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In-line Fourier-transform infrared spectroscopy as a versatile process analytical technology for preparative protein chromatography

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ABSTRACT

Fourier-transform infrared spectroscopy (FTIR) is a well-established spectroscopic method in the analysis of small molecules and protein secondary structure. However, FTIR is not commonly applied for in-line monitoring of protein chromatography. Here, the potential of in-line FTIR as a process analytical technology (PAT) in downstream processing was investigated in three case studies addressing the limits of currently applied spectroscopic PAT methods. A first case study exploited the secondary structural differences of monoclonal antibodies (mAbs) and lysozyme to selectively quantify the two proteins with partial least squares regression (PLS) giving root mean square errors of cross validation (RMSECV) of 2.42 g/l and 1.67 g/l, respectively. The corresponding Q^2 values are 0.92 and, respectively, 0.99, indicating robust models in the calibration range. Second, a process separating lysozyme and PEGylated lysozyme species was monitored giving an estimate of the PEGylation degree of currently eluting species with RMSECV of 2.35 g/l for lysozyme and 1.24 g/l for PEG with Q^2 of 0.96 and 0.94, respectively. Finally, Triton X-100 was added to a feed of lysozyme as a typical process-related impurity. It was shown that the species could be selectively quantified from the FTIR 3D field without PLS calibration. In summary, the proposed PAT tool has the potential to be used as a versatile option for monitoring protein chromatography. It may help to achieve a more complete implementation of the PAT initiative by mitigating limitations of currently used techniques.

1. Introduction

Preparative chromatography of biopharmaceuticals is typically monitored by measuring univariate signals such as pH, conductivity, pressure, and UV/Vis absorbance at a given wavelength [1,2]. Among these, especially single-wavelength UV/Vis spectroscopy has been a staple for process monitoring of biopharmaceutical chromatography due to its linear response to protein concentration as well as its broad dynamic range, sensitivity, and robustness. In spite of advantages, single-wavelength UV/Vis absorption measurements generally do not allow for selective quantification of multiple co-eluting proteins [3].

Even before the PAT initiative by the FDA in 2004 [4], research towards more selective monitoring methods for preparative chromatography was conducted. But the often small differences between biopharmaceutical product and protein as well as non-protein contaminants make this a nontrivial task [5,6]. As a possible solution, fast at- or on-line analytical methods, such as analytical chromatography, have been established. Discrete samples are taken from the process stream and analyzed on the spot. This approach has been proposed for controlling capture [7–9] and polishing steps [10,11]. However, at- or on-line analytical chromatography is complex in terms of equipment requiring a sampling module as well as an analytical chromatography system close to the process stream. Furthermore, the sampling and analysis time may be too long compared to the typical time frame available for taking process decisions.

An alternative approach exploits slight differences in UV/Vis absorption spectra of different components to selectively quantify different species by chemometric methods [6]. The approach yields results quickly enough to allow for real-time process decisions in chromatography [12–14] and works for minute spectral differences [15]. However, in the commonly measured spectral ranges, UV/Vis

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spectroscopy lacks sensitivity towards relevant aspects of protein structure, notably the secondary structure [16]. Furthermore, organic compounds are often not UV-active (e.g. sugars, polyols, and Polyethylene Glycol [PEG][17,18]) or they may obscure the protein signal (e.g. Triton X-100 [19] and benzyl alcohol [16]). Due to the high sensitivity, UV/Vis absorption spectroscopy is also prone to detector saturation [6,20].

FTIR allows to address several of these short-comings. Like UV/Vis spectroscopy, FTIR is a non-destructive, quantitative, and quick method which can be performed in-line [21–23]. FTIR measures the vibrational modes of samples and thereby provides a spectroscopic fingerprint for different organic molecules. Proteins absorb in the IR spectral range mainly due to vibrations of the polypeptide backbone [24,16,25]. Based on the backbone vibrations, FTIR grants insight into the secondary structure of the measured proteins. In consequence, FTIR is a widely used method for assessing the structural integrity of proteins during protein purification and formulation [16]. Furthermore, FTIR was previously used as an at-line PAT tool in downstream processing of biopharmaceuticals for quantifying product content, high molecular weight species (HMW), and host cell proteins (HCP) [26,27].

In this work, in-line FTIR as a PAT tool for preparative protein purification was implemented. An FTIR instrument was coupled to a lab-scale preparative chromatography system to perform the experiments. Three case studies were selected to investigate potential applications of FTIR as a PAT tool. First, a mixture of lysozyme and mAb was chosen due to the significant differences in secondary structure of the two proteins. While lysozyme mainly consists of alpha-helices (PDB ID 193L), mAb largely consists of beta-sheets (PDB ID 1HZH). The expected spectral differences can be used to selectively quantify the two proteins by PLS regression. Four linear-gradient elutions with varying gradient lengths were performed. Based on the results, a PLS model for each protein was optimized. The error of the PLS model was assessed by cross validation. Second, the preparative separation of PEGylated lysozyme was monitored. In contrast to UV/Vis spectroscopy, PEG gives a distinct signal in IR which can be used for quantification by PLS regression. Again, four linear gradient elutions were performed for the calibration of two PLS models. Finally, the potential to monitor process-related impurities using in-line FTIR was demonstrated by adding Triton X-100 to a feed solution of lysozyme. Triton X-100 is employed for virus inactivation in biopharmaceutical production and has to be removed from the product [19,28]. Based on an off-line calibration curve, mass-balancing of Triton X-100 in the flow-through during product loading was performed.

