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A Review on Quantitative Process Analytical Technology for Continuous Downstream Processing of Monoclonal Antibodies

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ABSTRACT

The competition in the biopharmaceutical market is increasing due to the market entry of biosimilars and rising costs in research and development of new drugs. Hence, continuous manufacturing gained significant attention due to its potential in reducing production cycle times and costs, as well as the possibility of real-time release testing. As a consequence, active monitoring and/or control systems are required for quantitative product quality measurements and in-process control. Process analytical technology emerged as a robust strategy for the development and implementation of in situ real-time testing, instead of the standard batch testing of end product. Through the evaluation of state-of-the-art applications, this review highlights future opportunities in the field of quantitative real-time analytical techniques for the characterization of monoclonal antibodies in continuous downstream biomanufacturing.

1 | Introduction

Process analytical technology (PAT), also referred to as in situ analytics, enables the collection of extensive empirical data, thereby facilitating process supervision, real-time feedback control, and quality by design (QdD) integration with the goal of ensuring final product quality (Chopda et al. 2022; Kessler and Kessler 2020). The US Food and Drug Administration (FDA) launched the initiative "Pharmaceutical CGMPs for the 21st Century: A Risk-Based Approach" in 2002. It aimed at improving the pharmaceutical sector through innovation derived from scientific and engineering knowledge on the development, analysis, and control of processes and products. A process and/or product is considered under control when all critical sources of variability are identified and managed by the process. This also includes understanding the impact on product quality within the established design space, which encompasses process parameters, manufacturing conditions, and materials used. Within that initiative, a "PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance" guidance was published, detailing a regulatory framework for the pharmaceutical industry, which was adopted in 2005 for worldwide use as "ICH Q8 Pharmaceutical development—Scientific guideline" (Menezes et al. 2009). A PAT tool, qualitative or quantitative in nature, can be integrated in-, on-, or at-line. To maintain uniformity in defining the placement of PAT tools, the definition used in this review aligns with the terminology published in the "PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing and Quality Assurance" guidance by the FDA in 2004 (Figure 1).

PAT can be applied throughout the entire biopharmaceutical development pipeline, from preclinical to commercial

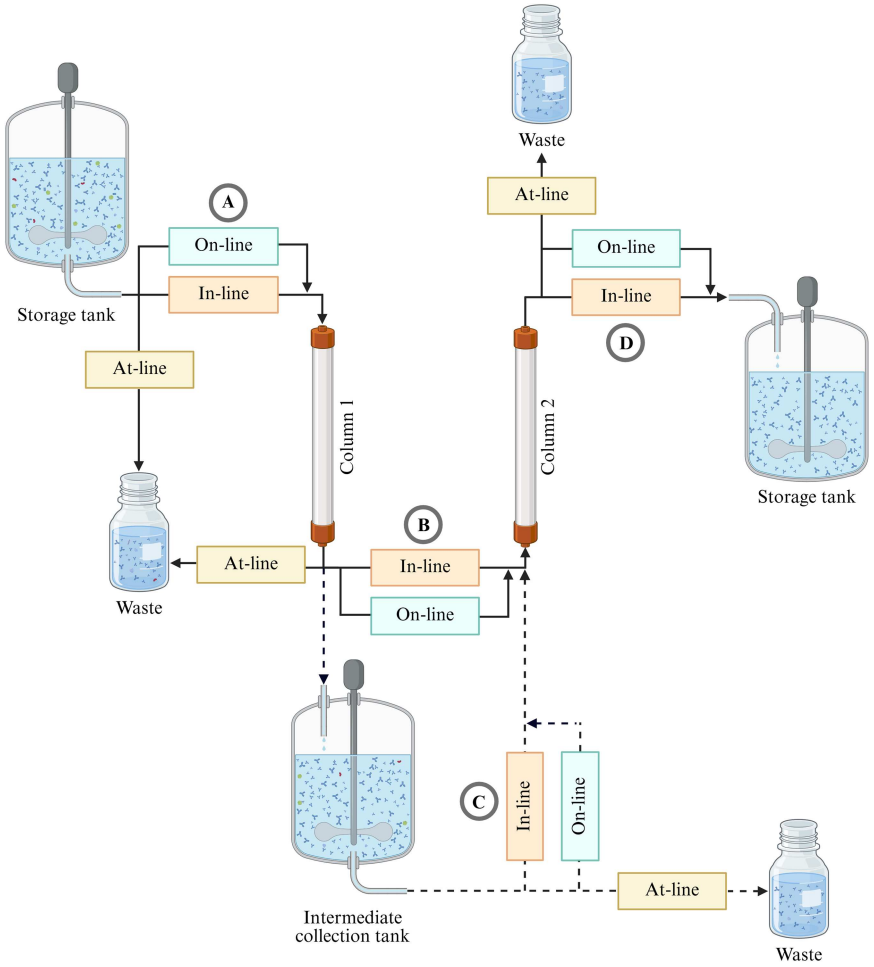

FIGURE 1 | Schematic representation of a multi-column chromatography-based step including surge tanks for (intermediate) product storage with multiple locations for process analytical technology (PAT) placement, following the definition from the FDA guidance on "PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing and Quality Assurance" (2004). Four scenarios for PAT placement are represented, where A refers to product testing before chromatography column 1, B represents product testing after chromatography column 1 and immediately before chromatography column 2, C denotes an alternative option for intermediate product collection and monitoring after chromatography column 1, and D illustrates product monitoring after chromatography column 2 and before storage. In-line refers to a measurement that takes place inside the process stream; on-line denotes a diverted sample from the system for analysis, which may be returned to the process stream afterwards; and at-line relates to the removal of a sample from the system for isolated analysis in close proximity to the process stream, being subsequently discarded. Figure created with BioRender.com.

manufacturing, enabling scientists to thoroughly examine and understand processes at each stage. When transferred to manufacturing, it may be used in different ways, namely for process monitoring, in-process control (IPC), and real-time release testing (RTRT) (Figure 2). It is important to note that process control implementation implies active decision-making based on the (near) real-time data, which is subject to additional regulatory requirements. Accordingly, assessments of the PAT tool regarding robustness and fit-for-purpose need to be conducted (Bordawekar et al. 2015; Glassey et al. 2011). PAT is also at the core of the transition of industry from batch to continuous processing (Chanda et al. 2015), which aims to reduce the manufacturing footprint and cost of goods, as well as increase efficiency and flexibility, while maintaining consistent product quality (Somasundaram et al. 2018).

Biomanufacturing processes require several upstream and downstream unit operations, comprising cell culture, purification, viral clearance, and formulation. In conventional batch processing, in-process samples are stored and analyzed off-line after each unit operation, resulting in long idle periods. Conversely, continuous processing integrates unit operations with constantly flowing process streams, enabling the same material throughput as batch processing while requiring a much smaller facility footprint (Rathore et al. 2023). Typical batch processing units can be adapted for continuous operation using systems equipped with multiple channels, pumps, and valves, such as those employed in periodic counter-current chromatography (PCC) and simulated moving bed (SMB) chromatography (Konoike et al. 2024; Rathore et al. 2023; Yang et al. 2020). Moreover, continuous processing relies on advanced process scheduling systems and surge tanks for intermediate product collection (Rathore et al. 2023; Thakur et al. 2021). Alternative unit operations were proposed for continuous processing, including aqueous two-phase extraction (ATPE), precipitation, and crystallization to enable integration with upstream processes and to be compatible with continuous flows (Yang et al. 2020). It is worth noting that PAT requirements for batch

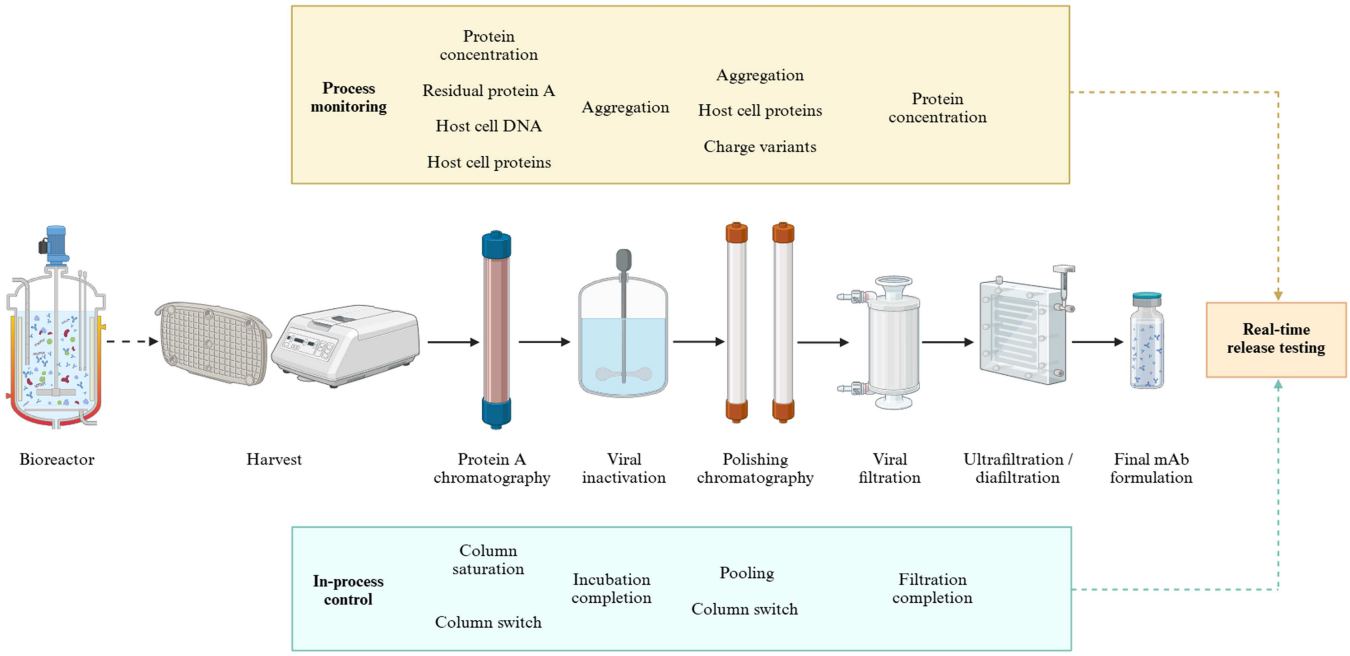

FIGURE 2 | Opportunities for process analytical technology (PAT) tools in typical downstream monoclonal antibody biomanufacturing platforms (mAbs) (Shukla et al. 2007), enabling continuous processing, through the implementation of process monitoring, in-process control (IPC), and real-time release testing (RTRT) (Bordawekar et al. 2015; Glassey et al. 2011). Figure created with BioRender.com.

and continuous processing unit operations can be similar, although continuous processing requires faster measurement times due to the limited time available for making decisions before proceeding to the next unit operation (Rathore et al. 2010).

The classical definition of PAT tools is based on the use of physical measuring devices, commonly referred to as hard sensors, that measure specific critical quality attributes (CQAs) of a raw material, an intermediate product, or a final product. These measurements can be obtained directly as an instant metric from the analytical tool, or indirectly, using models with correlations for variable prediction (Neugebauer et al. 2024). The latter is typically known as a soft sensor, where partial least squares (PLS) regression is widely used as an empirical predictive model in industry due its maturity within the chemometrics field, with development dating back to the late 1960s–1980s (Abdi and Williams 2013; Ferrer et al. 2008; Thissen et al. 2004). PLS is a linear multivariate method that derives latent variables to reduce the dimensionality of the predictor variables (independent variables) while retaining relevant information for predicting the response variables (dependent variables), thereby addressing multicollinearity. A major challenge of PLS is that it may experience inaccuracy when the dataset contains nonlinear correlations. Consequently, other nonlinear machine learning (ML) techniques, such as neural networks, are often applied (Abdi and Williams 2013).

The high complexity of biopharmaceutical products and the close chemical similarity of their contaminants present a challenge for the development and implementation of PAT, as opposed to small-molecule pharmaceuticals. Distinct CQAs play a role in the downstream processing (DSP) of monoclonal antibodies (mAbs), comprising the product, product-related impurities, and process-related impurities. Product-related impurities include size variants, such as aggregates and fragments, as well as charge variants resulting from post-translational modifications (PTMs). Process-related impurities consist of residual protein A from the affinity chromatography step, host cell DNA, and host cell proteins (HCPs) derived from the cell culture (Limpikirati et al. 2024). Each unit operation in the DSP cascade has its own targets and specifications that need to be tightly monitored and controlled during process development and, eventually, in manufacturing. Throughout the mAb DSP cascade, product and contaminant concentrations differ significantly depending on the platform process used. Typically, a mAb purification process results in a product recovery of 70% with high purity (> 95%). Moreover, purity targets specify < 100 ppm HCP, < 10 ng/dose host cell DNA, and < 5% aggregated content (Chon and Zarbis-Papastoitsis 2011; Limpikirati et al. 2024). A mAb concentration of < 10 g/L is generally expected after the protein A chromatography (Shukla et al. 2007). However, to cope with the increasing titers from intensified upstream processing (USP), the loading capacity of the protein A resin can reach up to 50 g/L (Chon and Zarbis-Papastoitsis 2011). By the end of the DSP cascade, mAb solutions are concentrated and a buffer exchange is performed, usually through ultrafiltration/diafiltration (UF/DF). The final product formulation requires high mAb concentrations, namely > 150 g/L for single-dose subcutaneous administration (Nadar et al. 2021). The combination of different compounds and concentrations means that proper PAT tool selection is essential to distinguish the product from product- and/or process-related contaminants in real time (Chanda et al. 2015; Rolinger et al. 2020).

