Laser-Based Mid-Infrared Spectroscopy Enables In-line Detection of Protein Secondary Structure from Preparative Liquid Chromatography

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www.cta.tuwien.ac.at/cavs/QCL-IR_of_proteins

Introduction

Liquid chromatography (LC) is an essential unit operation for purification of pharmaceutical proteins. Typically, the corresponding protein concentrations are monitored by single-wavelength UV/Vis spectroscopy, offering excellent sensitivity, high robustness and a broad linear range. One of the major drawbacks of these detectors is, however, that discrimination or quantitation of different co-eluting proteins is not possible.

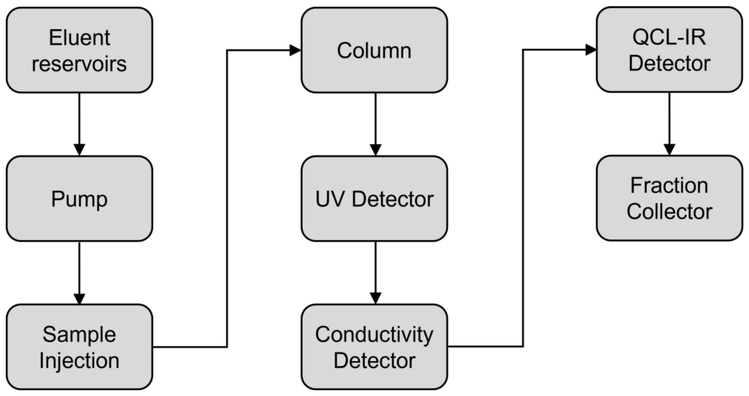
Mid-infrared absorption spectroscopy is a well-established technique for the analysis of proteins and polypeptides. The most powerful IR bands for protein quantification and secondary structure analysis are the amide I (1700-1600 cm^{-1}) and amide II (1600-1500 cm^{-1}) bands. A limiting difficulty of protein investigations in aqueous solutions is the strong IR absorbance of H_2O near 1640 cm^{-1} that overlaps with the amide I band. For routinely used Fourier-transform IR (FTIR) spectrometers employing low intensity thermal emitters as light source, suitable path lengths are restricted to <10 µm.

External cavity-quantum cascade laser (EC-QCL) based mid-IR spectroscopy is an emerging technology for the analysis of proteins. Higher optical powers compared to thermal light sources lead to increased sensitivity and larger applicable optical path lengths compared to conventional FTIR spectroscopy. This advantages open a wide range of possible applications, including near real-time protein monitoring from complex downstream operations [**1**].

Here, an EC-QCL based mid-IR spectrometer was coupled to a preparative liquid chromatography (LC) system. Two different model systems, based on ion-exchange chromatography (IEX) and size exclusion chromatography (SEC) were employed to demonstrate the high flexibility of LC-QCL-IR coupling. A significant challenge in IEX was caused by the overlapping bands of proteins and the applied sodium chloride (NaCl) gradient. Here, a novel approach for background compensation was devised to eliminate salt bands, leading to high-quality protein spectra. In case of SEC, proteins from overlapping chromatographic peaks, that cannot be distinguished with conventional UV/VIS detectors, were analyzed.

LC-QCL-IR Setup

- An ÄKTA pure system (Cytiva Life Sciences, MA, USA) equipped with an U9-M UV monitor, a C9 conductivity monitor and a F9-C fraction collector was used for the preparative-LC runs
- Laser-based mid-IR spectra were acquired with a ChemDetect Analyzer (Daylight Solutions Inc., San Diego, USA), equipped with an EC-QCL (1350-1750 cm^{-1}) and a 25 µm transmission cell [**2**]
- The collected fractions were additionally analyzed by reference analysis with reversed-phase high-performance liquid chromatography (RP-HPLC) [**3**]



References

[**1**] C. K. Akhgar, J. Ebner, O. Spadiut, A. Schwaighofer, B. Lendl "QCL-IR Spectroscopy for In-line Monitoring of Proteins from Preparative Ion-Exchange Chromatography," Anal. Chem., (Submitted).
[**2**] A. Schwaighofer, C. K. Akhgar, and B. Lendl, "Broadband laser-based mid-IR spectroscopy for analysis of proteins and monitoring of enzyme activity," Spectrochim. Acta A, 253, 119563 (2021).
[**3**] J. Kopp, F. B. Zauner, A. Pell, J. Hausjell, D. Humer, J. Ebner, C. Herwig, O. Spadiut, C. Slouka, R. Pell "Development of a generic reversed phase liquid chromatography method for protein quantification using analytical quality-by-design principles," J. Pharm. Biom. Anal., 188, 113412 (2020).