2. Materials and methods

2.1. Experimental setup

In-line FTIR measurements were performed using a Tensor 27 by Bruker Optics (Ettlingen, Germany) connected to an ÄKTApurifier system by GE Healthcare (Little Chalfort, UK). The chromatography system was equipped with a P-900 pump, a P-960 sample pump, UV-900 UV/Vis cell, and a Frac-950 fraction collector (all GE Healthcare). Unicorn 5.31 (GE Healthcare) was used to control the system. The FTIR was equipped with a liquid nitrogen-cooled Mercury Cadmium Telluride (MCT) detector and a BioATR II (Bruker Optics) with a flow-cell insert and a seven-reflections silicon crystal. The instrument was controlled by OPUS 7.2 (Bruker Optics).

In this setup, the effluent stream from the column outlet was diverted through the FTIR instrument and then back into the UV/Vis cell in the ÄKTApurifier system. The flowpath is illustrated in Fig. 1. The delay volume between the FTIR and the fraction collector was determined gravimetrically. As the flow rate was set in the chromatographic methods, the measurement of the delay volume enables the correlation of spectral data from the FTIR to collected fractions.

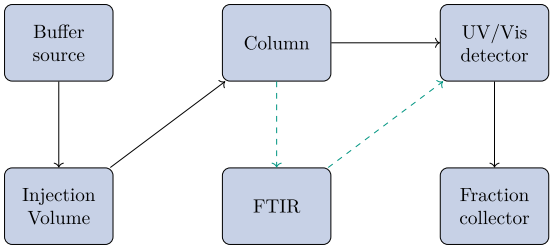

Fig. 1. Schematic representation of the flow path in the custom chromatography setup, solid lines represent the common flow path in the ÄKTApurifier while the dashed line represents the modification.

The interconnection between OPUS and Unicorn was achieved using a software solution developed in-house consisting of a Matlab (The Mathworks, Natrick, MA, United States) script and a VBScript in the built-in visual basic script engine of OPUS. The custom software enables start of a measurement at a time defined by Unicorn by sending a digital signal through the I/O port of the pump of the ÄKTApurifier System. The signal is captured by a USB-6008 data acquisition device (National Instruments, Austin, Tx, United States) controlled by Matlab which in turn triggers the measurement in OPUS.

2.2. Proteins and buffers

All solutions were prepared using water purified by a PURELAB Ultra water purification system by ELGA Labwater (High Wycombe, United Kingdom). Buffers were filtered using a 0.2 μm filter purchased from Sartorius (Göttingen, Germany) and degassed by sonification before use. All buffers were pH-adjusted using 32% HCl (Merck, Darmstadt, Germany).

Lysozyme was purchased from Hampton Research (Aliso Viejo, CA, United States). mAb was provided by Lek Pharmaceuticals d.d. (Mengeš, Slovenia) as a virus-inactivated Protein A eluate pool.

Preparative CEX chromatography runs in case studies I and III were conducted with a 50 mM sodium citrate buffer as equilibration buffer and with an added 500 mM NaCl as elution buffer. Both buffers were adjusted to pH 6.0. Sodium citrate tribasic dihydrate was purchased from Sigma-Aldrich (St. Louis, MO, United States), sodium chloride was purchased from Merck. For the CEX chromatography experiments in case study II, a 25 mM sodium acetate buffer (pH 5.0) was used as equilibration buffer. As elution buffer, a 25 mM sodium acetate buffer with 1 M NaCl (pH 5.0) was used. Sodium acetate trihydrate was purchased from Sigma-Aldrich. Batch-PEGylation of lysozyme was performed in a 25 mM sodium phosphate buffer at pH 7.2 using sodium phosphate monobasic dihydrate (Sigma-Aldrich) and di-sodium hydrogen phosphate dihydrate (Merck).

Analytical cation-exchange chromatography was carried out at pH 8.0 using a 20 mM Tris (Merck) buffer for equilibration and a 20 mM Tris buffer with 700 mM NaCl for elution.

2.2.1. PEGylation of lysozyme

The PEGylation protocol was adapted from [29]. Briefly, activated 5 kDa PEG was purchased as Methoxy-PEG-propionaldehyde (mPEG-aldehyde, Sunbright ME-050 AL) from NOF Corporation (Tokyo, Japan). Sodium cyanoborohydride ($NaCNBH_3$, Sigma Aldrich) was added to the reaction buffer to a concentration of 20 mM as reducing agent. mPEG-aldehyde was added to a molar PEG-to-protein ratio of 6.67. After 3 h, the mixture was diluted vol-

umetrically 7-fold using the acetate equilibration buffer and loaded onto the chromatography column.