As highlighted in the previous paragraph, this review focuses on the quantification of mAbs, which were first commercialized in 1986 (Ecker et al. 2015), and represent the dominant product class within the biopharmaceutical market until present days

(Walsh and Walsh 2022). However, the knowledge presented in this review may be transferable to other protein-based therapeutics, for instance hormones, enzymes, cytokines, clotting factors, and virus-like particles (VLPs) (van den Broek et al. 2013; Zeltins 2013). This can be attributed to their similar structural features, such as amino acid composition, PTMs, as well as secondary, tertiary, and quaternary structures (Orphanou and Gervais 2018). Research on innovative and versatile PAT systems is key to enabling and improving continuous biomanufacturing. In recent years, numerous relevant PAT applications were developed and thoroughly reviewed in the literature (Chopda et al. 2022; Dürauer et al. 2024; Gerzon et al. 2022; Gillespie et al. 2022; Jiang et al. 2017; Rathore and Kapoor 2015; Rolinger et al. 2020; Rüdt et al. 2017; São Pedro et al. 2022; Wasalathanthri et al. 2020). Nevertheless, a clear distinction between qualitative and quantitative PAT tools is lacking, despite the critical importance for IPC and RTRT strategies that support continuous DSP. Qualitative PAT tools are valuable but have limited applicability beyond early-stage process development for product and process understanding (Chanda et al. 2015). Hence, this review focuses on quantitative real-time monitoring and/or control analytics for continuous DSP of mAbs. The following section is intended to give an overview of currently available quantitative PAT tools for the detection and characterization of mAbs. A brief overview of the working principle of each technique will be provided, along with relevant examples of their applications. A summary of quantitative PAT is provided in Table 1, which includes key information such as CQAs, measurement mode (in-/on-/at-line), measurement time, real-time application, instrumentation, cost estimation, linear product concentration range, limit of detection (LOD) estimation for impurities, background matrix, data treatment modeling, predictive performance, technology readiness, scale-up implementation, sample requirements, robustness, and other challenges for each quantitative technique. Overall, this review can serve as a guide for the selection of fit-for-purpose real-time quantitative analysis of mAbs for continuous DSP applications.

2 | PAT Tools

2.1 | Ultraviolet/Visible Spectroscopy

Ultraviolet-visible (UV/Vis) spectroscopy is based on the attenuation of electromagnetic radiation in the visible and adjacent near-UV region (190–800 nm) due to absorption of the incident light (L.C. Passos and M.F.S. Saraiva 2019; Moldoveanu and David 2017). In general, it involves surrogate signals characterized by the absorbance at a specific wavelength. This is typically performed at 280 nm for protein characterization, which corresponds to a maximum absorption peak because of the aromatic side chains of tryptophan, tyrosine, and phenylalanine (Antosiewicz and Shugar 2016; Brestrich et al. 2018). This univariate UV/Vis measurement may encounter limitations at higher concentrations due to saturation of the UV signal and nonlinearity of the Beer-Lambert law (Ramakrishna et al. 2022). To circumvent these issues, alternative solutions were introduced, such as multi-wavelength and variable pathlength (VP) UV/Vis spectroscopy (Brestrich et al. 2018; Rathore and Kapoor 2015). VP spectroscopy is based on a mobile optical fiber that changes the path length (L) of the flow cell. In this context, L represents the distance the incident light travels through the sample before it reaches the detector, which is directly proportional to the amount of light that is absorbed by the sample.

A combination of VP and multi-wavelength UV/Vis spectroscopy was applied in conjunction with PLS regression for mAb monitoring in a preparative polishing chromatographic step (Brestrich et al. 2018). This work demonstrated the ability to selectively quantify mAb monomers and high molecular weight (HMW) species, even though the differences in their absorption spectra were relatively small. A commercially available in-line VP UV spectroscopy-based technology was used, namely a FlowVPE (Repligen Corporation, Massachusetts, US), and it was connected to an ÄKTA Pure 25 chromatographic system (Cytiva, Massachusetts, US). Highly loaded columns were utilized with product peak concentrations between 30 and 80 g/L and contaminant peak concentration ranging from 4– 20 g/L. Size exclusion chromatography (SEC) was conducted as the reference off-line analytical tool to analyze the collected chromatographic pools. Two PLS models were trained to predict mAb monomers and HMWs, with a root mean square error of prediction (RMSEP) of 1.26 and 0.50 g/L, respectively. Furthermore, this method was adopted to support pooling decisions by enabling the separation of the protein monomer and aggregates, based on the predicted concentrations of the PLS model. It was shown that the model accurately predicted a mAb purity of 94.4% compared to 94.2% measured with the off-line benchmark (Brestrich et al. 2018).

A multi-wavelength UV/Vis spectroscopy method was also applied to quantify mAbs in the effluent during the load phase of a preparative protein A chromatography (Rüdt et al. 2017). An UltiMate 3000 diode array detector (DAD) (Thermo Fisher Scientific, Massachusetts, US) featuring a semi-preparative flow cell with a 0.4 mm optical pathlength was used in-line to collect UV/Vis spectra. The DAD was connected to an ÄKTA Pure 25 chromatographic system (Cytiva, Massachusetts, US). PLS modeling was applied to correlate the absorption spectra with mAb concentration. The calibrated PLS model was applied in real-time to control the load phase of a protein A chromatographic step, with a target breakthrough concentration of 1.5 mg/mL, indicative of a 50% product breakthrough. Upon comparison with reference off-line protein A chromatography, the PLS model achieved an RMSEP of 0.06 mg/mL. This demonstrates how a real-time quantitative readout obtained with multiwavelength UV/Vis can be effectively applied to control the load phase in a protein A chromatography (Rüdt et al. 2017). Several other studies in literature combined a DAD with an ÄKTA chromatographic system (Ramakrishna et al. 2022; Rolinger et al. 2021; Zobel-Roos et al. 2017), reporting comparable predictive power for mAb quantification (R-squared $[R^2] \geq 0.98$). It is important to highlight that Rolinger et al. (2021) extended the work of Rüdt et al. (2017) by proposing a dynamic background subtraction method based on the conductivity signal. Their goal was to extend the applicability of the PLS models to encompass potential variations in the harvested cell culture fluid (HCCF). Five different feedstocks were used to vary the impurity profile in the protein A load material. Furthermore, Ramakrishna et al. (2022) reported a PLS model trained across a broad mAb concentration range of

TABLE 1 | Summary of quantitative process analytical technology (PAT) tools discussed in this review aimed at detecting and characterizing monoclonal antibodies (mAbs) for continuous downstream processing (DSP), including reported critical quality attributes (CQAs), measurement mode, measurement time, real-time application, instrumentation, cost estimation, linear product concentration range, limit of detection (LOD) estimation for impurities, background matrix, data treatment modeling, predictive performance, technology readiness, scale-up implementation, sample requirements, robustness, and other challenges for each technique.

	PAT tools							
	UV/Vis spectroscopy	**Refractive index**	**Raman spectroscopy**	**FTIR spectroscopy**	**NMR spectroscopy**	**Light scattering**	**Liquid chromatography**	**Mass spectrometry**
CQAs	Protein concentration, aggregation, fragmentation	Protein concentration	Protein concentration, aggregation, fragmentation	Protein concentration, aggregation, fragmentation, other impurities (e.g., HCPs)	Protein concentration, aggregation	Aggregation	Protein concentration, aggregation, fragmentation	Protein concentration, aggregation, fragmentation, PTMs, other impurities (e.g., HCPs)
Measurement mode	in-line, on-line	in-line, on-line	in-line, on-line, at-line	in-line, on-line, at-line	in-line	at-line	at-line	at-line
Measurement time	< 1 min	< 1 min	< 1 min[a]	< 1 min[b]	< 1 min	≥ 2 min	≥ 7 min	≥ 10 min
Real-time application	Protein A chromatography, ATPE, precipitation, polishing chromatography, UF/DF	Protein A chromatography, UF/DF, SPTFF	Protein A chromatography, ATPE, precipitation, polishing chromatography, UF/DF	Protein A chromatography, ATPE, precipitation, polishing chromatography, UF/DF	n.a.	n.a.	Polishing chromatography, VI, polishing chromatography	Protein A chromatography
Instrumentation	FlowVPE (Repligen Corporation), UltiMate 3000 DAD (Thermo Fisher Scientific), 1200 series DAD (Agilent Technologies), Smartline 2600 DAD (Knauer Wissenschaftliche Geräte GmbH)	K-Patents PR-43-PC probe (Vaisala), Optilab T-rEX detector (Wyatt Technology Corporation)	HyperFlux Pro Plus spectrometer (Tornado Spectral Systems) with commercial flow cell (Marqmetrix), Rxn2 analyzer (Kaiser Optical Systems) with custom-made flow cell, inVia microscope (Renishaw PLC), QE Pro spectrometer (Ocean Insight) with commercial probe (InPhotonics)	Tensor 27 spectrometer with BioATR II unit with flow cell insert (Bruker), Direct Detect spectrometer (Merck Millipore), Alpha II spectrometer (Bruker)	SpinTrack NMR (Resonance Systems) with custom-made flow tube	Zetasizer Nano ZS 90 (Malvern Panalytical)	DX-800 HPLC system (Dionex Corporation), Acquity UPLC system (Waters Corporation)	MALDI 4800 TOF/TOF spectrometer (SCIEX), Xevo G2-XS Q-TOF spectrometer with Acquity UPLC I-Class system (Waters Corporation)

(Continues)

TABLE 1 | (Continued)

	PAT tools							
	UV/Vis spectroscopy	**Refractive index**	**Raman spectroscopy**	**FTIR spectroscopy**	**NMR spectroscopy**	**Light scattering**	**Liquid chromatography**	**Mass spectrometry**
Cost estimation (EUR)	< 100k	< 50k	> 150k	< 100k	> 150k	< 100k	< 100k	> 200k
Linear product concentration range	0.8–100 g/L	0–100 g/L	0.7–100 g/L	0.7–210 g/L	6.25–50 g/L	1.17–37.5 g/L	0.15–10 g/L	0.001–1 g/L
LOD estimation impurities	0.02 g/L (aggregation), 12 g/L (fragmentation)	n.a.	1 g/L (aggregation), 12 g/L (fragmentation)	0.2 g/L (aggregation), 12 g/L (fragmentation), 700 ng/mL (HCPs)	0.2 g/L (aggregation)	1 g/L (aggregation)	0.02 g/L (aggregation), n.f. (fragmentation)	n.f. (aggregation), n.f. (fragmentation), n.f. (HCPs)
Background matrix	PBS (pH 7.4), 20 mM Na-Pb (pH 6.0), 20 mM Na-Pb 1 M NaCl (pH 6.0), 0.1 M glycine (pH 7.7), 20 mM CitAc (pH 3.6)	10 mM Na-Pb 40 mM NaCl (pH 6.5), 25 mM Na_3–Cit 50 mM NaCl 150 mM L-arginine (pH 5.8), 100 mM glycine-HCl (pH 3.5)	PBS (pH 7.4), 20-50 mM Na_3–Cit 0–0.5 M NaCl (pH 5.8–6.2)	PBS (pH 7.4), 50 mM Na_3–Cit 500 mM NaCl (pH 6.0)	PBS (pH 7.4)	100 mM PBS (pH 7.4), 100 mM Cit 0-100 mM NaCl (pH 3.0), 100 mM Ac 50–100 mM NaCl (pH 3.0), 100 mM glycine 100 mM NaCl (pH 3.0), 60 mM L-histidine HCl (pH 3.0-6.0)	K-Pb, Tris, NaCl Tris	DHAP ACN EtOH 0.1% TFA, ACN 0.1% FA
Data treatment modeling	Data-driven: PLS	n.a.	Data-driven: PLS, CNN, KNN, SVR, PCR; Hybrid: LKM-PLS	Data-driven: PLS	Data-driven: OLR	Data-driven: SVR, NN	Data-driven: OLR	n.a.
Predictive performance	High for protein concentration (R^2 from 0.93 to 0.99), Medium for aggregation (R^2 of 0.80), High for fragmentation (R^2 from 0.95 to 0.97)	n.a.	High for protein concentration (R^2 from 0.93 to 0.99), Low/Medium for aggregation (R^2 from 0.34 to 0.87), Low/Medium/High for fragmentation (R^2 from 0.34 to 0.97)	High for protein concentration (R^2 from 0.91 to 0.99), Medium/High for aggregation (R^2 from 0.82 to 0.94), High for fragmentation (R^2 from 0.94 to 0.98), Low/Medium for	High for protein concentration (R^2 from 0.95 to 0.99), High for aggregation (R^2 from 0.92 to 0.99)	High for aggregation (R^2 from 0.96 to 0.97)	High for protein concentration, aggregation, fragmentation (R^2 of 0.997)	n.a.

