins were chosen to exhibit different secondary structures which show characteristic spectral features in the investigated wavenumber range (Fig. 1A-C). Bovine serum albumin (BSA) mainly contains α-helical secondary structure and shows the corresponding band maximum at 1656 cm^{-1} in the amide I region as well as a narrow band at 1545 cm^{-1} in the amide II region [21,22]. γ-Globulin (γ-Gl) is predominantly composed of β-sheet structures resulting in an amide I band maximum at 1640 cm^{-1} and a broad amide II band with a maximum at 1550 cm^{-1} [23]. Lysozyme (Lys) comprises both α-helices and β-sheets leading to an amide I band maximum at 1656 cm^{-1} with shoulders at ~1640 cm^{-1} and ~1675 cm^{-1} and a narrow band with a maximum at 1545 cm^{-1} in the amide II region [23,24].

The spectra obtained by the laser-based spectrometer were compared with FTIR measurements (Fig. 1D-F). Evaluation of absorbance band shapes and positions reveal excellent agreement between laser-based and FTIR spectroscopy. It should be highlighted, that even minor spectral features such as the shoulders of the amide II band at approx. 1525 cm^{-1} (BSA, Lys) are well resolved in laser-based IR spectra. Even though not directly correlated to protein secondary structure assignment, the band with a maximum at 1465 cm^{-1} attributed to C-H bending vibrations show good congruence between the two spectroscopy methods [25].

For quantitative evaluation, the height of the band maxima in the amide I region was evaluated for protein solutions with seven different concentrations ranging between 0.1 and 10 mg mL^{-1}. The calibration curves in Fig. S1 show high linearity (r^2 > 0.999) down to a concentration of 0.1 mg mL^{-1}.

3.2. Performance comparison of the laser-based IR spectrometer to FTIR spectroscopy

After qualitative comparison of protein absorbance spectra, the overall performance of the laser-based spectrometer was benchmarked against FTIR instruments. For this purpose, typically the limit of detection (LOD)

$$LOD = \frac{3 \; x \; Noise_{RMS}}{slope \; of \; the \; calibration \; line}$$

is employed. This expression not only considers the noise level achieved by an instrument, but also the linear calibration function calculated from the height of the band maxima of protein solutions with different concentrations. According to Lambert − Beer's law, higher absorbance is obtained, when larger optical path lengths are employed for transmission measurements. Consequently, the short path length of 8 μm that needs to be used in FTIR spectroscopy not only considerably reduces the robustness of the measurement but also impairs its sensitivity. The RMS (root mean square)-noise is assessed from a 100% line, which is the absorbance spectrum calculated from two identical single channel spectra [26]. To this end, spectra were recorded with a water-filled transmission cell, followed by evaluation of the spectral region between 1600 and 1700 cm^{-1}. Under ideal conditions, the outcome would be a flat line at 100% transmittance, corresponding to zero absorbance. For the comparison of performance characteristics, a high-end FTIR spectrometer with a fast interferometer and a liquid nitrogen-cooled MCT detector as well as a routine FTIR instrument with a pyroelectric detector were used as a reference. To ensure valid comparison, the same data acquisition time and spectral resolution were set for measuring the 100% lines with the different instruments. For the laser-based IR spectrometer, the spectral resolution was determined by comparison of the band widths of the rotational bands of water vapor to FTIR spectra.