2.3. Preparative chromatography experiments

For all chromatography experiments, FTIR spectra were recorded continuously in the chromatography mode of OPUS with a resolution of 2 cm^{-1} in a range from 4000 cm^{-1} to 900 cm^{-1} without averaging multiple scans. In the given setup, each measurement took 3.22 s. Background measurements at the beginning of chromatographic runs were taken at the same resolution with 400 scans in equilibration buffer. All experiments were conducted twice, once with protein injection and once with buffer only as a blank run. The FTIR spectra from the blank runs were subsequently subtracted from the protein runs to account for spectral effects by the gradient.

2.3.1. Case study I: selective protein quantification

For case study I, a HiTrap column by GE Healthcare prepacked with SP Sepharose FF resin (Column Volume [CV] 5 ml) was used. The column was loaded to a density of 18.75 g/l, consisting of 12.5 g/l lysozyme and 6.25 g/l monoclonal antibody. The flow rate for all experiments was set to 0.5 ml/min. The column was equilibrated in a low-salt buffer for 5 CV before injection. The 50 ml sample was injected using a 50 ml superloop from GE Healthcare. Elution was carried out with a linear gradient from 0% to 100% high-salt buffer with gradient lengths of 1 CV, 2 CV, 3 CV, 4 CV. After elution, a high-salt wash of 8 CV was performed for column regeneration. The effluent was collected over the complete injection and elution in 500 μl fractions for offline analytics.

2.3.2. Case study II: separation of pegylated lysozyme species

The experiments with different PEGylated lysozyme species were conducted with Toyopearl Gigacap S-650M resin prepacked in a MiniChrom column (CV 5 ml) by Tosoh (Griesheim, Germany). The column was loaded to a density of 50 g/l of the heterogeneous batch PEGylation. The sample pump was run at 1 ml/min for loading. For the remaining chromatography run, the flow rate was set to 0.5 ml/min. The column was first equilibrated for 1 CV, followed by an injection of 57.6 CV of sample solution. Linear-gradient elutions from 0% to 100% high-salt buffer were conducted with gradients of 2 CV, 3 CV, 4 CV and 5 CV length, followed by a 2 CV high-salt rinse. The effluent was collected from the beginning of the gradient until the end of the high-salt rinse in 500 μl fractions for offline analytics.

In some of the collected fractions, unconjugated lysozyme started to precipitate after elution probably due to the low pH, high salt concentration or low temperature [30,29]. Fractions and the corresponding spectra showing signs of precipitation were excluded from PLS model calibration.

2.3.3. Case study III: process-related impurity

For the simulated process-related impurity experiments, a HiTrap column by GE Healthcare prepacked with SP Sepharose FF resin (CV 5 ml) was used. Triton X-100 Biochemica was purchased from AppliChem GmbH (Darmstadt, Germany). The column was loaded with 5 ml of 25 g/l lysozyme and 10 g/l Triton X-100 solution [28]. The elution step was set to 2 CV.

Reference samples were generated by diluting defined amounts of Triton X-100 in equilibration buffer at concentrations from 1.25 g/l to 10 g/l. To generate a calibration curve, the samples were manually applied onto the ATR crystal. FTIR measurements were performed with 400 scans for background and samples.

2.4. Analytical CEX chromatography

As reference analytics for case study I, analytical CEX chromatography was performed using a Dionex UltiMate 3000 liquid chromatography system by Thermo Fisher Scientific (Waltham, MA, United States). The system was composed of a HPG-3400RS pump, a WPS-3000TFC analytical autosampler, a TCC-3000RS column thermostat, and a DAD3000RS detector. The system was controlled by Chromeleon 6.80 (Thermo Fisher Scientific). Fractions from preparative CEX chromatography were analyzed off-line on a Proswift SCX-1S 4.6 mm × 50 mm column by Thermo Fisher Scientific. A flow rate of 1.5 ml/min was used. For each sample, the column was first equilibrated for 1.8 min with equilibration buffer. Next, 20 μl sample was injected into the system and washed for 0.5 min with equilibration buffer. A linear gradient was performed during the next 2 min from 0% to 50% followed by a step to 100% elution buffer which was maintained for 2 min.

For the experiments in case study II, a Vanquish UHPLC system (Thermo Fisher Scientific) was used. The Vanquish UHPLC System consisted of a Diode Array Detector HL, a Split Sampler FT, a Binary Pump F, and a Column Compartment H including a preheater and post-column cooler (all Thermo Fisher Scientific). The same buffers, column, and flow rate were used as for case study I. After injecting 5 μl of sample, the column was washed for 0.5 min. Subsequently, a bilinear gradient was performed from 0% to 50% elution buffer over 5 min and 50–100% elution buffer over 1.75 min. After the elution, a high-salt strip at 100% was run for 1 min. Calibration was performed by a dilution series of pure lysozyme. Since PEG does not absorb in UV/Vis, solely lysozyme contributes to the absorption signal. Peak identification with respect to the PEGylation degree was conducted using purified samples prepared according to [18]. From the molar concentration of PEGylated lysozyme species, the molar concentration of PEG was calculated.