(Continues)

TABLE 1 | (Continued)

	PAT tools							
	UV/Vis spectroscopy	**Refractive index**	**Raman spectroscopy**	**FTIR spectroscopy**	**NMR spectroscopy**	**Light scattering**	**Liquid chromatography**	**Mass spectrometry**
				HCPs (R^2 from 0.54 to 0.86)				
Technology readiness	High	High	Medium	Medium	Low	Low	High	High
Scale-up implementation	Yes	Yes	No	No	No	No	Yes	Yes
Sample requirements	n.a.	n.a.	n.a.	n.a.	n.a.	GMP sampling	GMP sampling and may need sample preparation	GMP sampling and need for sample preparation
Robustness	Medium	Medium	Low	Low	High	Medium	High	Medium
Other challenges	Need for improved model calibration to accurately predict aggregation	Sensitive to buffer changes, requiring appropriate matrix subtraction	Sensitive to buffer changes, requiring appropriate matrix subtraction; Need for improved model calibration to accurately predict aggregation and fragmentation	Sensitive to buffer changes, requiring appropriate matrix subtraction; Need for improved model calibration to accurately predict aggregation and HCPs	n.f.	May present limitations under varying protein concentrations	n.f.	n.f.
References	Bhangale et al. (2022); Gillespie et al. (2022); Helgers et al. (2021); Ramakrishna et al. (2022); Rolinger et al. (2021); Rüdt et al. (2017); Zobel-Roos et al. (2017)	Gillespie et al. (2022); Jiang et al. (2017); Walch et al. (2019); Webster et al. (2022)	Feidl et al. (2019); Goldrick et al. (2020); Helgers et al. (2021); Rolinger et al. (2023); J. Wang et al. (2023)	Capito et al. (2015); Großhans et al. (2018); Helgers et al. (2021); Thakur et al. (2021)	Taraban et al. (2019)	Gillespie et al. (2022); Shrivastava et al. (2023)	Dunn et al. (2023); Feidl et al. (2020); Gillespie et al. (2022); Lambiase et al. (2022); Rathore et al. (2010); Rathore, Wood, et al. (2008); Rathore, Yu, et al. (2008)	Dong et al. (2016); Gillespie et al. (2022); Hagman et al. (2008); Steinhoff et al. (2016)

Abbreviations: Ac, acetate; ACN, acetonitrile; ATPE, aqueous two-phase extraction; Cit, citrate; CitAc, citric acid; CNN, convolution neural network; DHAP, 2,5-dihydroxyacetophenone; EtOH, ethanol; FA, formic acid; FTIR, Fourier-transform infrared; GMP, good manufacturing practice; HCl, hydrochloric acid; HCPs, host cell proteins; KNN, k-Nearest Neighbor; K-Pb, potassium phosphate; LKM-PLS, lumped kinetic model-partial least squares; n.a., not applicable; Na_3–Cit, sodium citrate; NaCl, sodium chloride; Na-Pb, sodium phosphate; n.f., not found; NMR, nuclear magnetic resonance; NN, neural network; OLR, ordinary linear regression; PBS, phosphate-buffered saline; PCR, principal component analysis regressor; PLS, partial least squares; PTMs, post-translational modifications; R^2, R-squared; SPTFF, single pass tangential flow filtration; SVR, support vector regressor; TFA, trifluoroacetic acid; Tris, tris (hydroxymethyl)aminomethane; UF/DF, ultrafiltration/diafiltration; UV/Vis, ultraviolet/visible; VI, viral inactivation.

[a]Measurement time might change depending on the excitation laser used.

[b]Measurement time might change depending on the number of scans selected.

0.8–100 g/L. Additionally, Zobel-Roos et al. (2017) demonstrated the possibility of predicting IgG monomer and dimer concentrations using PLS modeling. The DAD is usually commercialized as part of a high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) system and, therefore, it is often available in (bio)analytical laboratories. The detector can be detached from the liquid chromatography (LC) unit and used separately, which makes it a convenient option. Furthermore, an example of its application in a DSP cascade was demonstrated by Helgers et al. (2021), where a DAD was used to monitor multiple unit operations, including ATPE, precipitation, integrated counter current chromatography (ICCC), and UF/DF. It should be noted that the precipitation unit employed a two-step approach, combining precipitation and dissolution. The former used the light phase (LP) of the ATPE unit together with a precipitating agent to induce mAb precipitation. The latter was accomplished by mixing the recovered mAb precipitates, obtained using a hollow fiber module, with a buffered solution. Hence, the DAD was utilized exclusively to monitor dissolved components. The multi-wavelength UV/Vis spectroscopy method enabled the characterization of protein concentration, aggregation, and fragmentation (Helgers et al. 2021).

In short, UV/Vis spectroscopy combined with multivariate statistical models, namely PLS, is a suitable method for in-/on-line quantitative measurements (Brestrich et al. 2018; Helgers et al. 2021; Ramakrishna et al. 2022; Rolinger et al. 2021; Rüdt et al. 2017; Zobel-Roos et al. 2017). An acquisition time of 0.01–30 s is typically achieved (Rüdt et al. 2017), which supports real-time monitoring and control of mAb monomers, protein aggregation, and fragmentation over a wide range of concentrations. This PAT tool is typically integrated in chromatographic separations and used for automated pooling decisions but could also be applied to column switching in continuous chromatography applications (Brestrich et al. 2018). Moreover, it was shown to be effective in a continuous DSP cascade to monitor multiple unit operations (Helgers et al. 2021). Future challenges for this technology are associated with method robustness because of the employed statistical models, which require extensive calibration (Brestrich et al. 2018; Rüdt et al. 2017).

2.2 | Refractive Index (RI)

RI measures the bending of a light path that occurs due to refraction when light travels through a medium, such as a protein solution, which arises from a change in the speed of the light rays. The RI changes proportionally with the concentration of a solute in the bulk solution and it can be used to monitor the concentration of a wide range of proteins. RI measurements over a concentration range results in protein-specific linear slope responses, which allows it to be applied in several industries, namely sugar, chemical, and biotechnology (Webster et al. 2022; Zhao et al. 2011).

For mAbs, in-line RI was applied to monitor protein bulk concentrations in the retentate and permeate stream during UF/DF and single pass tangential flow filtration (SPTFF) operations (Webster et al. 2022). A K-Patents PR-43-PC RI probe with a temperature-controlled flow cell (Vaisala, Vantaa, Finland) was used, allowing for measurements every second. Off-line mAb concentrations were measured using a SoloVPE at 280 nm (Repligen Corporation, Massachusetts, US). IgG1 and IgG4 concentrations were quantified throughout the UF/DF step, both with initial concentrations of 5 g/L, reaching concentration targets from 10 to 30 g/L after the first UF/DF and 30–100 g/L after the second UF/DF. Initial concentrations of IgG1 and IgG4 of 5 and 15 g/L were employed during the SPTFF step, respectively, with a target volumetric concentration factor (CF) of 3. Product concentrations were calculated by correcting the RI signal of the retentate for the contributions of the buffers, using the permeate RI as reference. This indicates that RI is highly sensitive to buffer changes and therefore requires appropriate matrix subtraction (Jiang et al. 2017; Walch et al. 2019). Mean absolute percentage error (MAPE) values for the IgG1 and IgG4 concentration estimates ranged from 2.25% to 3.29% and 4.44% to 7.64% during UF/DF, and reached 5.01% and 25.27% during SPTFF, respectively. The high error observed for the IgG4 SPTFF concentration prediction was attributed to protein fouling at the surface of the RI probe, leading to an overestimation of product concentration. Hence, probe placement needs to be carefully considered within the process to ensure accurate sensor readings, which should be done with adequate fluid mixing (Webster et al. 2022). Another application of RI for mAbs was reported in a multi-sensor proof-of-principle study, including on-line prediction of mAb concentration during a Protein A chromatography step (Walch et al. 2019). An Optilab T-rEX RI detector (Wyatt Technology Corporation, California, US) was integrated in an ÄKTA Pure 25 system (Cytiva, Uppsala, Sweden). Enhanced CQA quantification was achieved through a combination of sensors including attenuated total reflectance Fourier-transform infrared (ATR-FTIR), fluorescence, and multi-angle light scattering (MALS), along with a conventional triple wavelength UV detector (214, 260, and 280 nm), pH, and conductivity probes.

2.3 | Raman Spectroscopy

Raman spectroscopy relates to the interaction of electromagnetic radiation with matter, which is associated with molecular vibrations. Upon light-matter interaction, a small fraction of the incoming laser light undergoes inelastic scattering. Inelastic scattering can be classified as Stokes or anti-Stokes, where the wavelength of the scattered light becomes longer or shorter than the wavelength of the incident laser, respectively. The loss or gain of energy corresponds to molecular vibrations, such as bending, stretching, and twisting. Large molecules, such as proteins, exhibit different vibrations associated with specific bonds within the molecule (Esmonde-White et al. 2022; Vandenabeele 2013). The amide bands, including amide A, B, and I–VII, arise from vibrations of the peptide bond, i.e., CONH group. More specifically, the amide I, corresponding to the stretching vibration of C=O, and the amide II and III, associated with coupled C–N stretching and N–H bending vibrations, are of particular interest in protein conformational studies, particularly with respect to secondary structure (Rygula et al. 2013). Furthermore, Raman spectroscopy can provide information regarding amino acid side chains and disulfide bonds, which are linked to the tertiary structure of proteins (Kuhar et al. 2021).

Wang et al. (2023) reported Raman spectroscopy-based regression models that enable real-time monitoring of mAb size variants, namely aggregation and fragmentation. These regression models include convolution neural network (CNN), k-Nearest Neighbor (KNN), support vector regressor (SVR) with a nonlinear radial basis function kernel, principal component analysis regressor (PCR), and PLS regressor. A protein A chromatographic step with a 60 mg/mL column load density was performed on an ÄKTA Avant 150 system (Cytiva, Uppsala, Sweden). Moreover, a HyperFlux Pro Plus Raman spectrometer (Tornado Spectral Systems, Ontario, Canada) and a commercially available flow cell with a flow path volume of 200 μL (Marqmetrix, Washington, US) were utilized. Measurements were carried out in-line and data acquisition was achieved every 38 s. SEC was employed as a reference analytical technique to analyze size variants. In the test dataset used for the regression models, aggregate content ranged from 1.3 ± 0.2 to 5.9 ± 0.4%, and fragment content ranged from 1.9 ± 0.05 to 5.4 ± 0.7%. The KNN model outperformed all other regression models in quantitatively predicting aggregation and fragmentation, revealing the lowest mean absolute error (MAE) values. More specifically, a MAE of 0.53% was obtained for HMW species and 0.35% for low molecular weight (LMW) species, proving the applicability of such a model for in-line monitoring of the eluate from a protein A chromatographic step (J. Wang et al. 2023).

In addition, a hybrid modeling study was conducted to integrate Raman spectroscopy into a protein A chromatographic system (Feidl et al. 2019). This type of model combines mechanistic modeling based on deterministic knowledge derived from physical, chemical, or biological principles with data-driven modeling, such as PLS. A flow cell was connected to a Kaiser Raman Rxn2 analyzer (Kaiser Optical Systems, Michigan, US) and a ChromaCon CUBE chromatography system (ChromaCon, Zurich, Switzerland). Breakthrough curves from a low titer harvest of < 0.42 mg/mL, typical for perfusion bioreactors, were monitored in-line through the estimation of mAb concentration. An extended Kalman filter (EKF) was applied to estimate antibody concentration by combining the information of the mechanistic lumped kinetic model (LKM) with the real-time information derived from Raman-based PLS. An RMSEP of 0.026 mg/mL was obtained when comparing the predicted mAb concentrations with off-line measurements obtained by protein A chromatography. Moreover, the RMSEP value was comparable to the analytical standard deviation of 0.01 mg/mL of the off-line technique, indicating that the prediction error is similar to the variability of the reference measurements. It is important to carefully consider the detection limits of Raman spectroscopy, which can become a bottleneck at low protein concentrations as applied in the mentioned study. Nonetheless, it was shown that this technique is sufficient to monitor and control a protein A chromatographic step with a 70% breakthrough, approaching column saturation (Feidl et al. 2019).