The noise level obtained for high-end FTIR spectroscopy is better by a factor of approx. 2 compared to the laser-based IR spectrometer (see Table 1). However, because of the larger employable path length (factor ~ 3), the LOD of the laser-based IR spectrometer is better by a factor of approx. 1.5. Since the Chem-Detect spectrometer does not require liquid nitrogen cooling, the performance is best compared to a routine FTIR spectrometer. Here, the less sensitive pyroelectric detector and the associated slower scanner velocity result in a lower number of recorded scans
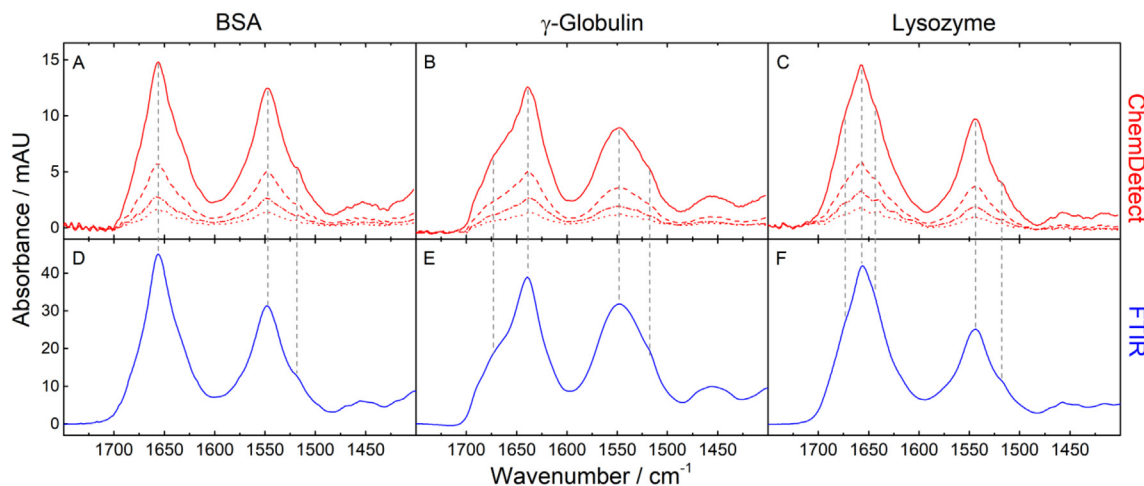

Fig. 1. Mid-IR absorbance spectra of 2.5 (red solid line), 1.0 (red dashed line), 0.5 (red dash-dotted line and 0.25 mg mL^{-1} (red dotted line) protein solutions acquired with the laser-based spectrometer (A-C) and 20 mg mL^{-1} (blue solid line) protein solution recorded by FTIR spectroscopy (D-F). Gray dashed lines highlight the high overlap of the spectral features between the mid-IR spectra acquired by the laser-based spectrometer and FTIR spectroscopy.

Table 1

Comparison of characteristic parameters and performance between the laser-based IR spectrometer and conventional FTIR spectroscopy.

	Meas. time [sec]/scans	RMS-noise[a] 10^{-5} [AU]	Path length [μm]	LOD [mg mL^{-1}]	Length × width × height/Volume [dm/dm^3]
ChemDetect	45/91	4.89	25	0.026	2.9 × 2.3 × 1.1/7
High-end FTIR (Vertex 80v)	45/341	2.25	8	0.034	8.5 × 7.1 × 2.8/169
Routine FTIR (Tensor 37)	45/48	25.36	8	0.383	6.6 × 4.5 × 2.8/83

[a] Determined in the spectral region between 1600 and 1700 cm^{-1} from the 100% line of a water-filled transmission cell. The noise level is stated in absorbance units (AU).

during the same measurement time and consequently in a higher noise level. Hence, the lower achieved noise level (factor ~ 5) of the laser-based IR spectrometer combined with the larger applicable optical path results in a LOD that is better by a factor of ~ 15 than the one of the routine FTIR spectrometer. Finally, it should be noted that FTIR spectroscopy offers the advantage of spectral coverage across the entire mid-IR region.

3.3. Monitoring of an enzymatic reaction

The laser-based IR spectrometer was employed to monitor an enzymatic reaction. For this purpose, the hydrolysis of the triacetateester triacetin to glycerin and acetate was chosen (Fig. 2A), which is catalyzed by the enzyme lipase [27]. Triacetin was used as substrate because it is sufficiently soluble in water. Fig. 2 shows the dynamic laser-based IR spectra recorded at a substrate concentration of 100 mM. In the accessible spectral region, multiple changes occur that can be attributed to the consumption of substrate and formation of the enzymatic reaction product over time. The negative bands at 1733 cm^{-1} and 1374 cm^{-1} are assigned to the C=O stretching of the ester moiety and symmetric CH$_3$ deformation vibration, respectively, of the substrate triacetin [28]. The emerging bands at 1553 cm^{-1} and 1415 cm^{-1} are attributed to the asymmetric and symmetric stretching vibrations of the carboxylate group in acetate [29].