2.5. Data analysis

All data analysis was performed in Matlab. For case studies I and II, the data was first preprocessed and subsequently fitted with PLS-1 models by the SIMPLS algorithm [31]. Preprocessing consisted of linearly interpolating off-line analytics to be on the same time scale as the FTIR spectra. For case studies I and II, spectral data above 2000 cm^{-1} resp. above 3100 cm^{-1} was discarded. Next, a Savitzky-Golay filter with a second-order polynomial was applied on the spectra and optionally, the first or second derivative was taken [32]. Cross-validation was performed by excluding one chromatography run, calibrating a PLS model on the remaining runs, and calculating a residual sum of squares on the excluded run. This procedure was repeated until all runs had been excluded once. All residual sums of squares for the different submodels were subsequently summed yielding the Predictive Residual Sum of Squares (PRESS). The PRESS was scaled according to Wold et al. by the number of samples and latent variables used in the PLS model [33]. Based on the scaled PRESS, an optimization was performed using the built-in genetic algorithm of Matlab for integers [34]. The genetic algorithm optimized the window width of the Savitzky-Golay filter, the order of derivative, as well as the number of latent variables for the PLS-1 model. The RMSECV was calculated from the PRESS by dividing by the total number of samples. The Q^2 values were calculated by dividing the PRESS by the summed squares of the response corrected to the mean [33].

For case study III, spectral data was smoothed both in direction of time and wavenumber using a Savitzky-Golay filter with a second-order polynomial and a frame length of 17 and 51, respectively. A linear baseline was calculated and subtracted for each spectrum individually to account for a non-horizontal non-zero baseline. The baseline subtraction was performed on the reference spectra as well as on the spectra from the chromatography experiment. Based on the area under the spectrum between wavenumbers 1007–1170 cm^{-1}, a mass balance for Triton X-100 was calculated from the spectral data of the chromatography run.

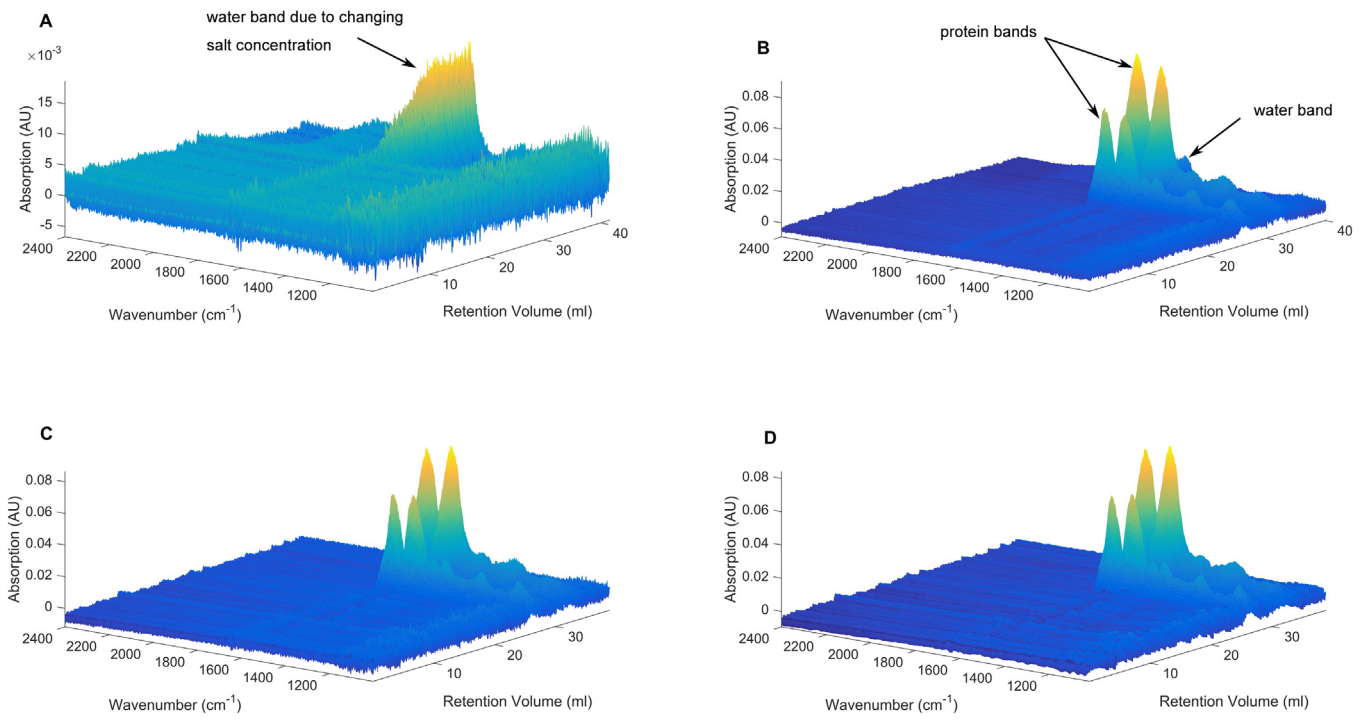

Fig. 2. Work flow for data treatment of chromatography spectra illustrated with data from case study I, 4 CV run: background run – salt gradient without protein (A); raw spectra of the run with protein (B); spectral data after the background has been subtracted (C); data after smoothing by Savitzky–Golay algorithm (D).