The use of high-throughput (HT) Raman spectroscopy was analyzed by Goldrick et al. (2020) as an option to circumvent the high cost associated with individual probes and the difficulty of measuring low sample volumes. Specifically, a Renishaw inVia Raman microscope (Renishaw PLC, Gloucestershire, UK) was combined with a cation exchange (CEX) chromatographic step at-line. The chromatographic experiments were performed with an ÄKTA Avant (Cytiva, Massachusetts, US). The total protein concentration and monomer purity of an Fc-fusion protein were investigated under different buffer conditions. Concentration ranges of 0.7–19.41 mg/mL and 1.7–33.20 mg/mL were used for the product concentrations in the two test datasets. Monomer purity varied from 70% to 100%, where HMW and LMW species each reached up to 20%. PLS models were employed for data analysis, revealing accurate predictions of total protein concentration with R^2 values ≥ 0.98. Also, RMSEP values of 1.09 and 3.62 mg/mL were obtained comparing total protein concentrations predicted by the model with reference values measured using off-line SEC. However, monomer purity predictions resulted in low R^2 (0.86 and 0.34) and high RMSEP values (4.27% and 13.68%). The model predictions are significantly worse for the second tested dataset, likely because most samples had an aggregate content below 5%, which corresponds to concentrations of around 0.03–1.66 mg/mL. The authors stated that, based on previous experiments, the LOD is about 1 mg/mL. Consequently, a significant portion of the aggregate content in the samples was likely below the LOD and the sensitivity of the set-up was insufficient to detect these HMW species. Furthermore, the elution buffer was changed for the second tested dataset, potentially resulting in alterations to the background spectra due to the modified sample matrix. These two reasons may have contributed to lower accuracy in monomer purity predictions for the second dataset (Goldrick et al. 2020).

Multiple studies report the use of Raman spectroscopy as a PAT tool, enabling in-, on-, or at-line quantitative measurements (Feidl et al. 2019; Goldrick et al. 2020; J. Wang et al. 2023). While most studies report its application in monitoring chromatographic steps, its use within a continuous DSP cascade, including ATPE, precipitation, ICCC, and UF/DF, was also demonstrated (Helgers et al. 2021). As mentioned before, this study incorporated a two-step approach where precipitation and dissolution were combined. Therefore, Raman spectroscopy was employed solely to monitor dissolved components. Various CQAs, such as protein concentration, aggregation, and fragmentation, can be characterized using this technique. A measurement time of around 38 s can be achieved (J. Wang et al. 2023), but the acquisition time ultimately depends on the excitation laser used (Rüdt et al. 2017). Additionally, the use of Raman spectroscopy for future monitoring of other CQAs, including HCPs and PTMs, was suggested by Wang et al. (2023) and McAvan et al. (2020), respectively. Several chemometric models were proven to be applicable for analyzing Raman spectroscopy data, ranging from a PLS regression model (Goldrick et al. 2020) to other ML approaches, such as CNN, KNN, SVR, PCR (J. Wang et al. 2023), and mechanistic models, namely LKM combined with PLS (Feidl et al. 2019). The major challenges with regards to Raman spectroscopy are related to background matrix correction, detection limits, and technology adoption in late-stage biopharmaceutical process development and commercialization (Feidl et al. 2019; Goldrick et al. 2020).

2.4 | FTIR Spectroscopy

FTIR spectroscopy is based on the absorption of infrared radiation (IR), triggering vibrational transitions by the change in the molecular dipole moment, leading to a spectroscopic fingerprint

of the analyzed sample. These transitions depend on the strength and polarity of the vibrating bonds, subject to intramolecular and intermolecular interactions, as seen for Raman spectroscopy. The IR region is subdivided into near-infrared (NIR, 750 nm to 2.5 μm), mid-infrared (MIR, 2.5–50 μm), and far-infrared (FIR, 50–1000 μm). FTIR became an established technique for the characterization of proteins, based on nine distinct infrared (IR) absorption bands, i.e., amide A, amide B, and amide I–VII. Specifically, the amide I band, positioned at a wavenumber of around 1650 cm^{-1}, and the amide II band, around 1550 cm^{-1}, are the most prominent bands, which provide information about the secondary structure of proteins (Barth 2007; Kafle et al. 2023). Moreover, the ability to study a protein's tertiary structures was reported, through the analysis of disulfide bridges and amino acid residues, such as tyrosine and tryptophan (Mankova et al. 2022).

Großhans et al. (2018) described the use of ATR-FTIR for in-line monitoring of a protein CEX chromatography. Two PLS models were optimized to selectively distinguish and quantify mAb and lysozyme. Spectroscopic measurements were conducted using a Tensor 27 FTIR spectrometer with a BioATR II unit (Bruker, Ettlingen, Germany), including a flow cell insert and a seven-reflections silicon crystal, connected to an ÄKTA chromatographic system (GE HealthCare, Buckinghamshire, UK). Fractions from preparative CEX chromatography were analyzed off-line using an HPLC with an ion exchange column. Spectra were continuously acquired in the chromatographic system with a measurement time of 3.23 s. A high concentration range was covered, i.e., 35–75 g/L and 40–115 g/L of mAb and lysozyme, respectively. The PLS models showed accurate results and enabled the identification and quantification of the two proteins based on their secondary structure. Particularly, R^2 values of 0.92 and 0.99, as well as RMSEP values of 2.42 and 1.67 g/L were obtained for mAb and lysozyme, respectively (Großhans et al. 2018).

Another application of FTIR spectroscopy was aimed at monitoring samples from different DSP unit operations, including protein A chromatography, chromatography-based polishing steps, and UF/DF (Capito et al. 2015). Multiple CQAs were monitored, namely HCPs, mAb monomer, and mAb aggregates. An at-line Direct Detect FTIR spectrometer (Merck Millipore, Massachusetts, US) with a deuterated triglycine sulfate (DTGS) detector was used. Sample preparation was required as the described technique made use of a filter card membrane, where the samples were dried to ensure that no residual water was present. PLS models were applied to analyze the spectral data, which were calibrated to minimize sample matrix effects of the buffers used in the DSP unit operations. UV spectroscopy at 280 nm and protein A chromatography were used as reference analytics to predict mAb concentration. Moreover, SEC was applied to characterize aggregated mAb species, and enzyme linked immunosorbent assay (ELISA) was used to detect the HCP content. FTIR enabled the quantification of mAb over a concentration range of 0.7–26.8 mg/mL, HCPs from 0 to 3000 ng/mL, and aggregate content from 0% to 5.4%. The prediction of mAb concentration showed R^2 values ranging from 0.95 to 0.99, as well as RMSEP values from 0.640 to 0.243 g/L. For the HCP estimation, R^2 values between 0.54 and 0.86, and RMSEP values from 587 to 288 ng/mL were obtained. The low predictive performance may be attributed to the inability of FTIR to detect low concentrations of HCPs present in the samples. Hence, the proposed HCP quantification approach is likely limited to early unit operations in the purification cascade, such as protein A chromatography, where residual HCP levels typically vary from several hundred to several thousand ppm (approximately 10^5–10^6 ng/mL). The current limit of quantification for HPCs was stated to be around 700 ng/mL. Lastly, R^2 values ranging from 0.82 to 0.91, and RMSEP values from 0.579% to 0.383% for the quantification of mAb aggregates were reported (Capito et al. 2015).

Helgers et al. (2021) reported an application of FTIR for in-/on-line multiple-parameter monitoring, including mAb monomer, HMW impurities, and LMW impurities. An alternative DSP cascade composed of ATPE, precipitation, ICCC, and UF/DF was used, where FTIR was applied exclusively to monitor dissolved components. Data were acquired using an Alpha II FTIR spectrometer (Bruker, Massachusetts, US), set to automatically average a total of 24 spectra, each with 1 s of integration time. The spectral data was used to correlate changes in component concentration with the spectral intensity of the amide I region using PLS regression. Protein A chromatography for mAb concentration estimation and SEC for size variant characterization were conducted as reference off-line techniques. Protein monomer concentrations ranged from 0.3 to 20 g/L throughout the DSP platform process. Additionally, HMW and LMW species concentrations were around ≤ 2 and ≤ 22 g/L, respectively. During the ATPE step, R^2 values were 0.94, 0.94, and 0.98 with RMSEP values of 0.167, 0.013, and 0.5 g/L for mAb, HMW, and LMW concentrations, respectively. On the precipitation flowthrough, R^2 values of 0.91 and 0.94 as well as RMSEPs of 0.03 and 0.43 g/L were obtained for the estimation of mAb monomer and LMW species, respectively. As for the chromatographic step, an R^2 value of 0.92 with an RMSEP of 0.24 g/L was reported for the determination of the mAb monomer concentration. Lastly, after the UF/DF step, mAb concentration was determined with an R^2 of 0.95 and an RMSEP of 1.09 g/L (Helgers et al. 2021).

Several in-, on-, and at-line quantitative applications of FTIR spectroscopy for multi-parameter monitoring in different steps of the downstream cascade were demonstrated, enabling the characterization of CQAs, such as product concentration, size variants, and HCPs (Capito et al. 2015; Großhans et al. 2018; Helgers et al. 2021). In addition to CQAs, an in-column FTIR spectroscopic setup to evaluate cleaning-in-place (CIP) protocols and protein A resin fouling was reported in a protein A chromatographic step (Boulet-Audet et al. 2016). A single scan may require a time frame of 0.5–4 s, with the total acquisition time corresponding to the sum of all specified scans (Rüdt et al. 2017). In terms of data analysis, the most applied quantification model for FTIR spectra is PLS regression. Challenges of FTIR spectroscopy are related to background matrix correction, detection limits (Capito et al. 2015), and setup compatibility with the production environment, including devices without liquid nitrogen cooling and the application of in-line fiber optic probes (Großhans et al. 2018).

2.5 | Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy relies on the principle that atoms have electrically charged nuclei. When an external magnetic field is

supplied, energy transfer occurs from lower to higher energy levels through absorption at a specified radio frequency. This radio frequency varies depending on different nucleus-related factors, namely its type (e.g., ^{1}H, ^{13}C, or ^{15}N), chemical environment, and typical location in the magnetic field when the field is not uniform (Singh and Singh 2022). Having in mind its application to biomacromolecules, NMR spectroscopy enables the characterization of its atomic-level structure (i.e., amino acid residues). Furthermore, the NMR peak intensity can be correlated with the relaxation of nuclear spin, corresponding to the equilibrium state after perturbation by an external magnetic field, which provides information on protein structure dynamics (Ma et al. 2023).

Taraban et al. (2019) investigated the use of NMR spectroscopy for in-line monitoring of protein concentration and aggregate content. Specifically, water NMR (wNMR) was used, which is a subtype of ^{1}H NMR. In wNMR, the signal originates from water protons rather than the entire sample, as in conventional ^{1}H NMR, where all ^{1}H nuclei are detected. This article does not report on the direct application of NMR as a PAT tool during a downstream process, but rather proof of its potential. Forced degradation studies by thermal stress were performed to induce protein aggregation. The protein concentration ranged from 6.25 to 50 g/L, obtained through sequential dilutions, while the aggregated species content ranged approximately from 1% to 8%. SEC was used as reference method for the quantification of mAb aggregates. A custom-made flow NMR was used to analyze changes of the proton transverse relaxation rate [$R_2(^{1}H_2O)$]. The setup included a benchtop wide through-bore time-domain low-field SpinTrack NMR (Resonance Systems, Kirchheim unter Teck, Germany) with a resonance frequency of 15.9 MHz. The proton transverse relaxation time, $T_2(^{1}H_2O)$, which is the inverse of $R_2(^{1}H_2O)$, corresponds to the time it takes for the NMR signal to decay after excitation, which is influenced by the molecular environment of water protons. Ultimately, this information can be translated into the protein concentration and aggregation, since the abundance in the sample influences the relaxation behavior of water protons. Different flow rates, up to 50 mL/min, were tested to mimic the settings used for continuous biomanufacturing, and the total measurement time was about 50 s for each $R_2(^{1}H_2O)$. Linear relationships were found for the protein concentrations when plotted against its corresponding $R_2(^{1}H_2O)$ values for the distinct flow rates tested, resulting in R^2 values ranging from 0.95 to 0.99. Similarly, for the aggregate content a linear fit was reported with R^2 values between 0.92 and 0.99. Furthermore, the results showed that the investigated setup is sensitive to quantify small changes in protein concentration ($\pm$ 1 mg/mL) and aggregate content ($\pm$ 1%) (Taraban et al. 2019). This proof-of-concept study indicated how commercially available benchtop NMR spectroscopy can be integrated in DSP as a potential in-line PAT tool for quantitative monitoring of mAb products and product-related impurities. Nevertheless, more research is needed to demonstrate real-time monitoring and/or control in DSP unit operations.