Ion Exchange Chromatography (IEX)

- IEX runs were performed using a 1 mL HiTrap Capto Q column, flow rate of 0.5 mL/min, elution buffer A of 50 mM Tris/HCl (pH 8.5) and elution buffer B with additionally 1 M NaCl
- A reference blank run (no proteins) and sample runs (2 proteins) with different gradients were performed:

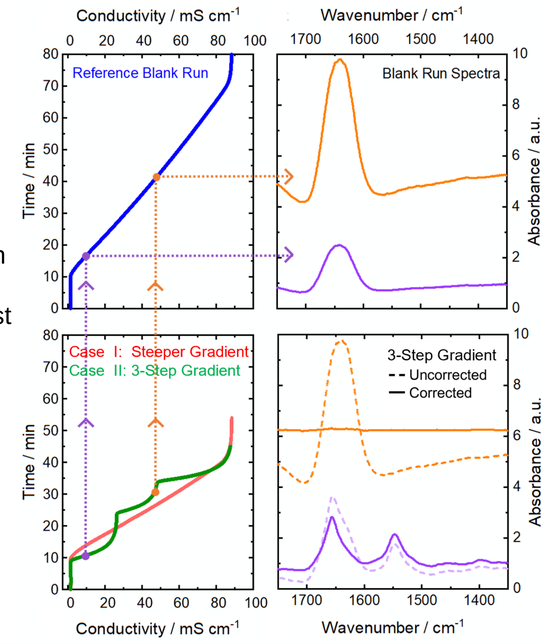
  Blank run: linear gradient within 60 min from 0 to 1 M NaCl

  Case I: steeper linear gradient within 30 min from 0 to 1 M NaCl

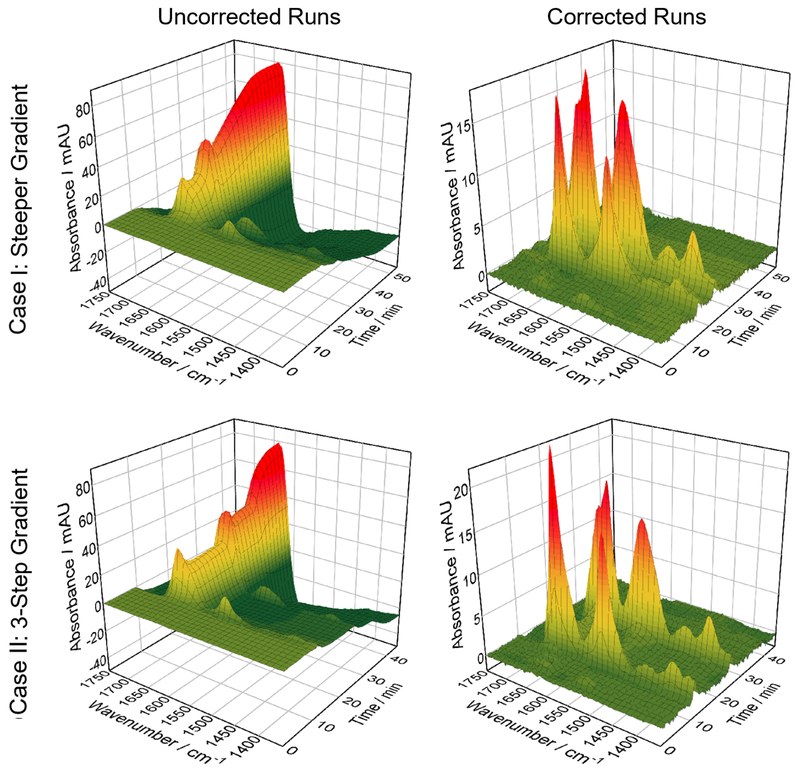
  Case II: 3-step gradient: 0.25 M, 0.5 M, 1 M NaCl

A significant challenge was caused by overlapping IR absorbances of proteins and NaCl gradient. Thus, a novel background correction approach, using the following algorithm was applied:

1) Relate conductivity detector signal to QCL-IR spectra of blank and sample runs
2) Take sample run spectrum and search for reference spectrum with closest conductivity value
3) Subtract selected reference spectrum from sample spectrum
4) Continue with next sample spectrum from 2)

