Hyphenation of Preparative Liquid Chromatography to Laser-Based Mid-Infrared Spectroscopy for Monitoring of Proteins in Chromatographic Effluents

Christopher K. Akhgar,[1] Julian Ebner,[2] Mirta R. Alcaraz,[3,4] Julian Kopp,[2] Héctor Goicoechea,[3,4] Oliver Spadiut,[2] Andreas Schwaighofer[1] and Bernhard Lendl[1]

[1]Institute of Chemical Technologies and Analytics, Technische Universität Wien, Getreidemarkt 9, 1060 Vienna, Austria
[2]Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, Getreidemarkt 9, 1060 Vienna, Austria
[3]Laboratorio de Desarrollo Analítico y Quimiometría (LADAQ), Cátedra de Química Analítica I, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Santa Fe, S3000ZAA, Argentina
[4]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290, CABA, C1425FQB, Argentinas

Website

Email

Introduction

Mid-infrared spectroscopy provides detailed information about proteins that cannot be obtained with conventional UV detectors. 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 pronounced challenge in measurements of aqueous protein solutions is the strong absorption of H_2O at approx. 1640 cm^{-1} that overlaps with the amide I band. In conventional Fourier-transform infrared (FTIR) spectroscopy, the optical path-length is restricted to <10 µm to avoid total IR absorption. These small path lengths are unsuitable for flow-through operations due to low robustness.

External cavity-quantum cascade lasers (EC-QCLs) offer higher optical powers compared to the thermal sources used in FTIR spectroscopy, leading to increased sensitivity and larger applicable optical path lengths. These advantages open a wide range of possible applications, including near real-time protein monitoring from downstream operations.

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.

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 [2]
- 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

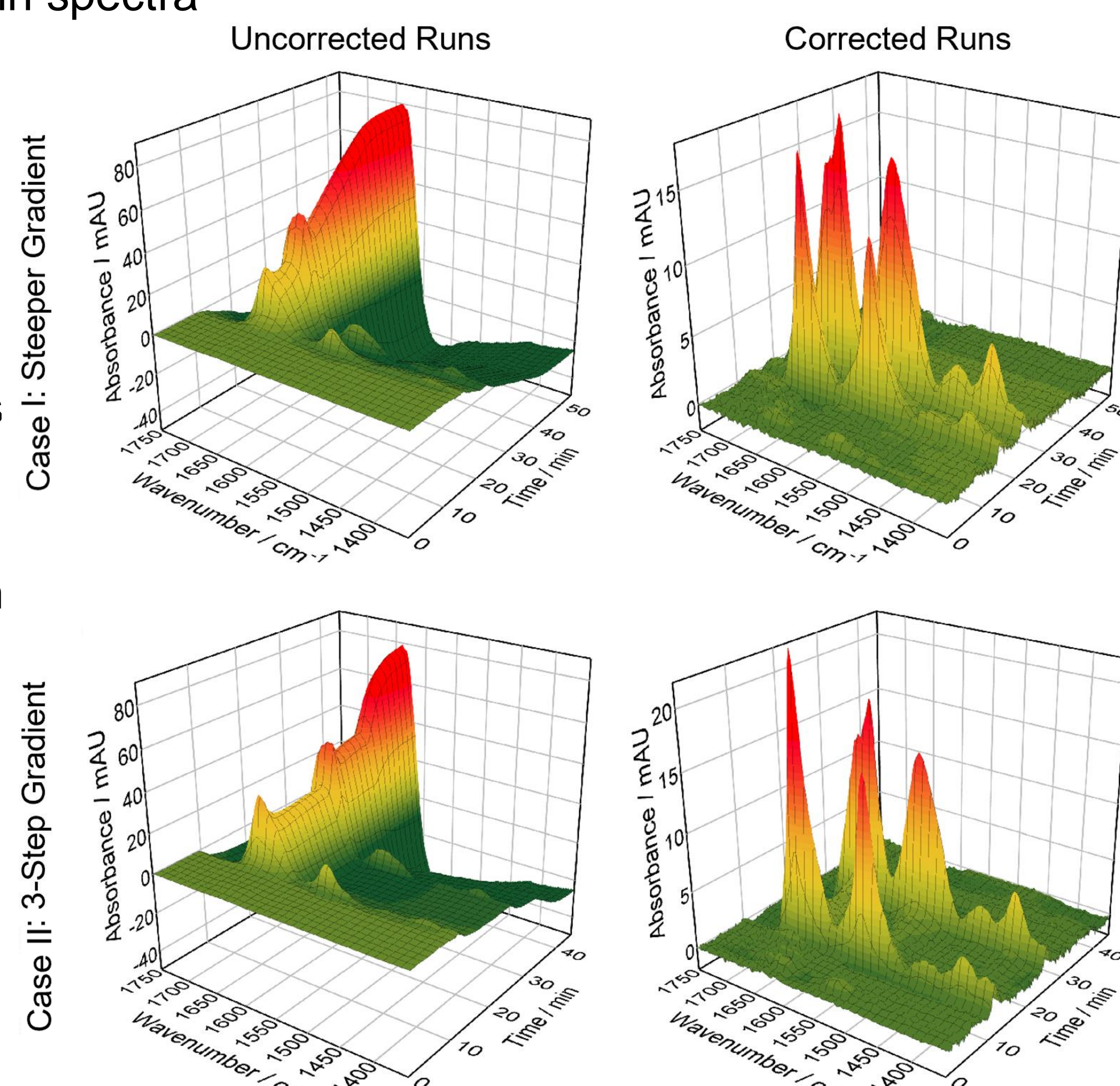
  Case I: steeper linear gradient within 30 min from 0 to 1 M NaCl