2.6 | Light Scattering (LS)

The working principle of LS is based on the interaction that occurs between particles and incident light. Two different mechanisms can take place, namely absorption or scattering. Particles can scatter light through reflection, refraction, and/or diffraction (Diebold et al. 2022). In colloidal suspensions, such as samples of HMW biotherapeutics (e.g., mAbs), light can be scattered in different directions depending on the Brownian motion of particles in solution. Therefore, scattering data contains valuable information that can be translated into particle sizes, orientation, and position coordinates (Dhont 1996). For quantitative characterization of biopharmaceuticals, two different LS techniques are typically applied. First, static light scattering (SLS) or MALS measures the intensity of the scattered light as a function of the angle. The second technique is dynamic light scattering (DLS) or quasi-elastic light scattering (QELS), where time-dependent fluctuations in the scattered light signal are recorded. LS is used to determine protein molecular weight and concentration, as well as the particle size distribution (Shrivastava et al. 2023; Some and Kenrick 2012).

Shrivastava et al. (2023) coupled DLS with ML for the rapid estimation of size-based heterogeneity in mAb samples. This application of LS for the characterization of biopharmaceuticals is not a direct PAT implementation, rather it is proof of its capabilities that may lead to future application in DSP. The proposed at-line DLS-ML technique allowed for a 2-min acquisition time. ML algorithms, namely support vector machine (SVM) and neural network (NN), were used to predict the amount of monomer and multimer species, including dimers, trimers, and tetramers, within a size range of 10–100 nm. A Zetasizer Nano ZS 90 equipped with a He–Ne laser (Malvern Panalytical, Worcestershire, UK) was used to acquire the complete particle size distribution based on the scattered light intensity. Aggregation was induced through pH, thermal, and/or photo stress, leading to mAb aggregate concentrations ranging from 1 to 21 mg/mL. SEC was used as the reference analytics for aggregate quantification. The DLS-ML results showed R^2 values of 0.97 and 0.96 with RMSEP values of 5.20% and 6.75% for NN and SVR algorithms, respectively (Shrivastava et al. 2023). The application of this novel approach combining LS and ML algorithms demonstrated the suitability of DLS for the accurate quantification of the aggregate content. Nevertheless, LS can present some limitations, such as lack of particle identification, as well as multiple scattering and particle number fluctuations, leading to poor resolution, reproducibility, and accuracy in determining the content of monomers and multimers, particularly under varying protein concentrations (Patel et al. 2018; Sharma et al. 2023). Further exploration of data-driven modeling approaches combined with LS, and the integration with control strategies, is of high interest to reach the full potential for quantitative mAb monitoring.

2.7 | Liquid Chromatography

LC allows for high resolution measurements of the molecular weight of mAbs, as well as the identification of size variants, charge variants, and other impurities, such as host cell DNA (Gilardoni and Regazzoni 2022). There are several LC modes, such as size exclusion, affinity, ion-exchange, reversed-phase, and adsorption chromatography. Each LC mode is based on a distinct basic principle to separate analytes, either by size or specific biochemical interactions, including electrostatic and binding interactions (Meyer 2004a, 2004b). LC is fundamentally

a separation technique, usually coupled to optical detectors, where UV and RI are commonly used detectors (Yeung and Synovec 1986).

Rathore, Yu, et al. (2008) developed a SEC setup for real-time chromatographic pooling decision-making. The feasibility of using a commercially available process analyzer was demonstrated, using a DX-800 HPLC system (Dionex Corporation, Sunnyvale, CA) in combination with an ÄKTA Explorer process chromatography system (GE Healthcare, Uppsala, Sweden) equipped with a hydroxyapatite (HA) column. The HA column was loaded at a protein concentration from 20 to 30 g/L and fractions with a purity profile varying from 5% to 100% were collected. This technique is mostly suited for at-line integration, as samples were withdrawn from the process for analysis in a continuous fashion but not returned to the process stream. The described system enabled an 11-min SEC assay with UV absorbance detection. As a means of comparison, a reference off-line SEC assay tool using an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) was also performed, leading to a 30-min measurement time. The comparison of the estimated purity from the two methods (i.e., at-line and off-line SEC) resulted in an R^2 of 0.997, revealing a high degree of correlation (Rathore, Yu, et al. 2008). Moreover, the possibility of scaling up this approach was studied by Rathore et al. (2010), making use of a pilot plant and a manufacturing plant that involved a 2294- and 45930-fold scale-up, respectively. It was possible to conclude that the system delivers consistent pool purity, highlighting the scalability of the at-line HPLC tool (Rathore et al. 2010).

Another study reported on the feasibility of a different commercially available analytical tool, namely an Acquity UPLC system (Waters Corporation, Massachusetts, US) (Rathore, Wood, et al. 2008). It enabled real-time pooling decisions during a preparative polishing step, where the UPLC was integrated with an ÄKTA Explorer 100 chromatographic system (GE Healthcare, Uppsala, Sweden). An assay time of 7 min was achieved using UV absorbance detection. However, the UPLC system could not withdraw samples continuously and it would, therefore, need an external sampling device. This poses a potential adoption hurdle, as automated sampling devices are not widely accepted by the biopharmaceutical community, mainly due to GMP concerns (Jenzsch et al. 2018). Nevertheless, multiple commercially available options for automated sampling are available, including the BaychroMAT (Bayer Technology Services, Leverkusen, Germany), MAST (Merck Millipore, Massachusetts, US), Numera (Securecell AG, Urdorf, Switzerland), and SegFlow (Flownamics, Wisconsin, US) (Hofer et al. 2020; Jenzsch et al. 2018; Klutz et al. 2015; Wasalathanthri et al. 2020).

The use of LC coupled to optical detectors as at-line quantitative PAT tool for mAb characterization was demonstrated, where this technique supported pooling decisions for preparative chromatography at different production scales (Rathore et al. 2010; Rathore et al. 2008; Rathore, Yu, et al. 2008). Moreover, a holistic approach showcasing the application of at-line HPLC to a whole USP/DSP cascade was reported in literature (Feidl et al. 2020). This included a continuous manufacturing platform with a perfusion bioreactor, two-column protein A chromatography unit, viral inactivation (VI), and two chromatography-based polishing steps. There are several relevant commercially available solutions, both in terms of analytical equipment as well as valves and sampling devices to adapt pre-existing off-line systems. For example, Tiwari et al. (2018) showed how to transform an off-line existing HPLC into an at-line system through the integration of a software controlled two position/six port valve, namely a 1260 infinity valve drive (Agilent Technologies, Santa Clara, CA) (Tiwari et al. 2018). It is important to note that LC might not be suitable for DSP applications that demand very short data acquisition times of seconds to a few minutes, as the average reported measurement time is in the order of tens of minutes. Hence, its application should be carefully considered having in mind the requirements for process monitoring and/or control.

2.8 | Mass Spectrometry (MS)

MS enables the analysis of several product characteristics, including molecular weight, amino acid sequence, and PTMs, such as glycosylation, oxidation, and deamidation. Furthermore, MS is useful to analyze process-related impurities, namely HCPs (Rogstad et al. 2017). Mass spectrometers measure the mass-to-charge (m/z) ratio of analytes such as peptides, peptide fragments, or proteins (Mann et al. 2001). There are three different working principles that can be employed to accomplish the mass separation, namely separation by quadrupole electric fields generated by metal rods (quadrupole MS), separation on the basis of time-of-flight (TOF MS), and separation by selective ejection of ions from a three-dimensional trapping field (ion trap or Fourier-transform ion cyclotron MS) (Mann et al. 2001). The most used ionization methods that allow gentle ionization of large biomolecules include electrospray (ES) and matrix-assisted laser desorption ionization (MALDI), both of which can be coupled to any of the above-mentioned methods of separation (Mann et al. 2001).

Steinhoff et al. (2016) described the use of an at-line HT microarray-based MALDI-TOF MS to quantitatively determine light chains and heavy chains, with an acquisition time of < 1 min, excluding sample preparation time. It enabled the study of antibody integrity and the presence of HCP contaminants, such as histones and ribosomal proteins. This application of MS is not a direct PAT implementation yet, rather it is a proof of its capabilities for the characterization of biologics, which could result in future application in DSP. Here, a MALDI 4800 TOF/TOF mass spectrometer (SCIEX, Massachusetts, US) and a silicon wafer (Si-Mat Silicon Materials, Landsberg, Germany) for the microarray were utilized. Protein A chromatography and SEC were used as reference analytics to determine mAb concentration and aggregate content. For the extracellular mAb content characterization, a 6-day batch and perfusion reactor were used, allowing for a mAb concentration ranging from 0.02 to 0.25 g/L and an average concentration of 0.2 ± 0.07 g/L, respectively. These results were determined using both MALDI-MS and analytical protein A chromatography, revealing similar results. Moreover, it was found that MALDI-MS lead to an increasing detection of mAb aggregates (4.4%) compared to the reference off-line analytical tool (1.8%), which could be attributed to protein adsorption to the SEC column or the crystallization process during MALDI sample preparation (Steinhoff et al. 2016).

Other work demonstrated a HT and automated purification platform using LC-MS (Dong et al. 2016). A Xevo G2-XS Q-TOF

mass spectrometer (Waters Corporation, Massachusetts, US) coupled with an Acquity UPLC I-Class system (Waters Corporation, Massachusetts, US) was used as an at-line quantitative analytical tool for monitoring PTMs, such as protein glycosylation and glycation. Prior to the LC-MS analysis, a protein A chromatographic step was performed coupled to an automated liquid handling workstation, particularly a Freedom EVO (Tecan Trading AG, Männedorf, Switzerland). The purified samples were subsequently analyzed via LC-MS, comprising of a 10-min reversed-phase (RP) UPLC, to separate light chains and heavy chains of mAbs, and a reduced mass analysis for the characterization of the heavy chains glycoforms. Regarding the glycosylation study, it was found that some glycoforms in the mAb Fc domain, namely G0F and G1F, varied during cell culture. More specifically, G0F values ranged from 55–60 to 85%, and G1F from 35 to 6%–11% under all DoE conditions, where pH, dissolved oxygen, glucose target, temperature, and seeding density were varied. Additionally, glycation levels that resulted from the sum of three antibody domains, light chain, Fd′, and scFc, were evaluated. Values ranged from 7% to 9% on Day 0, decreasing under all tested conditions until Day 8 of cultivation. In the beginning of Day 8 of cultivation, changes in glycation levels were directly correlated with changes in the glucose feed target. Furthermore, the authors suggested the feasibility of such a technique for real-time feedback control strategies, provided the models are further optimized to capture the relationships between CQAs and critical process parameters (CPPs). More specifically, the PAT tool output (e.g., glycation level) could be used to detect shifts in product quality, enabling adjustments to CPPs, namely glucose feeding strategies, thereby ensuring consistent product quality (Dong et al. 2016).

In summary, MS is a relevant tool for at-line quantitative characterization of mAbs (Dong et al. 2016; Steinhoff et al. 2016). A measurement time of less than 1 min can be achieved with MALDI-MS, excluding sample preparation time (Steinhoff et al. 2016), and around 10 min with LC-MS (Dong et al. 2016). However, the required sample preparation for MS-based analysis, such as the proteolytic digestion of mAb samples, is a complex and time-consuming step that may introduce sample variability (Wojtkiewicz et al. 2021). An emerging alternative for sample preparation was demonstrated by Toth et. al (2019), where continuous on-column digestion was achieved as proteins passed through the stationary phase (Toth et al. 2019). However, this is a complex technology that can lead to problems regarding the life cycle assessment of the column and method validation (Wasalathanthri et al. 2020).