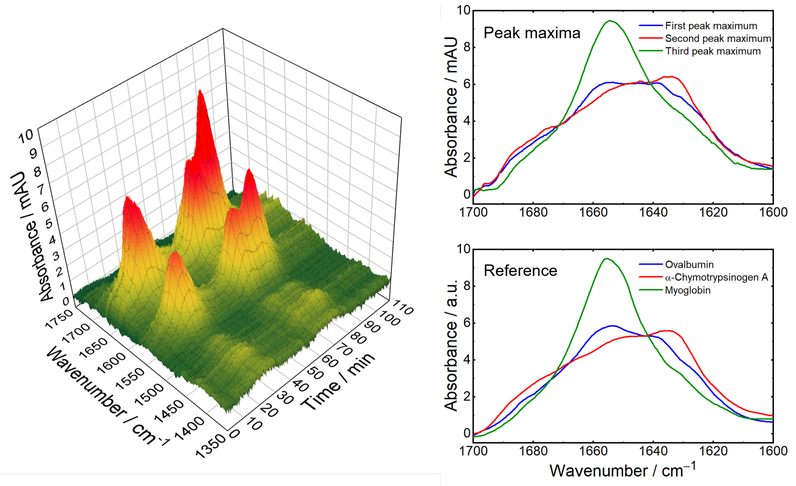


- Even though amide I and II bands were visible in spectra of uncorrected runs, the effect of the NaCl gradient was clearly dominating
- The corrected runs showed stable baselines and distinct amide I and amide II bands with secondary structure specific information about the eluted proteins
- A single blank run was sufficient for correcting the sample runs of highly different gradient profiles

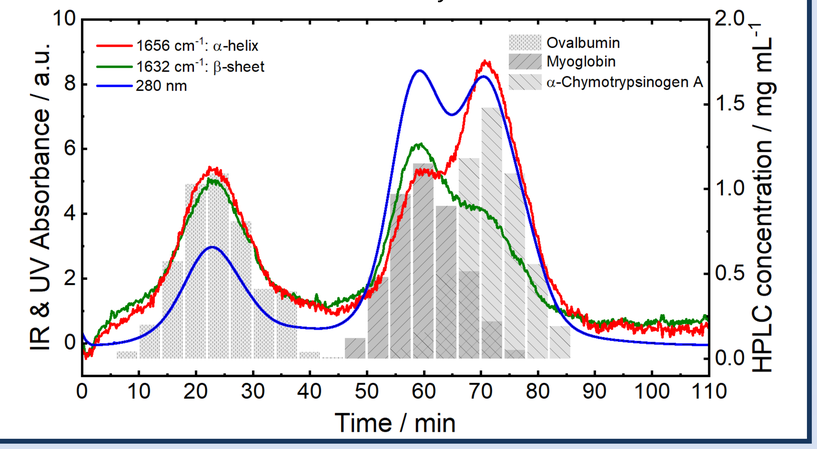


Size Exclusion Chromatography (SEC)

- A SEC run was performed using a HiLoad 16/60 Superdex 200 pg column and isocratic elutation with 50 mM phosphate buffer (pH=7.4) and 0.25 mL/min flow rate
- Three model proteins with different secondary structure and molecular weights were injected into the system
- QCL-IR spectra showed a stable baseline across the chromatographic run and specific amide I and amide II bands for the three proteins
- Absorption spectra, extracted from the peak maxima agreed well with reference off-line spectra of pure protein solutions



- IR signals at specific wavenumbers across the chromatographic run were compared to the response of the UV detector, showing excellent agreement of peak shapes and positions
- IR absorbances at 1656 and 1632 cm^{-1} provided information about the secondary structure which could not be obtained with UV detector
- The profile of secondary structure specific information agreed well with reference RP-HPLC off-line analysis of the collected fractions



Conclusions

- A QCL-IR spectrometer was succesfully coupled to a preparative LC system for in-line monitoring of protein secondary structure
- A novel gradient compensation approach for IEX was introduced. Here, a single reference blank run was sufficient for correcting various gradient profiles
- In SEC, secondary structure specific information of co-eluting proteins was obtained which is not provided by UV detectors
- LC-QCL-IR coupling holds high potential for complementing laborious and time-consuming off-line methods

This work has received funding from the COMET Center CHASE (project No 868615), which is funded within the framework of COMET (Competence Centers for Excellent Technologies) by BMVIT, BMDW, and the Federal Provinces of Upper Austria and Vienna. The COMET program is run by the Austrian Research Promotion Agency (FFG). Further funding was received by the European Union's Horizon 2020 research and innovation program through NUTRISHIELD project under grant agreement no. 818110.