For monitoring of the enzymatic activity at different substrate concentrations, the decrease in absorbance at 1733 cm^{-1} was evaluated. The position of this carbonyl stretching band is not prone to pH variations in contrast to the band position of the reaction product [30]. In Fig. 3A, the band heights were plotted versus the reaction time for substrate concentrations between 5 and 100 mM. In the next step, the initial slope of the progression curve was determined by a linear fit between 3 and 5 min of the reaction time.
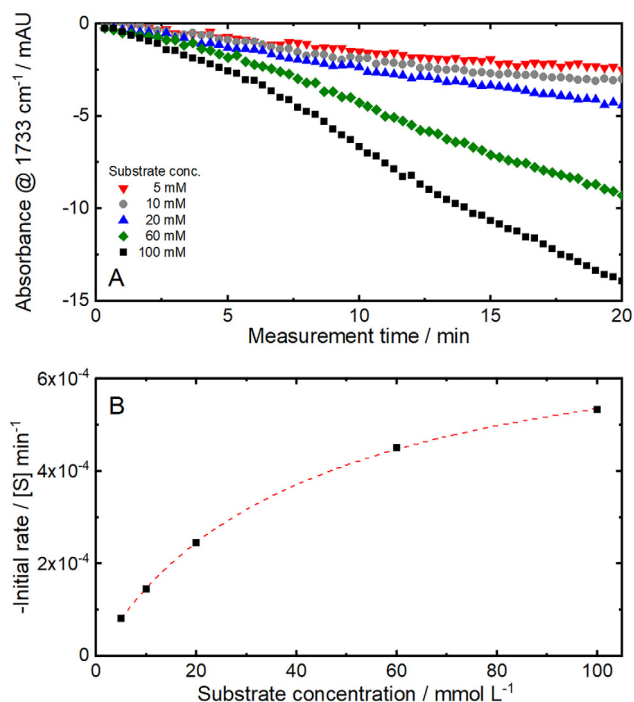

Fig. 2. (A) Scheme of the hydrolysis reaction of triacetin to glycerin and acetate. (B) Laser-based IR spectrum of the enzymatic reaction of lipase with 100 mM triacetin between 0 min (black) and 20 min (red) reaction time.

Fig. 3. (A) Progression curve of the enzymatic reaction performed at different substrate concentrations. (B) Initial rate vs substrate concentration follows the Michaelis-Menten relationship (dashed red line).

Subsequently the initial rate was plotted versus the substrate concentration and showed a good fit (r^2 > 0.9998) to the Michaelis-Menten equation (Fig. 3B), which is typically used to describe enzyme reactions under conditions of zero-order kinetics at the beginning of the reaction [31]. Based on this evaluation, a Michaelis-Menten constant (K_M) of 41.8 mmol L^{-1} was determined, which agrees with reported values of (K_M) for lipase catalyzed hydrolysis reactions [32].

4. Conclusion

In this work, we employed a commercial broadband laser-based IR spectrometer for analysis of protein secondary structure. Characteristic spectral features of proteins with different secondary structure were successfully measured at concentrations as low as 0.25 mg mL^{-1}. The ChemDetect spectrometer is equipped with a thermoelectrically-cooled detector and requires a small external water-cooling unit to thermally stabilize the laser head during operation. This equipment allows for low maintenance and durable operation under room-temperature environment. Performance comparison with a FTIR instrument under comparable working conditions (i.e. room-temperature operable pyroelectric detector) revealed a LOD better by a factor of ~ 15 for the laser-based IR spectrometer at similar spectra acquisition times. A further important parameter for versatile and application-oriented usage of a spectrometer is its portability in terms of weight and footprint. Also regarding these parameters, the laser-based spectrometer is highly attractive (see Table 1).
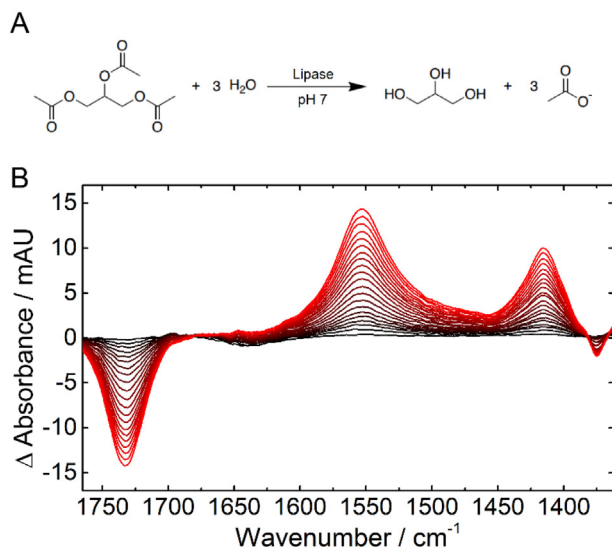
The ChemDetect spectrometer was further employed to monitor dynamic spectral changes during the hydrolysis of triacetin by the enzyme lipase. In this regard, the large accessible spectral range proved to be of importance for following the progression of multiple absorbance bands of the substrate as well as the product throughout the enzymatic reaction. Here, the spectral coverage of 400 cm^{-1} exceeds the possibilities previously accomplishable by laser-based IR spectroscopy employing for the analysis of liquids. Furthermore, for monitoring dynamic processes, fast measurement times are an important factor. In this context, the ChemDetect spectrometer enables spectra acquisition rates < 1 Hz allowing to observe fast reaction progressions that take place within a few minutes. In the present study multiple spectra are co-added in order to improve the noise level by spectra averaging.