  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. Even though amide I and II bands were visible in spectra of uncorrected runs, the effect of the NaCl gradient was clearly dominating
- A novel background correction approach was applied, where a single blank run is sufficient for correcting sample runs of highly different gradient profiles, showing excelling protein spectra

Background compensation:

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

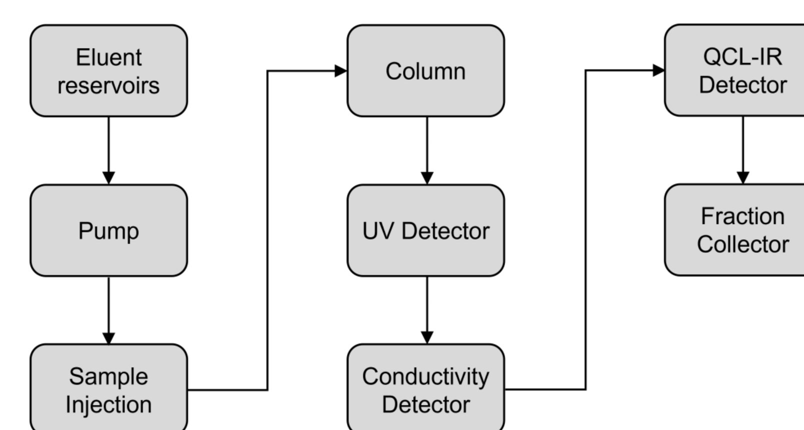


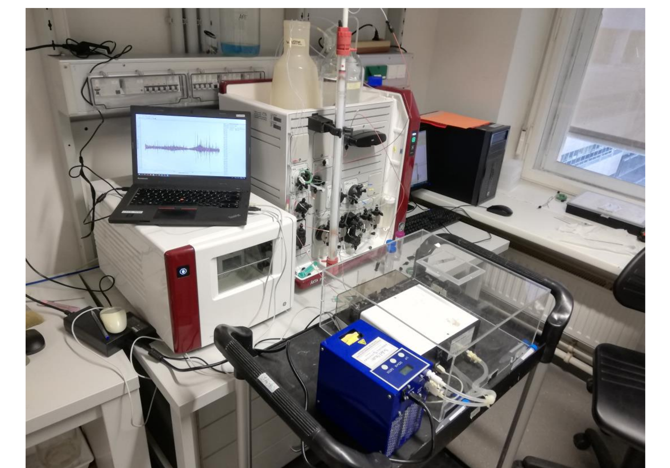
Conclusions & Outlook

- A QCL-IR spectrometer was succesfully coupled to a preparative LC system for in-line monitoring of protein secondary structure
- For IEX, a novel gradient compensation approach was introduced. Here, a single reference blank run was sufficient for correcting various gradient profiles
- In SEC, qualitative and quantitative information about individual proteins from overlapping chromatographic peaks was obtained
- LC-QCL-IR coupling holds high potential for complementing laborious and time-consuming off-line methods

LC-QCL-IR Setup

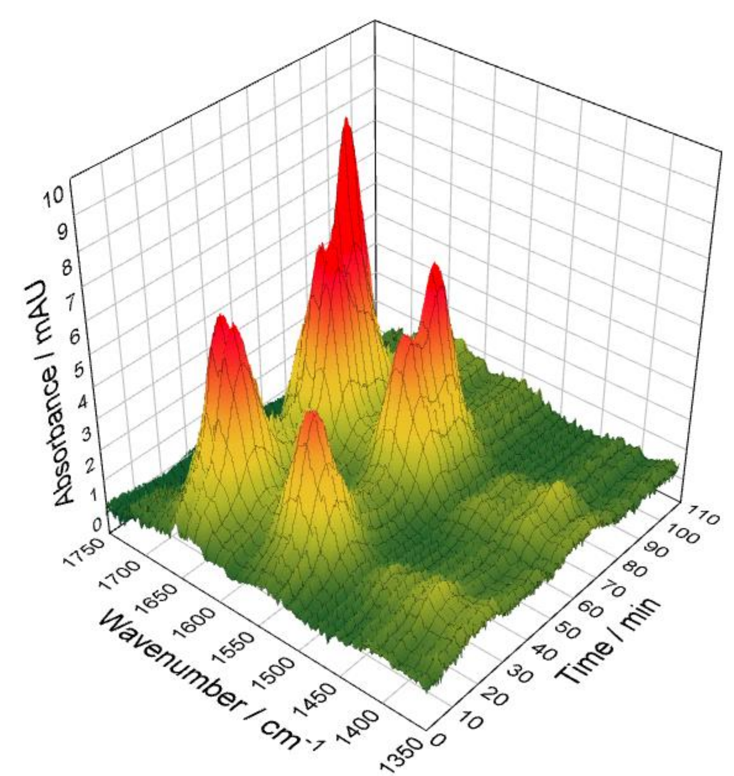
- ÄKTA pure system (Cytiva Life Sciences, MA, USA) equipped with an U9-M UV monitor, a C9 conductivity monitor and a F9-C fraction collector
- ChemDetect Analyzer (Daylight Solutions Inc., San Diego, USA), equipped with an EC-QCL (1350-1750 cm^{-1}) and a 25 µm transmission cell [1]
- The collected fractions were additionally analyzed by reference analysis with high-performance liquid chromatography (HPLC)