3 | Discussion and Outlook

A thorough analysis of quantitative PAT applied for the detection and characterization of mAbs in continuous DSP applications was performed in this review. Table 1 in combination with an exemplary decision tree (Figure 3) can help prioritize PAT tool(s) when considering key analytical requirements for continuous DSP processing, namely the measurement mode (in-/on-/at-line), CQAs, scale-up implementation, predictive performance, and cost. The focus of this review is on mAb quantification, but the content may be applied to other protein-based therapeutics. Moreover, knowledge of PAT tools applied in other biopharmaceutical products may constitute an important asset on the investigation and development of future PAT-related applications for mAbs. The cumulative information gathered in this review highlights several knowledge gaps and

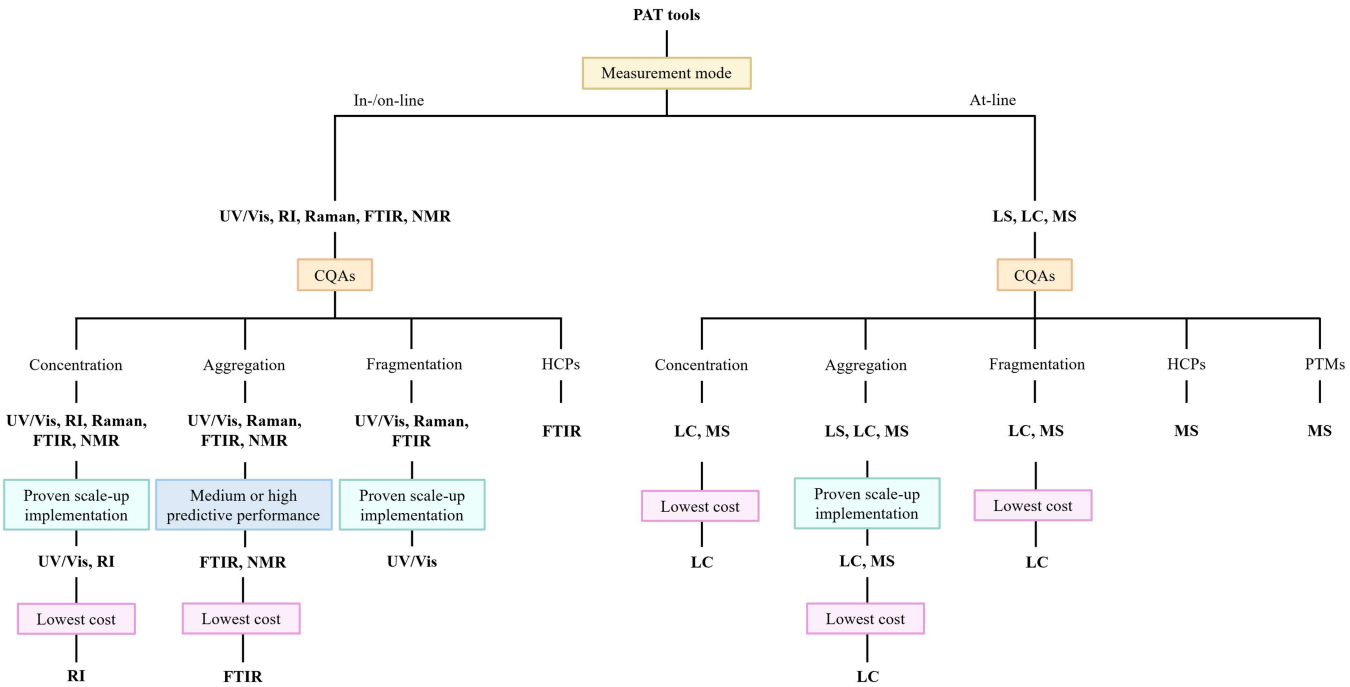

FIGURE 3 | Exemplary decision tree for the selection of quantitative process analytical technology (PAT) for the application in continuous downstream processing (DSP) of monoclonal antibodies (mAbs). It features key parameters to enable continuous processing, namely measurement mode (in-/on-/at-line), critical quality attributes (CQAs), scale-up implementation, predictive performance, and cost. FTIR, Fourier-transform infrared; LC, liquid chromatography; LS, light scattering; MS, mass spectrometry; NMR, nuclear magnetic resonance; UV/Vis, ultraviolet/visible.

challenges in the field of PAT for continuous DSP of mAbs, which will be discussed in more detail in this section.

PAT applications in DSP are mostly reported for chromatographic steps in literature, namely protein A capture and polishing. Therefore, expanding research to include additional conventional mAb DSP unit operations, such as VI, viral filtration (VF), and UF/DF, alongside emerging continuous downstream alternatives, namely ATPE and precipitation, could further support the transition towards end-to-end continuous DSP. This is also important from an analytical perspective, as matrices and the dynamic range of analytes vary significantly throughout the DSP train. Only assessing the performance in chromatography unit operations does not provide a holistic view on the potential of PAT to enable continuous DSP.

Spectroscopy is the most widely applied type of PAT tool due to the non-destructive nature and fast measurements of seconds to a few minutes. However, a commonly described bottleneck is the detection limit of spectroscopic tools, where product and/or contaminant concentration thresholds cannot be detected. Innovations within the optical engineering field could foster the commercialization of equipment with improved light sources, internal circuits, and detectors, thereby increasing the detection range. On the other hand, techniques such as LC and MS, or combinations thereof, should not be overlooked as these present advantages, namely reduced matrix interference and minimal data modeling requirements. Therefore, research and development efforts should focus on minimizing LC and/or MS measurement times and extending its (near) real-time application, also by including unit operations beyond chromatography.

PAT applications reported in literature were mainly conducted in small-scale (lab-scale) setups, lacking a demonstrated application in a scaled-up system and/or GMP environment, such as pilot- or manufacturing-scale operations. Hence, even though PAT was introduced in the early 2000s, its industrial implementation remains notably limited for IPC and RTRT strategies. This can be attributed to a conservative environment, driven by the increasingly competitive nature and regulatory hurdles, where the latter includes the absence of clear guidelines for managing process deviations, equipment failures, and out-of-specification products produced via continuous manufacturing, as well as limited guidance on regulatory approvals for multivariate methods and artificial intelligence (AI)-based control systems. While cost reduction and faster market entry are essential for commercial success of mAb products, it also restricts investments in advanced analytical and manufacturing technologies at production scale. This may be interpreted as counterintuitive, as multiple economic analyses and case studies published projected reductions of $>80\%$ in capital costs and $>60\%$ in cost of goods when shifting from batch to continuous manufacturing (Rathore et al. 2023). A potential way to overcome this challenge is to strengthen collaboration between academia and industry to assess PAT tool feasibility in industry-relevant conditions. This could be done using representative product or reference material, e.g., NISTmAb (Schiel and Turner 2018), and process specifications, as well as sharing expert knowledge on the GMP requirements and optimization possibilities within an existing or new platform process. Another solution would be the publication of additional case studies by regulatory bodies, where effective implementation of PAT for continuous biomanufacturing could be demonstrated. The interaction between industry, regulatory, and academia represents a powerful combination that could unlock the full potential of continuous manufacturing for biologics (Rathore et al. 2023).

PAT for continuous manufacturing requires integration of hardware and software, resulting in the need of proper IT infrastructure for handling large datasets and in-house expertise on model building, including mechanistic, data-driven, and/or hybrid modeling. Regarding analytical equipment that requires calibrated quantification models, namely UV/Vis, Raman, and FTIR spectroscopy, it is important to note that calibration often leads to resource issues as recalibration may be needed for different products, changes in impurity profiles, and/or the variety of matrices per unit operation. This results in a demand for generalized quantification models and in silico spectral generation that could reduce the calibration burden for platform processes. Moreover, the development of hardware–software frameworks is tightly connected to the concept of Industrial Internet of Things (IIoT), which contributes to the Industry 4.0 (I4.0) paradigm shift. This is often referred to as Bioprocessing 4.0 in the biomanufacturing industry and it represents the evolution of bioprocesses towards manufacturing systems with increased levels of automation, digitalization, and flexibility (Isoko et al. 2024). Several advanced technologies and/or tools are being implemented in research institutes and companies, such as digital twins (Schmidt et al. 2022, 2024), AI (Thon et al. 2021), robot-assisted systems (Khandagale 2024), and additive manufacturing (3D printing) (Muhindo et al. 2023). Hence, these technologies lie at the core of the transition toward continuous manufacturing, and their ongoing developments should be considered when selecting appropriate PAT tools (Isoko et al. 2024).

Overall, the DSP cascade of mAbs consists of multiple unit operations, each with unique requirements in terms of monitoring and control that need to be considered when designing a continuous mode of operation. Thus, PAT tool selection should be done carefully, as there is no one-size-fits-all solution for quantitative measurements that are essential for IPC and RTRT strategies. The content of this review may serve as a basis for PAT orientation and selection, while also indicating valuable opportunities to improve and innovate real-time quantitative monitoring and/or control of mAbs in continuous downstream biomanufacturing.

Author Contributions

Mariana Carvalho: conceptualization, investigation, visualization, writing – original draft. **Ana Cruz:** supervision. **Cees Haringa:** writing – review and editing, supervision, funding acquisition. **Marcel Ottens:** writing – review and editing, supervision. **Marieke Klijn:** conceptualization, writing – review and editing, supervision, administration, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

References

Abdi, H., and L. J. Williams. 2013. "Partial Least Squares Methods: Partial Least Squares Correlation and Partial Least Square Regression." In *Computational Toxicology: Volume II*, edited by B. Reisfeld and A. N. Mayeno, 549–579. Humana Press. https://doi.org/10.1007/978-1-62703-059-5_23.

Antosiewicz, J. M., and D. Shugar. 2016. "UV–Vis Spectroscopy of Tyrosine Side-Groups in Studies of Protein Structure. Part 2: Selected Applications." *Biophysical Reviews* 8, no. 2: 163–177. https://doi.org/10.1007/s12551-016-0197-7.

Barth, A. 2007. "Infrared Spectroscopy of Proteins." *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1767, no. 9: 1073–1101. https://doi.org/10.1016/j.bbabio.2007.06.004.

Bhangale, R. P., R. Ye, T. B. Lindsey, and L. S. Wolfe. 2022. "Application of Inline Variable Pathlength Technology for Rapid Determination of Dynamic Binding Capacity in Downstream Process Development of Biopharmaceuticals." *Biotechnology Progress* 38, no. 2: e3236. https://doi.org/10.1002/btpr.3236.

Bordawekar, S., A. Chanda, A. M. Daly, et al. 2015. "Industry Perspectives on Process Analytical Technology: Tools and Applications in API Manufacturing." *Organic Process Research & Development* 19, no. 9: 1174–1185. https://doi.org/10.1021/acs.oprd.5b00088.

Boulet-Audet, M., S. G. Kazarian, and B. Byrne. 2016. "In-Column ATR-FTIR Spectroscopy to Monitor Affinity Chromatography Purification of Monoclonal Antibodies." *Scientific Reports* 6, no. 1: 30526. https://doi.org/10.1038/srep30526.

Brestrich, N., M. Rüdt, D. Büchler, and J. Hubbuch. 2018. "Selective Protein Quantification for Preparative Chromatography Using Variable Pathlength UV/Vis Spectroscopy and Partial Least Squares Regression." *Chemical Engineering Science* 176: 157–164. https://doi.org/10.1016/j.ces.2017.10.030.

van den Broek, I., W. M. A. Niessen, and W. D. van Dongen. 2013. "Bioanalytical LC–MS/MS of Protein-Based Biopharmaceuticals." *Journal of Chromatography B* 929: 161–179. https://doi.org/10.1016/j.jchromb.2013.04.030.

Capito, F., R. Skudas, H. Kolmar, and C. Hunzinger. 2015. "At-Line Mid Infrared Spectroscopy for Monitoring Downstream Processing Unit Operations." *Process Biochemistry* 50, no. 6: 997–1005. https://doi.org/10.1016/j.procbio.2015.03.005.

Chanda, A., A. M. Daly, D. A. Foley, et al. 2015. "Industry Perspectives on Process Analytical Technology: Tools and Applications in API Development." *Organic Process Research & Development* 19, no. 1: 63–83. https://doi.org/10.1021/op400358b.

Chon, J. H., and G. Zarbis-Papastoitsis. 2011. "Advances in the Production and Downstream Processing of Antibodies." *New Biotechnology* 28, no. 5: 458–463. https://doi.org/10.1016/j.nbt.2011.03.015.

Chopda, V., A. Gyorgypal, O. Yang, et al. 2022. "Recent Advances in Integrated Process Analytical Techniques, Modeling, and Control Strategies to Enable Continuous Biomanufacturing of Monoclonal Antibodies." *Journal of Chemical Technology & Biotechnology* 97, no. 9: 2317–2335. https://doi.org/10.1002/jctb.6765.

Dhont, J. 1996. "Chapter 3 - Light Scattering." In *Studies in Interface Science* 2, 107–170. Elsevier. https://doi.org/10.1016/S1383-7303(96)80005-6.

Diebold, M., S. D. Backer, P. M. Niedenzu, B. R. Hester, and F. Vanhecke. 2022. "Light Scattering 1—The Physics of Light Scattering." In *Pigments, Extenders, and Particles in Surface Coatings and Plastics: Fundamentals and Applications to Coatings, Plastics and Paper Laminate Formulation*, edited by M. Diebold, S. De Backer, P. M. Niedenzu, B. R. Hester, and F. A. C. Vanhecke, 81–99. Springer International Publishing. https://doi.org/10.1007/978-3-030-99083-1_3.

Dong, J., N. Migliore, S. J. Mehrman, J. Cunningham, M. J. Lewis, and P. Hu. 2016. "High-Throughput, Automated Protein A Purification Platform With Multiattribute LC–MS Analysis for Advanced Cell Culture Process Monitoring." *Analytical Chemistry* 88, no. 17: 8673–8679. https://doi.org/10.1021/acs.analchem.6b01956.

Dunn, Z. D., P. Bohman, A. Quinteros, et al. 2023. "Automated Online-Sampling Multidimensional Liquid Chromatography With Feedback-Control Capability as a Framework for Real-Time Monitoring of mAb Critical Quality Attributes in Multiple Bioreactors." *Analytical Chemistry* 95, no. 49: 18130–18138. https://doi.org/10.1021/acs.analchem.3c03528.