The results presented here confirm laser-based IR spectroscopy as a fast, sensitive and rugged analysis technique. In this respect, the employed ChemDetect spectrometer poses a versatile and ready-to-use device that can be readily employed in diverse fields of applications. Envisioned future application are industrial process control and hyphenation with chromatographic separation systems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2021.119563.

References

[1] A. Barth, Infrared spectroscopy of proteins, Biochim. Biophys. Acta, Bioenerg. 1767 (2007) 1073–1101.

[2] H. Fabian, W. Mäntele, Infrared Spectroscopy of Proteins, Handbook of Vibrational Spectroscopy, John Wiley & Sons Ltd, Hoboken, NJ, USA, 2006.

[3] H. Yang, S. Yang, J. Kong, A. Dong, S. Yu, Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy, Nat. Protoc. 10 (2015) 382–396.

[4] S. Bal Ram, Basic Aspects of the Technique and Applications of Infrared Spectroscopy of Peptides and Proteins, in: Infrared Analysis of Peptides and Proteins, American Chemical Society, 1999, pp. 2–37.

[5] J. Faist, F. Capasso, D.L. Sivco, C. Sirtori, A.L. Hutchinson, A.Y. Cho, Quantum Cascade Laser, Science 264 (1994) 553–556.

[6] A. Schwaighofer, M. Brandstetter, B. Lendl, Quantum cascade lasers (QCLs) in biomedical spectroscopy, Chem. Soc. Rev. 46 (2017) 5903–5924.

[7] A. Schwaighofer, B. Lendl, Quantum cascade laser-based infrared transmission spectroscopy of proteins in solution, in: Y. Ozaki, M. Baranska, I.K. Lednev, B.R. Wood (Eds.), Vibrational Spectroscopy in Protein Research, Academic Press, 2020, pp. 59–88.

[8] M. Brandstetter, L. Volgger, A. Genner, C. Jungbauer, B. Lendl, Direct determination of glucose, lactate and triglycerides in blood serum by a tunable quantum cascade laser-based mid-IR sensor, Appl. Phys. B: Lasers Opt. 110 (2013) 233–239.

[9] M.R. Alcaráz, A. Schwaighofer, C. Kristament, G. Ramer, M. Brandstetter, H. Goicoechea, B. Lendl, External cavity-quantum cascade laser spectroscopy for mid-IR transmission measurements of proteins in aqueous solution, Anal. Chem. 87 (2015) 6980–6987.

[10] A. Schwaighofer, M.R. Alcaráz, C. Araman, H. Goicoechea, B. Lendl, External cavity-quantum cascade laser infrared spectroscopy for secondary structure analysis of proteins at low concentrations, Sci. Rep. 6 (2016) 33556.

[11] A. Schwaighofer, M. Montemurro, S. Freitag, C. Kristament, M.J. Culzoni, B. Lendl, Beyond FT-IR spectroscopy: EC-QCL based mid-IR transmission spectroscopy of proteins in the amide I and amide II region, Anal. Chem. 90 (2018) 7072–7079.

[12] C.K. Akhgar, G. Ramer, M. Żbik, A. Trajnerowicz, J. Pawluczyk, A. Schwaighofer, B. Lendl, The Next Generation of IR Spectroscopy: EC-QCL-Based Mid-IR Transmission Spectroscopy of Proteins with Balanced Detection, Anal. Chem. 92 (2020) 9901–9907.

[13] M.R. Alcaráz, A. Schwaighofer, H. Goicoechea, B. Lendl, EC-QCL mid-IR transmission spectroscopy for monitoring dynamic changes of protein secondary structure in aqueous solution on the example of beta-aggregation in alcohol-denaturated alpha-chymotrypsin, Anal. Bioanal. Chem. 408 (2016) 3933–3941.

[14] A. Schwaighofer, M.R. Alcaraz, L. Lux, B. Lendl, pH titration of β-lactoglobulin monitored by laser-based Mid-IR transmission spectroscopy coupled to chemometric analysis, Spectrochim. Acta, Part A 226 (2020) 117636.