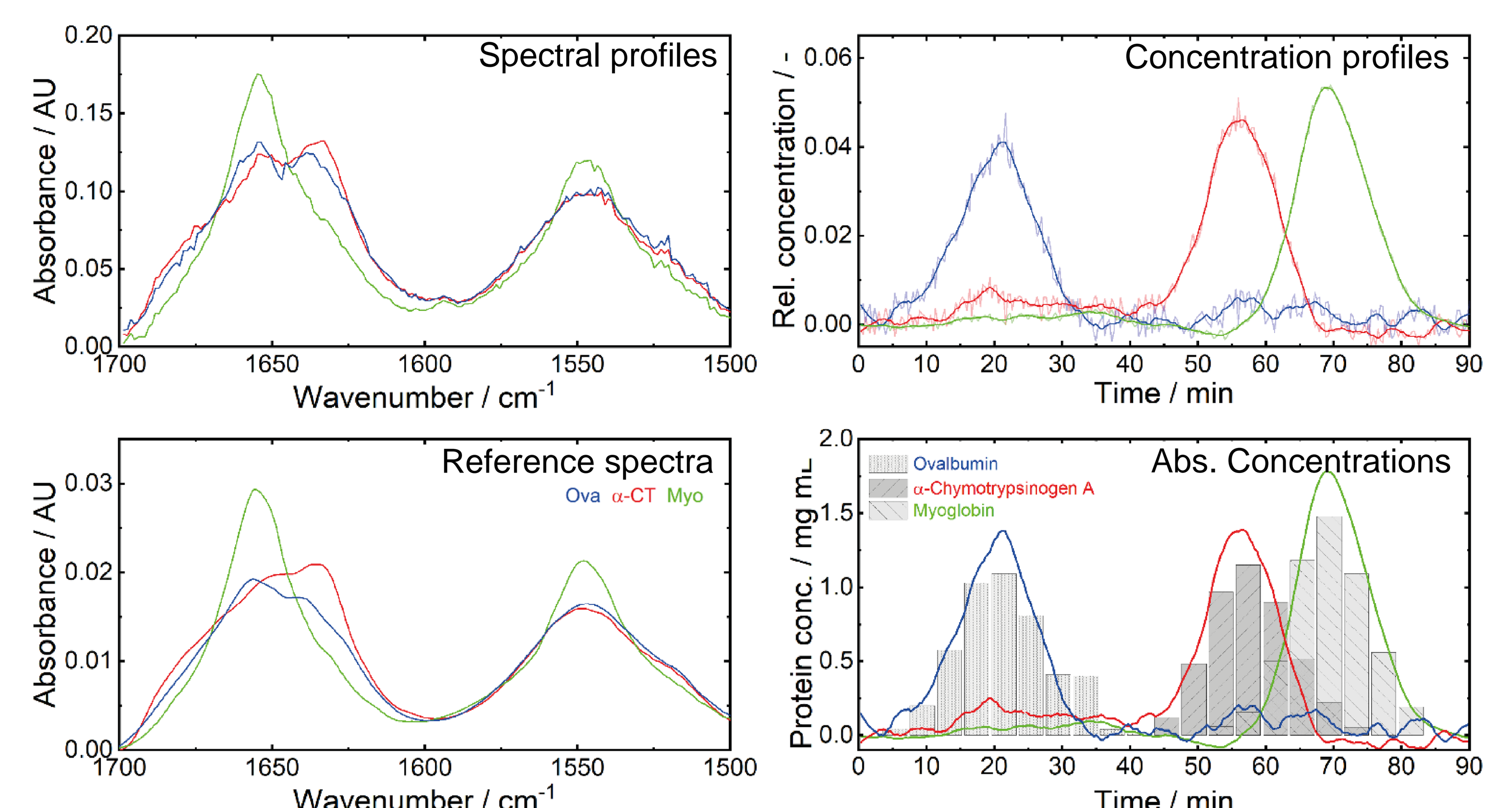


Size Exclusion Chromatography (SEC)

- 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 [3]
- Three model proteins with different secondary structures 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



- Chemometric analysis based on Simple-to-use Interactive Self-modeling Mixture Analysis (SIMPLISMA) and Multivariate Curve Resolution (MCR) was applied
- Spectral and concentration profiles of the individual proteins were obtained without initial knowledge
- Chemometric spectral profiles showed excellent agreement in band shape & position to reference off-line IR spectra of pure protein solutions
- Absolute protein concentrations were calculated via Beer-Lambert law, showing excellent agreement to HPLC off-line measurements



References

[1] 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).
[2] 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., 94, 5583-5590 (2022).
[3] C. K. Akhgar, J. Ebner, O. Spadiut, A. Schwaighofer, B. Lendl, “Laser-based mid-infrared spectroscopy enables in-line detection of protein secondary structure from preparative liquid chromatography” Proc. SPIE, 11957 (2022).

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.