Dürauer, A., A. Jungbauer, and T. Scharl. 2024. "Sensors and Chemometrics in Downstream Processing." *Biotechnology and Bioengineering* 121, no. 8: 2347–2364. https://doi.org/10.1002/bit.28499.

Ecker, D. M., S. D. Jones, and H. L. Levine. 2015. "The Therapeutic Monoclonal Antibody Market." *mAbs* 7, no. 1: 9–14. https://doi.org/10.4161/19420862.2015.989042.

Esmonde-White, K. A., M. Cuellar, and I. R. Lewis. 2022. "The Role of Raman Spectroscopy in Biopharmaceuticals From Development to Manufacturing." *Analytical and Bioanalytical Chemistry* 414, no. 2: 969–991. https://doi.org/10.1007/s00216-021-03727-4.

Feidl, F., S. Garbellini, M. F. Luna, et al. 2019. "Combining Mechanistic Modeling and Raman Spectroscopy for Monitoring Antibody Chromatographic Purification." *Processes* 7, no. 10: 683. https://doi.org/10.3390/pr7100683.

Feidl, F., S. Vogg, M. Wolf, et al. 2020. "Process-Wide Control and Automation of an Integrated Continuous Manufacturing Platform for Antibodies." *Biotechnology and Bioengineering* 117, no. 5: 1367–1380. https://doi.org/10.1002/bit.27296.

Ferrer, A., D. Aguado, S. Vidal-Puig, J. M. Prats, and M. Zarzo. 2008. "PLS: A Versatile Tool for Industrial Process Improvement and Optimization." *Applied Stochastic Models in Business and Industry* 24, no. 6: 551–567. https://doi.org/10.1002/asmb.716.

Gerzon, G., Y. Sheng, and M. Kirkitadze. 2022. "Process Analytical Technologies – Advances in Bioprocess Integration and Future Perspectives." *Journal of Pharmaceutical and Biomedical Analysis* 207: 114379. https://doi.org/10.1016/j.jpba.2021.114379.

Gilardoni, E., and L. Regazzoni. 2022. "Liquid Phase Separation Techniques for the Characterization of Monoclonal Antibodies and Bioconjugates." *Journal of Chromatography Open* 2: 100034. https://doi.org/10.1016/j.jcoa.2022.100034.

Gillespie, C., D. P. Wasalathanthri, D. B. Ritz, et al. 2022. "Systematic Assessment of Process Analytical Technologies for Biologics." *Biotechnology and Bioengineering* 119, no. 2: 423–434. https://doi.org/10.1002/bit.27990.

Glassey, J., K. V. Gernaey, C. Clemens, et al. 2011. "Process Analytical Technology (PAT) for Biopharmaceuticals." *Biotechnology Journal* 6, no. 4: 369–377. https://doi.org/10.1002/biot.201000356.

Goldrick, S., A. Umprecht, A. Tang, et al. 2020. "High-Throughput Raman Spectroscopy Combined With Innovate Data Analysis Workflow to Enhance Biopharmaceutical Process Development." *Processes* 8, no. 9: 1179. https://doi.org/10.3390/pr8091179.

Großhans, S., M. Rüdt, A. Sanden, et al. 2018. "In-Line Fourier-Transform Infrared Spectroscopy as a Versatile Process Analytical Technology for Preparative Protein Chromatography." *Journal of Chromatography A* 1547: 37–44. https://doi.org/10.1016/j.chroma.2018.03.005.

Hagman, C., D. Ricke, S. Ewert, S. Bek, R. Falchetto, and F. Bitsch. 2008. "Absolute Quantification of Monoclonal Antibodies in Biofluids by Liquid Chromatography–Tandem Mass Spectrometry." *Analytical Chemistry* 80, no. 4: 1290–1296. https://doi.org/10.1021/ac702115b.

Helgers, H., A. Schmidt, L. J. Lohmann, et al. 2021. "Towards Autonomous Operation by Advanced Process Control—Process Analytical Technology for Continuous Biologics Antibody Manufacturing." *Processes* 9, no. 1: 172. https://doi.org/10.3390/pr9010172.

Hofer, A., P. Kroll, M. Barmettler, and C. Herwig. 2020. "A Reliable Automated Sampling System for On-Line and Real-Time Monitoring of CHO Cultures." *Processes* 8, no. 6: 637. https://doi.org/10.3390/pr8060637.

Isoko, K., J. L. Cordiner, Z. Kis, and P. Z. Moghadam. 2024. "Bioprocessing 4.0: A Pragmatic Review and Future Perspectives." *Digital Discovery* 3, no. 9: 1662–1681. https://doi.org/10.1039/D4DD00127C.

Jenzsch, M., C. Bell, S. Buziol, F. Kepert, H. Wegele, and C. Hakemeyer. 2018. "Trends in Process Analytical Technology: Present State in Bioprocessing." In *New Bioprocessing Strategies: Development and Manufacturing of Recombinant Antibodies and Proteins*, edited by B. Kiss, U. Gottschalk, and M. Pohlscheidt, 211–252. Springer International Publishing. https://doi.org/10.1007/10_2017_18.

Jiang, M., K. A. Severson, J. C. Love, et al. 2017. "Opportunities and Challenges of Real-Time Release Testing in Biopharmaceutical Manufacturing." *Biotechnology and Bioengineering* 114, no. 11: 2445–2456. https://doi.org/10.1002/bit.26383.

Kafle, B., U. Böcker, S. G. Wubshet, et al. 2023. "Fourier-Transform Infrared Spectroscopy for Characterization of Liquid Protein Solutions: A Comparison of Two Sampling Techniques." *Vibrational Spectroscopy* 124: 103490. https://doi.org/10.1016/j.vibspec.2022.103490.

Kessler, R. W., and W. Kessler. 2020. "4.10 - Best Practice and Performance of Hardware in Process Analytical Technology (PAT)." In *Comprehensive Chemometrics (Second Edition)*, edited by S. Brown, R. Tauler, and B. Walczak, 237–274. Elsevier. https://doi.org/10.1016/B978-0-12-409547-2.14611-6.

Khandagale, S. S., N. O. Bappasaheb, A. S. Khose, P. Kshirsagar, P. Nemane, and R. H. Thube. 2024. "Role of Pharmaceutical Automation and Robotics in Pharmaceutical Industry: A Review." *Systematic Reviews in Pharmacy* 15: 131–135.

Klutz, S., J. Magnus, M. Lobedann, et al. 2015. "Developing the Biofacility of the Future Based on Continuous Processing and Single-Use Technology." *Journal of Biotechnology* 213: 120–130. https://doi.org/10.1016/j.jbiotec.2015.06.388.

Konoike, F., M. Taniguchi, and S. Yamamoto. 2024. "Integrated Continuous Downstream Process of Monoclonal Antibody Developed by Converting the Batch Platform Process Based on the Process Characterization." *Biotechnology and Bioengineering* 121, no. 8: 2269–2277. https://doi.org/10.1002/bit.28537.

Kuhar, N., S. Sil, and S. Umapathy. 2021. "Potential of Raman Spectroscopic Techniques to Study Proteins." *Spectrochimica Acta, Part A: Molecular and Biomolecular Spectroscopy* 258: 119712. https://doi.org/10.1016/j.saa.2021.119712.

Lambiase, G., S. E. Inman, M. Muroni, V. Lindo, M. J. Dickman, and D. C. James. 2022. "High-Throughput Multiplex Analysis of mAb Aggregates and Charge Variants by Automated Two-Dimensional Size Exclusion-Cation Exchange Chromatography Coupled to Mass Spectrometry." *Journal of Chromatography A* 1670: 462944. https://doi.org/10.1016/j.chroma.2022.462944.

L. C. Passos, M., and M. L. M.F.S. Saraiva. 2019. "Detection in UV-Visible Spectrophotometry: Detectors, Detection Systems, and Detection Strategies." *Measurement* 135: 896–904. https://doi.org/10.1016/j.measurement.2018.12.045.

Limpikirati, P. K., S. Mongkoltipparat, T. Denchaipradit, et al. 2024. "Basic Regulatory Science Behind Drug Substance and Drug Product Specifications of Monoclonal Antibodies and Other Protein Therapeutics." *Journal of Pharmaceutical Analysis* 14, no. 6: 100916. https://doi.org/10.1016/j.jpha.2023.12.006.

Ma, J., C. Pathirana, D. Q. Liu, and S. A. Miller. 2023. "NMR Spectroscopy as a Characterization Tool Enabling Biologics Formulation Development." *Journal of Pharmaceutical and Biomedical Analysis* 223: 115110. https://doi.org/10.1016/j.jpba.2022.115110.

Mankova, A. A., A. I. Nagaeva, N. N. Brandt, and A. Y. Chikishev. 2022. "Comparison of Vibrational Spectra of Proteins With Similar Secondary and Different Tertiary Structures." *Vibrational Spectroscopy* 120: 103375. https://doi.org/10.1016/j.vibspec.2022.103375.

Mann, M., R. C. Hendrickson, and A. Pandey. 2001. "Analysis of Proteins and Proteomes by Mass Spectrometry." *Annual Review of Biochemistry* 70, no. 1: 437–473. https://doi.org/10.1146/annurev.biochem.70.1.437.

McAvan, B. S., A. P. France, B. Bellina, P. E. Barran, R. Goodacre, and A. J. Doig. 2020. "Quantification of Protein Glycation Using Vibrational Spectroscopy." *Analyst* 145, no. 10: 3686–3696. https://doi.org/10.1039/c9an02318f.

Menezes, J. C., A. P. Ferreira, L. O. Rodrigues, L. P. Brás, and T. P. Alves. 2009. "4.10 - Chemometrics Role Within the PAT Context: Examples From Primary Pharmaceutical Manufacturing." In *Comprehensive Chemometrics*, edited by S. D. Brown, R. Tauler, and B. Walczak, 313–355. Elsevier. https://doi.org/10.1016/B978-044452701-1.00012-0.

Meyer, V. R. 2004a. "Introduction." In *Practical High-Performance Liquid Chromatography*, 4–13. https://doi.org/10.1002/0470032677.ch1.

Meyer, V. R. 2004b. "Theoretical Principles." In *Practical High-Performance Liquid Chromatography*, 14–51. https://doi.org/10.1002/0470032677.ch2.

Moldoveanu, S. C., and V. David. 2017. "Chapter 2 - Short Overviews of Analytical Techniques Not Containing an Independent Separation Step." In *Selection of the HPLC Method in Chemical Analysis*, edited by S. C. Moldoveanu and V. David, 31–53. Elsevier. https://doi.org/10.1016/B978-0-12-803684-6.00002-0.

Muhindo, D., R. Elkanayati, P. Srinivasan, M. A. Repka, and E. A. Ashour. 2023. "Recent Advances in the Applications of Additive Manufacturing (3D Printing) in Drug Delivery: A Comprehensive Review." *AAPS PharmSciTech* 24, no. 2: 57. https://doi.org/10.1208/s12249-023-02524-9.

Nadar, S., G. Shooter, B. Somasundaram, E. Shave, K. Baker, and L. H. L. Lua. 2021. "Intensified Downstream Processing of Monoclonal Antibodies Using Membrane Technology." *Biotechnology Journal* 16, no. 3: 2000309. https://doi.org/10.1002/biot.202000309.

Neugebauer, P., M. Zettl, D. Moser, J. Poms, L. Kuchler, and S. Sacher. 2024. "Process Analytical Technology in Downstream-Processing of Drug Substances– A Review." *International Journal of Pharmaceutics* 661: 124412. https://doi.org/10.1016/j.ijpharm.2024.124412.

Orphanou, C., and D. Gervais. 2018. "Higher-Order Structure and Conformational Change in Biopharmaceuticals." *Journal of Chemical Technology & Biotechnology* 93, no. 9: 2477–2485. https://doi.org/10.1002/jctb.5682.

Patel, B. A., A. Gospodarek, M. Larkin, et al. 2018. "Multi-Angle Light Scattering as a Process Analytical Technology Measuring Real-Time Molecular Weight for Downstream Process Control." *mAbs* 10, no. 7: 945–950. https://doi.org/10.1080/19420862.2018.1505178.

Ramakrishna, A., V. Prathap, V. Maranholkar, and A. S. Rathore. 2022. "Multi-Wavelength UV-Based PAT Tool for Measuring Protein Concentration." *Journal of Pharmaceutical and Biomedical Analysis* 207: 114394. https://doi.org/10.1016/j.jpba.2021.114394.

Rathore, A. S., R. Bhambure, and V. Ghare. 2010. "Process Analytical Technology (PAT) for Biopharmaceutical Products." *Analytical and Bioanalytical Chemistry* 398, no. 1: 137–154. https://doi.org/10.1007/s00216-010-3781-x.