The volume represented by each spectrum was calculated from the recording time and the volumetric flow rate of the experiment. Triton X-100 masses in each segment were calculated utilizing the calibration curve and summed up over time.

3. Results and discussion

In-line FTIR measurements were applied as a PAT tool for different preparative chromatographic protein separations. In three different case studies, FTIR was used for selective quantification of different species. First, background correction of the FTIR chromatograms is discussed which was necessary for further data processing. In a first case study, the capability of FTIR to measure differences in secondary structure in-line and utilize the differences for selective quantification of mAb and lysozyme was demonstrated. A second case study made use of the absorption of PEG in IR to monitor the PEGylation degree of eluting PEGylated lysozyme species. Finally, the third case study used the selectivity of FTIR to selectively quantify Triton-X 100, a detergent used for viral inactivation.

3.1. Background subtraction and spectral preprocessing

Background subtraction for in-line FTIR measurements is of major importance as water has an absorption band around 1600 cm^{-1} (cf. Fig. 2A) which coincides with the most prominent protein band amide I. The spectral processing workflow is illustrated in Fig. 2 using data from case study I. Specifically the elution of mAb and lysozyme using a 4 CV gradient is shown. Most of the water absorption can be eliminated by taking a background with the equilibration buffer at the beginning of each chromatographic run. The water band is, however, also influenced by the salt content of the buffer around 1650 cm^{-1}. Salt gradients therefore cause a change in absorption over the run (cf. Fig. 2A and B). To reduce buffer effects, it is important to find a suitable dynamic background correction. An approach based on reference spectra matrices and chemometric correlations was not implemented due to the overlap of water and protein bands [35]. Instead, an alternative approach was chosen. Based on the retention time, a blank run without protein but including the salt gradient was subtracted from the actual preparative run (cf. Fig. 2C). The resulting chromatogram provided a smooth baseline over the whole experiment. After baseline correction, additional data preprocessing was performed. The single scan spectra were smoothed by a Savitzky-Golay filter to reduce random noise (cf. Fig. 2D) and to take derivatives on the spectral data.

3.2. Case study I: selective protein quantification

mAb and lysozyme feature significant differences in secondary structure. While mAb consists largely of beta-sheets (PDB ID 1HZH), lysozyme mainly contains alpha-helices (PDB ID 193L). These differences make the two proteins simple model components to study the performance of in-line FTIR for selectively quantifying proteins. The bands visible between 1200 cm^{-1} and 1700 cm^{-1} in Fig. 2D are characteristic amide bands associated with the protein backbone [16,24,25]. Especially the amide I band is frequently used for assessing the secondary structure of proteins. For PLS calibration, all wavenumbers below 2000 cm^{-1} were taken into account to include all protein bands without interference at the boundary due to the Savitzky-Golay filter.

Based on four CEX runs, two PLS-1 models were optimized for selective quantification of mAb and lysozyme, respectively. The resulting model parameters are listed in Table 1. Fig. 3 shows a comparison from off-line analytics and the prediction of PLS models. Both PLS models match peak maxima and peak widths well and are able to discern the two components. For mAb, a root-mean-square error of cross validation (RMSECV) of 2.42 g/l was reached. For lysozyme, the RMSECV was 1.67 g/l. The corresponding Q^2 values were 0.92 and 0.99, respectively. The high Q^2 values show, that a large part of the variation in the off-line concentration measurements could be explained by the PLS model. The differentiation

Table 1
Model parameters for case studies I and II are listed below including the parameters for the Savitzky–Golay filter and the latent variables of the PLS-1 model. Additionally, the RMSECV for each model is listed.

	Case study I		Case study II	
	mAb	lysozyme	lysozyme	PEG
Savitzky–Golay Window	215	21	101	361
Derivative	0	0	2	2
Latent variables	3	7	6	8
RMSECV (g/l)	2.41	1.63	2.35	1.24