[15] L.L. Liu, L. Wang, J. Zonderman, J.C. Rouse, H.-Y. Kim, Automated, High-Throughput Infrared Spectroscopy for Secondary Structure Analysis of Protein Biopharmaceuticals, J. Pharm. Sci. 109 (2020) 3223–3230.

[16] S. Riedi, A. Hugi, A. Bismuto, M. Beck, J. Faist, Broadband external cavity tuning in the 3–4 mu m window, Appl. Phys. Lett. 103 (2013) 031108.

[17] A. Hugi, R. Maulini, J. Faist, External cavity quantum cascade laser, Semicond. Sci. Technol. 25 (2010) 083001.

[18] M. Montemurro, A. Schwaighofer, A. Schmidt, M.J. Culzoni, H.K. Mayer, B. Lendl, High-throughput quantitation of bovine milk proteins and discrimination of commercial milk types by external cavity-quantum cascade laser spectroscopy and chemometrics, Analyst 144 (2019) 5571–5579.

[19] M.R. Alcaráz, A. Schwaighofer, H. Goicoechea, B. Lendl, Application of MCR-ALS to reveal intermediate conformations in the thermally induced α-β transition of poly-l-lysine monitored by FT-IR spectroscopy, Spectrochim. Acta, Part A 185 (2017) 304–309.

[20] S. Navea, R. Tauler, E. Goormaghtigh, A. de Juan, Chemometric tools for classification and elucidation of protein secondary structure from infrared and circular dichroism spectroscopic measurements, Proteins: Struct., Funct., Genet. 63 (2006) 527–541.

[21] J. Grdadolink, Y. Marechal, Bovine serum albumin observed by infrared spectrometry. I. Methodology, structural investigation, and water uptake, Biopolymers 62 (2001) 40–53.

[22] F. Dousseau, M. Pezolet, Determination of the secondary structure content of proteins in aqueous solutions from their amide I and amide II infrared bands, Comparison between classical and partial least-squares methods, Biochemistry 29 (1990) 8771–8779.

[23] M. van de Weert, P.I. Haris, W.E. Hennink, D.J.A. Crommelin, Fourier transform infrared spectrometric analysis of protein conformation: Effect of sampling method and stress factors, Anal. Biochem. 297 (2001) 160–169.

[24] M. Levitt, J. Greer, Automatic Identification of Secondary Structure in Globular Proteins, J. Mol. Biol. 114 (1977) 181–239.

[25] V. Balan, C.T. Mihai, F.D. Cojocaru, C.M. Uritu, G. Dodi, D. Botezat, I. Gardikiotis, Vibrational Spectroscopy Fingerprinting in Medicine: from Molecular to Clinical Practice, Materials 12 (2019) 2884.

[26] P.R. Griffiths, J.A. de Haseth, Fourier Transform Infrared Spectrometry, John Wiley & Sons Inc, Hoboken, NJ, USA, 2006.

[27] N. Lopez, M.A. Pernas, L.M. Pastrana, A. Sanchez, F. Valero, M.L. Rua, Reactivity of pure Candida rugosa lipase isoenzymes (Lip1, Lip2, and Lip3) in aqueous and organic media. Influence of the isoenzymatic profile on the lipase performance in organic media, Biotechnol. Progr. 20 (2004) 65–73.

[28] S. Armenta, W. Tomischko, B. Lendl, A Mid-Infrared Flow-Through Sensor for Label-Free Monitoring of Enzyme Inhibition, Appl. Spectrosc. 62 (2008) 1322–1325.

[29] M. Kansiz, J.R. Gapes, D. McNaughton, B. Lendl, K.C. Schuster, Mid-infrared spectroscopy coupled to sequential injection analysis for the on-line monitoring of the acetone-butanol fermentation process, Anal. Chim. Acta 438 (2001) 175–186.

[30] P. Domínguez de María, J.M. Sánchez-Montero, J.V. Sinisterra, A.R. Alcántara, Understanding Candida rugosa lipases: An overview, Biotechnol. Adv. 24 (2006) 180–196.

[31] H. Bisswanger, Enzyme assays, Perspect. Sci. 1 (2014) 41–55.

[32] Z.D. Knezevic, S.S. Siler-Marinkovic, L.V. Mojovic, Kinetics of lipase-catalyzed hydrolysis of palm oil in lecithin/izooctane reversed micellles, Appl. Microbiol. Biot. 49 (1998) 267–271.