Rathore, A. S., and G. Kapoor. 2015. "Application of Process Analytical Technology for Downstream Purification of Biotherapeutics." *Journal of Chemical Technology & Biotechnology* 90, no. 2: 228–236. https://doi.org/10.1002/jctb.4447.

Rathore, A. S., L. Parr, S. Dermawan, K. Lawson, and Y. Lu. 2010. "Large Scale Demonstration of a Process Analytical Technology Application in Bioprocessing: Use of On-Line High Performance Liquid Chromatography for Making Real Time Pooling Decisions for Process Chromatography." *Biotechnology Progress* 26, no. 2: 448–457. https://doi.org/10.1002/btpr.320.

Rathore, A. S., G. Thakur, and N. Kateja. 2023. "Continuous Integrated Manufacturing for Biopharmaceuticals: A New Paradigm or an Empty Promise?" *Biotechnology and Bioengineering* 120, no. 2: 333–351. https://doi.org/10.1002/bit.28235.

Rathore, A. S., R. Wood, A. Sharma, and S. Dermawan. 2008. "Case Study and Application of Process Analytical Technology (PAT) Towards Bioprocessing: II. Use of Ultra-Performance Liquid Chromatography (UPLC) for Making Real-Time Pooling Decisions for Process Chromatography." *Biotechnology and Bioengineering* 101, no. 6: 1366–1374. https://doi.org/10.1002/bit.21982.

Rathore, A. S., M. Yu, S. Yeboah, and A. Sharma. 2008. "Case Study and Application of Process Analytical Technology (PAT) Towards Bioprocessing: Use of on-Line High-Performance Liquid Chromatography (HPLC) for Making Real-Time Pooling Decisions for Process Chromatography." *Biotechnology and Bioengineering* 100, no. 2: 306–316. https://doi.org/10.1002/bit.21759.

Rogstad, S., A. Faustino, A. Ruth, D. Keire, M. Boyne, and J. Park. 2017. "A Retrospective Evaluation of the Use of Mass Spectrometry in FDA Biologics License Applications." *Journal of the American Society for Mass Spectrometry* 28, no. 5: 786–794. https://doi.org/10.1007/s13361-016-1531-9.

Rolinger, L., J. Hubbuch, and M. Rüdt. 2023. "Monitoring of Ultra- and Diafiltration Processes by Kalman-Filtered Raman Measurements." *Analytical and Bioanalytical Chemistry* 415, no. 5: 841–854. https://doi.org/10.1007/s00216-022-04477-7.

Rolinger, L., M. Rüdt, and J. Hubbuch. 2020. "A Critical Review of Recent Trends, and a Future Perspective of Optical Spectroscopy as PAT in Biopharmaceutical Downstream Processing." *Analytical and Bioanalytical Chemistry* 412, no. 9: 2047–2064. https://doi.org/10.1007/s00216-020-02407-z.

Rolinger, L., M. Rüdt, and J. Hubbuch. 2021. "A Multisensor Approach for Improved Protein A Load Phase Monitoring by Conductivity-Based Background Subtraction of UV Spectra." *Biotechnology and Bioengineering* 118, no. 2: 905–917. https://doi.org/10.1002/bit.27616.

Rüdt, M., N. Brestrich, L. Rolinger, and J. Hubbuch. 2017. "Real-Time Monitoring and Control of the Load Phase of a Protein A Capture Step." *Biotechnology and Bioengineering* 114, no. 2: 368–373. https://doi.org/10.1002/bit.26078.

Rüdt, M., T. Briskot, and J. Hubbuch. 2017. "Advances in Downstream Processing of Biologics – Spectroscopy: An Emerging Process Analytical Technology." *Journal of Chromatography A* 1490: 2–9. https://doi.org/10.1016/j.chroma.2016.11.010.

Rygula, A., K. Majzner, K. M. Marzec, A. Kaczor, M. Pilarczyk, and M. Baranska. 2013. "Raman Spectroscopy of Proteins: A Review." *Journal of Raman Spectroscopy* 44, no. 8: 1061–1076. https://doi.org/10.1002/jrs.4335.

São Pedro, M. N., M. E. Klijn, M. H. M. Eppink, and M. Ottens. 2022. "Process Analytical Technique (PAT) Miniaturization for Monoclonal Antibody Aggregate Detection in Continuous Downstream Processing." *Journal of Chemical Technology & Biotechnology* 97, no. 9: 2347–2364. https://doi.org/10.1002/jctb.6920.

Schiel, J. E., and A. Turner. 2018. "The NISTmAb Reference Material 8671 Lifecycle Management and Quality Plan." *Analytical and Bioanalytical Chemistry* 410, no. 8: 2067–2078. https://doi.org/10.1007/s00216-017-0844-2.

Schmidt, A., H. Helgers, L. J. Lohmann, et al. 2022. "Process Analytical Technology as Key-Enabler for Digital Twins in Continuous Biomanufacturing." *Journal of Chemical Technology & Biotechnology* 97, no. 9: 2336–2346. https://doi.org/10.1002/jctb.7008.

Schmidt, A., A. Hengelbrock, and J. Strube. 2024. "Continuous Biomanufacturing in Upstream and Downstream Processing." *Physical Sciences Reviews* 9: 3167–3222. https://doi.org/10.1515/psr-2022-0106.

Sharma, A., J. Beirne, D. Khamar, C. Maguire, A. Hayden, and H. Hughes. 2023. "Evaluation and Screening of Biopharmaceuticals Using Multi-Angle Dynamic Light Scattering." *AAPS PharmSciTech* 24, no. 4: 84. https://doi.org/10.1208/s12249-023-02529-4.

Shrivastava, A., S. Mandal, S. K. Pattanayek, and A. S. Rathore. 2023. "Rapid Estimation of Size-Based Heterogeneity in Monoclonal Antibodies by Machine Learning-Enhanced Dynamic Light Scattering." *Analytical Chemistry* 95, no. 21: 8299–8309. https://doi.org/10.1021/acs.analchem.3c00650.

Shukla, A. A., B. Hubbard, T. Tressel, S. Guhan, and D. Low. 2007. "Downstream Processing of Monoclonal Antibodies—Application of Platform Approaches." *Journal of Chromatography B* 848, no. 1: 28–39. https://doi.org/10.1016/j.jchromb.2006.09.026.

Singh, M. K., and A. Singh. 2022. "Chapter 14 - Nuclear Magnetic Resonance Spectroscopy." In *Characterization of Polymers and Fibres*, edited by M. K. Singh and A. Singh, 321–339. Woodhead Publishing. https://doi.org/10.1016/B978-0-12-823986-5.00011-7.

Somasundaram, B., K. Pleitt, E. Shave, K. Baker, and L. H. L. Lua. 2018. "Progression of Continuous Downstream Processing of Monoclonal Antibodies: Current Trends and Challenges." *Biotechnology and Bioengineering* 115, no. 12: 2893–2907. https://doi.org/10.1002/bit.26812.

Some, D., and S. Kenrick. 2012. *Characterization of Protein-Protein Interactions via Static and Dynamic Light Scattering*. INTECH Open Access Publisher.

Steinhoff, R. F., D. J. Karst, F. Steinebach, et al. 2016. "Microarray-Based MALDI-TOF Mass Spectrometry Enables Monitoring of Monoclonal Antibody Production in Batch and Perfusion Cell Cultures." *Methods* 104: 33–40. https://doi.org/10.1016/j.ymeth.2015.12.011.

Taraban, M. B., K. T. Briggs, P. Merkel, and Y. B. Yu. 2019. "Flow Water Proton NMR: In-Line Process Analytical Technology for Continuous Biomanufacturing." *Analytical Chemistry* 91, no. 21: 13538–13546. https://doi.org/10.1021/acs.analchem.9b02622.

Thakur, G., V. Hebbi, and A. S. Rathore. 2021. "Near Infrared Spectroscopy as a PAT Tool for Monitoring and Control of Protein and Excipient Concentration in Ultrafiltration of Highly Concentrated Antibody Formulations." *International Journal of Pharmaceutics* 600: 120456. https://doi.org/10.1016/j.ijpharm.2021.120456.

Thakur, G., S. Nikita, A. Tiwari, and A. S. Rathore. 2021. "Control of Surge Tanks for Continuous Manufacturing of Monoclonal Antibodies." *Biotechnology and Bioengineering* 118, no. 5: 1913–1931. https://doi.org/10.1002/bit.27706.

Thissen, U., M. Pepers, B. Üstün, W. J. Melssen, and L. M. C. Buydens. 2004. "Comparing Support Vector Machines to PLS for Spectral Regression Applications." *Chemometrics and Intelligent Laboratory Systems* 73, no. 2: 169–179. https://doi.org/10.1016/j.chemolab.2004.01.002.

Thon, C., B. Finke, A. Kwade, and C. Schilde. 2021. "Artificial Intelligence in Process Engineering." *Advanced Intelligent Systems* 3, no. 6: 2000261. https://doi.org/10.1002/aisy.202000261.

Tiwari, A., N. Kateja, S. Chanana, and A. S. Rathore. 2018. "Use of HPLC as an Enabler of Process Analytical Technology in Process Chromatography." *Analytical Chemistry* 90, no. 13: 7824–7829. https://doi.org/10.1021/acs.analchem.8b00897.

Toth, C. A., Z. Kuklenyik, and J. R. Barr. 2019. "Nuts and Bolts of Protein Quantification by Online Trypsin Digestion Coupled LC-MS/MS Analysis." In *Functional Proteomics: Methods and Protocols*, edited by X. Wang and M. Kuruc, 295–311. Springer New York. https://doi.org/10.1007/978-1-4939-8814-3_19.

Vandenabeele, P. 2013. "Theoretical Aspects." In *Practical Raman Spectroscopy – An Introduction*, 1–38. https://doi.org/10.1002/9781119961284.ch1.

Walch, N., T. Scharl, E. Felföldi, et al. 2019. "Prediction of the Quantity and Purity of an Antibody Capture Process in Real Time." *Biotechnology Journal* 14, no. 7: 1800521. https://doi.org/10.1002/biot.201800521.

Walsh, G., and E. Walsh. 2022. "Biopharmaceutical Benchmarks 2022." *Nature Biotechnology* 40, no. 12: 1722–1760. https://doi.org/10.1038/s41587-022-01582-x.

Wang, J., J. Chen, J. Studts, and G. Wang. 2023. "In-Line Product Quality Monitoring During Biopharmaceutical Manufacturing Using Computational Raman Spectroscopy." *mAbs* 15, no. 1: 2220149. https://doi.org/10.1080/19420862.2023.2220149.

Wasalathanthri, D. P., M. S. Rehmann, Y. Song, et al. 2020. "Technology Outlook for Real-Time Quality Attribute and Process Parameter Monitoring in Biopharmaceutical Development—A Review." *Biotechnology and Bioengineering* 117, no. 10: 3182–3198. https://doi.org/10.1002/bit.27461.

Webster, T. A., K. Turner, C. DuBois, R. MacDougall, and C. Mason. 2022. "Continuous Background Correction of Refractive Index Signal to Improve Monoclonal Antibody Concentration Monitoring During UF/DF and SPTFF Operations." *Bioprocess and Biosystems Engineering* 45, no. 4: 647–657. https://doi.org/10.1007/s00449-021-02683-8.

Wojtkiewicz, M., L. Berg Luecke, M. I. Kelly, and R. L. Gundry. 2021. "Facile Preparation of Peptides for Mass Spectrometry Analysis in Bottom-up Proteomics Workflows." *Current Protocols* 1, no. 3: e85. https://doi.org/10.1002/cpz1.85.

Yang, O., M. Qadan, and M. Ierapetritou. 2020. "Economic Analysis of Batch and Continuous Biopharmaceutical Antibody Production: A Review." *Journal of Pharmaceutical Innovation* 15, no. 1: 182–200. https://doi.org/10.1007/s12247-018-09370-4.

Yeung, E. S., and R. E. Synovec. 1986. "Detectors for Liquid Chromatography." *Analytical Chemistry* 58, no. 12: 1237A–1256A.

Zeltins, A. 2013. "Construction and Characterization of Virus-Like Particles: A Review." *Molecular Biotechnology* 53, no. 1: 92–107. https://doi.org/10.1007/s12033-012-9598-4.

Zhao, H., P. H. Brown, and P. Schuck. 2011. "On the Distribution of Protein Refractive Index Increments." *Biophysical Journal* 100, no. 9: 2309–2317. https://doi.org/10.1016/j.bpj.2011.03.004.

Zobel-Roos, S., M. Mouellef, C. Siemers, and J. Strube. 2017. "Process Analytical Approach Towards Quality Controlled Process Automation for the Downstream of Protein Mixtures by Inline Concentration Measurements Based on Ultraviolet/Visible Light (UV/VIS) Spectral Analysis." *Antibodies* 6, no. 4: 24. https://doi.org/10.3390/antib6040024.