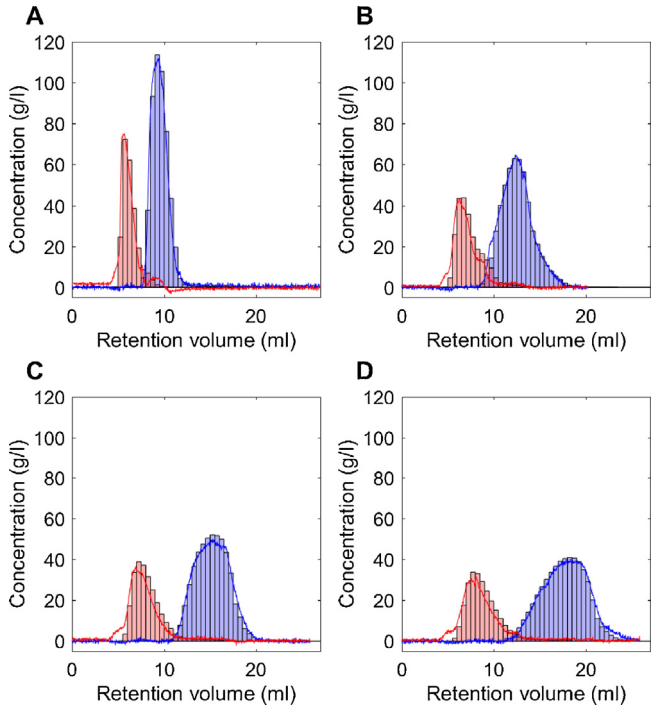

Fig. 3. Four chromatographic runs are shown for in-line FTIR measurements and selective quantification of mAb and lysozyme. The red bars and lines refer to the mAb off-line measurement and mAb PLS prediction, respectively. The blue bars and lines refer to the lysozyme off-line measurement and lysozyme PLS prediction, respectively. The different subplots show different gradient lengths: A 1 CV, B 2 CV, C 3 CV, D 4 CV. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

between different proteins may however become more challenging for smaller differences in secondary structure. Interestingly, the combination of Savitzky-Golay filtering and PLS modeling allowed to reduce the measurement noise compared to single-wavelength measurements. As shown by Figs. 2C and 3, the measurement noise in the IR spectra is higher than the noise observed in the PLS prediction. By filtering and projecting the spectra to latent variables, random noise is reduced [32,33]. Furthermore, 3.23 s measurement time makes FTIR quick enough for monitoring most practical preparative chromatography applications in real-time. In-line FTIR spectroscopy allowed to cover high concentration ranges. The predicted concentration of lysozyme during the 1 CV run reaches 112 g/l without any interference from detector saturation. The measurement setup therefore covers all concentrations typically occurring in preparative protein chromatography.

In summary, the results show that FTIR in conjunction with PLS modeling can differentiate in-line between proteins based on their secondary structure and has the potential to be applied for real-time monitoring and control of preparative chromatography.

3.3. Case study II: separation of PEGylated lysozyme species

In conventional chromatography systems, the separation of differently PEGylated species cannot be monitored holistically as PEG does not absorb in UV. Contrary to this, PEG produces a number of prominent bands in IR. A strong band around 1090 cm^{-1} with multiple shoulders is characteristic of C–O stretching [36]. Due to symmetric CH_2 stretching, PEG furthermore generates a doublet at 2884 cm^{-1} and 2922 cm^{-1}. Bands occurring between 1200 cm^{-1} and 1700 cm^{-1} are related to the protein backbone with some interference from PEG C–H bending.

Fig. 4 shows a typical chromatographic separation of PEGylated lysozyme species. During the elution, the ratio between PEG and protein bands decreases. First, with a retention volume of 6.8 ml, the absorption of the C–O band at 1090 cm^{-1} (denoted as CO_1 in Fig. 4) exceeds the absorption of amide I band (AI_1). For the second peak with a retention volume of 10.3 ml, the absorption of the amide I (AI_2) is higher than for the C–O stretching band (CO_2). The last peak does not show characteristic PEG bands, i.e. consists of unconjugated lysozyme. The order of elution followed a descending degree of PEGylation which is in line with previous publications [18,37,38].

Based on the evaluation of IR absorption bands, it was decided to include all wavenumbers from 900 cm^{-1} to 3100 cm^{-1} into PLS model calibration. Initial PLS calibration on the concentration of the different PEGylated lysozyme species showed that the conjugation did not cause large enough band shifts to allow for selective quantification of the different PEGylated lysozyme species. Instead, two PLS models were fitted on the total PEG resp. lysozyme concentration independently. PEG concentration was calculated by weighting the off-line lysozyme concentration according to the PEGylation degree. In Table 1, the optimization results are summarized. Fig. 5 compares the PLS prediction with off-line analytics. RMSECV values of 1.24 g/l and 2.35 g/l were reached for the PEG and lysozyme concentration, respectively. The corresponding Q^2 values were respectively 0.96 and 0.94 showing that the PLS models predicted the responses well. Based on the PEG and lysozyme concentrations, a molar ratio could be calculated corresponding to the current average PEGylation degree. To simplify visual interpretation, the molar ratio was only plotted if the lysozyme concentration exceeded its RMSECV 3-fold.

The predicted PEG and lysozyme concentrations accurately followed the concentrations measured by off-line analytics. Furthermore, the molar ratio gives a suitable tool for in-line monitoring of the elution of different PEG species. Interestingly, the two PLS models are able to extend their prediction over the calibration range, i.e. to perform a weak extrapolation. This can be seen as the PEG-to-lysozyme ratio exceeds the value of two, which limits the calibration range spanned by off-line analytics. Higher PEGylated species of lysozyme do however occur and could be measured by the FTIR [18,39].

In summary, FTIR allows to monitor not only the protein and PEG concentration but also the PEGylation degree during chromatographic separations.

3.4. Case study III: quantification of a process-related impurity

Triton X-100 is used for viral inactivation of biopharmaceuticals if pH treatment has to be circumvented, e.g. for Factor VIII or pH-sensitive mAbs [19,28]. To achieve viral inactivation, Triton X-100 concentration needs to be above a minimal level. Typically, a concentration of 1% (w/V) is used. Here, Triton X-100 concentration of a mock virus inactivation batch was monitored during the subsequent load phase onto a chromatographic column. During the chromatographic run, in-line FTIR measurements were perform (cf. Fig. 6).

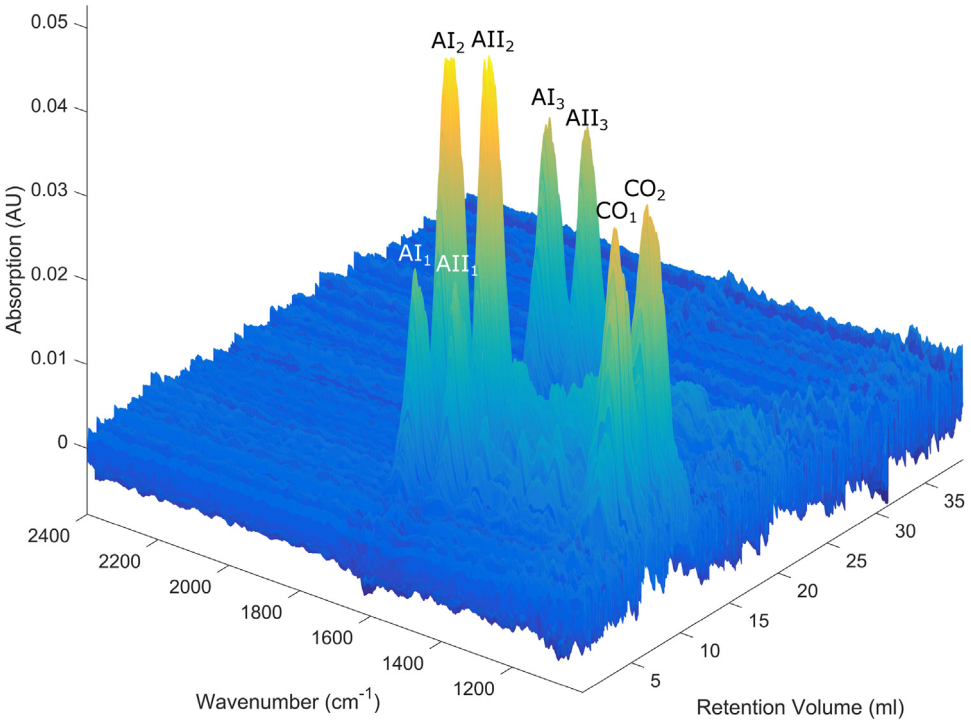

Fig. 4. Elution of PEGylated lysozyme species from a CEX column with a gradient length of 5 CV. Bands visible between wavenumbers 1200–1700 cm^{-1} are the characteristic amide bands associated with protein. The major protein bands amide I and amide II are marked as AI and AII, respectively. The band at approximately 1100 cm^{-1} is characteristic of PEG (C–O stretching, marked as CO). The subscript numerals refer to the elution order.

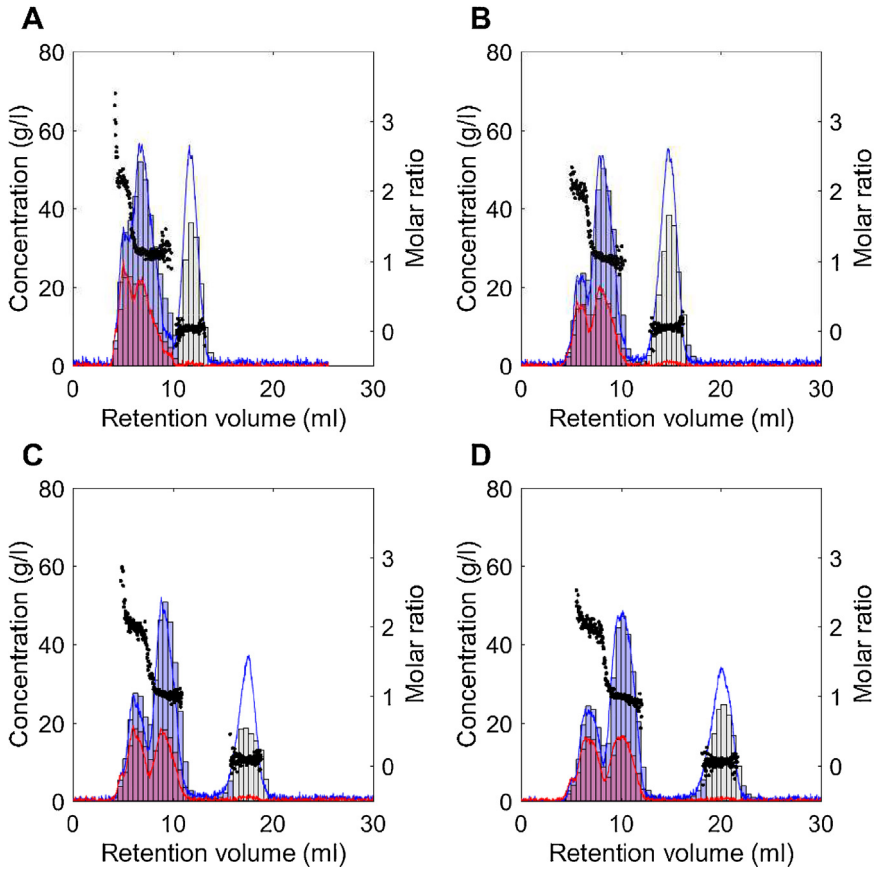

Fig. 5. Four chromatographic runs are shown for in-line FTIR measurements and selective quantification of PEG and lysozyme. The red bars and lines refer to the PEG off-line measurement and PEG PLS prediction, respectively. The blue bars and lines refer to the lysozyme off-line measurement and lysozyme PLS prediction, respectively. Grey bars correspond to measured protein concentrations on partially precipitated samples. Black dots show the molar ratio between PEG and lysozyme, i.e. the current mean PEGylation degree. The different subplots show different gradient lengths: A 2 CV, B 3 CV, C 4 CV, D 5 CV. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

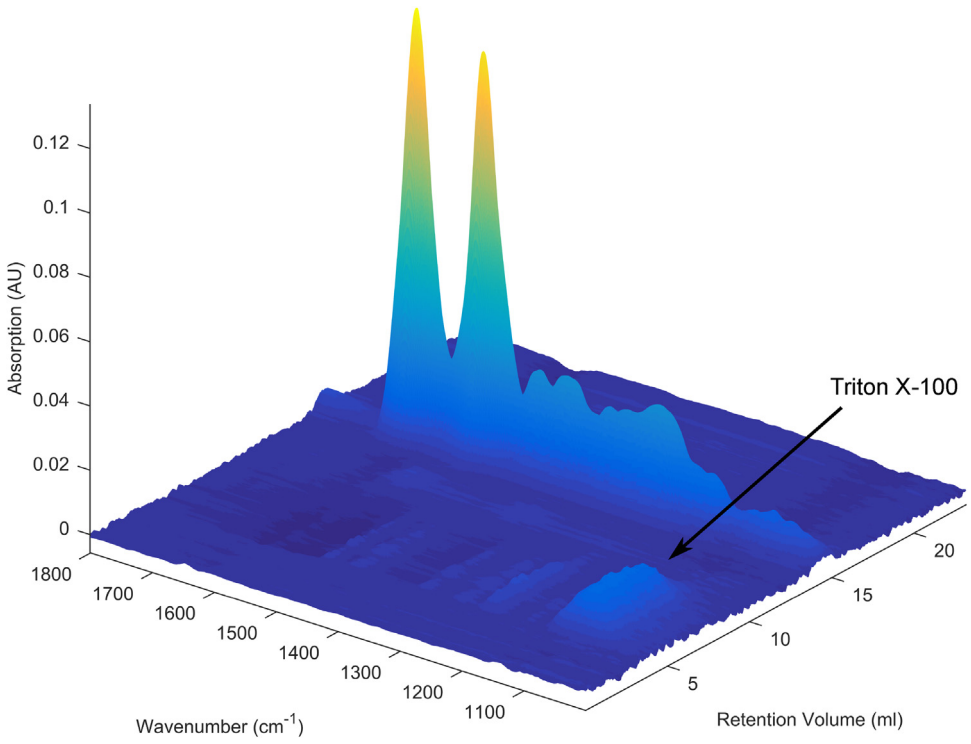

Fig. 6. Triton X-100 as a process-related impurity can be seen in the flow-through of the cation-exchange experiment from 5.5 ml to 11 ml at 1090 cm^{-1}.

In IR, Triton X-100 causes a characteristic band due to C–O stretching at 1090 cm^{-1}. By comparison of the blank run and the actual experiment, it was concluded that Triton X-100 is not retained on the column and is mainly present in the flow-through. The flow-through occurred between 5.5 ml and 11 ml As Triton X-100 and protein spectra only weakly interfere with each other, the Triton X-100 content was measured by simply correlating the band area of C–O stretching from 1007 cm^{-1} to 1170 cm^{-1} to the Triton X-100 concentration. A linear regression for the calibration curve resulted in a $R^2 > 0.9997$. Based on the calibration curve, in-line mass-balancing could be performed. The mass balance for Triton X-100 showed a recovery rate of 94.12% in the flow-through. This shows that it is possible to selectively quantify Triton X-100 content during the chromatographic load phase.

4. Conclusion and outlook

FTIR spectroscopy was successfully implemented in-line as a PAT tool for biopharmaceutical purification processes. It was demonstrated that FTIR is able to distinguish and selectively quantify proteins in-line based on their secondary structure. Furthermore, FTIR presents a powerful tool for monitoring different chemical components such as PEG or Triton X-100. Based on selective in-line quantification of PEG and protein, PEGylation degrees could be measured in-line. Selective mass balancing was performed on the process-related contaminant Triton X-100. In summary, FTIR provides orthogonal information to the typically measured UV/Vis spectra. It therefore is potentially interesting for monitoring process attributes which have been previously hidden. FTIR may help to achieve a more complete implementation of the PAT initiative.

Future research should be directed towards making the setup more compatible with the production environment. Challenges include the use of detectors without liquid nitrogen cooling and the application of fiber optics for in-line process probes.

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