



Universiteit  
Leiden

The Netherlands

## Unraveling proteoform complexity by native liquid chromatography-mass spectrometry

Schaick, G. van

### Citation

Schaick, G. van. (2023, January 24). *Unraveling proteoform complexity by native liquid chromatography-mass spectrometry*. Retrieved from <https://hdl.handle.net/1887/3512617>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/3512617>

**Note:** To cite this publication please use the final published version (if applicable).

## Summary

Proteins are widely known as key players that fulfill crucial roles at the molecular level in the human body but also for their involvement in many processes in everyday life. For example, proteins can be used as medicine in health care or for their enzymatic function in the food industry. All these proteins have in common that they do not exist as single species but rather as a complex mixture of structural variants, so-called proteoforms. This heterogeneity results mainly from the presence of post-translational modifications (PTMs), such as glycosylation and glycation. To further complicate this matter, these PTMs can induce structural as well as functional changes (**Chapter 1**). Therefore, it is of great importance that new analytical methods are developed to unravel these complex structures and to establish their influence on function. The coupling of native separations with mass spectrometry (MS) has emerged as a powerful approach to reliably study these aspects (**Chapter 2**). This work in this thesis describes the (further) development and application of such methodologies for biopharmaceutical and biotechnological products.

First, the hyphenation of native separations with MS is improved by the implementation of dopant-enriched nitrogen gas to increase ionization efficiency and thereby, aid the characterization of (highly) glycosylated proteins (**Chapter 3**). In **Chapters 4** and **5**, charge-based separations are developed to monitor charge variants of biotherapeutic proteins. Anion exchange (AEX) and cation exchange chromatography (CEX) have been employed for the characterization of acidic and basic proteins, respectively. In **Chapter 4**, the AEX-MS analysis of biopharmaceutical erythropoietin results in the assignment of ~350 compositions mainly differing in their glycosylation pattern (i.e., levels of sialylation and number of LacNAC repeats). Additionally, also minor modifications, such as deamidation and O-acetylation of sialic acids, can be monitored using this methodology. Altogether, more than 100 additional compositions are assigned using AEX-MS compared to standalone MS detection. Moreover, the AEX-MS method obtains the same information as standard quality control methods with substantially less sample treatment, saving time and costs. In **Chapter 5**, CEX-MS methods were applied to the separation of therapeutic monoclonal antibodies (mAbs). In addition, the influence of PTMs on mAb conformation is investigated by online coupling of CEX with ion mobility and collision induced unfolding (CIU). This novel approach enables mAb subclass classification as well as investigation of gas-phase stability of mAbs in a glycoform-specific manner.

The next part of this thesis focuses on the establishment of structure-function relationships of biotechnological products, such as industrial enzymes. For therapeutic proteins, functional data is often widely available, but this is not the case for industrial enzymes. In **Chapters 6, 7, and 8** methods are developed using native separations together with (online) MS detection and parallel activity measurements to address questions related to the function of particular proteoforms. In **Chapter 6**, a novel AEX-MS method enables the separation, detection, and fractionation of proteoforms based on their level of phosphorylated glycans offering detailed structural characterization and opening possibilities for subsequent functional assignment. Investigation of the collected fractions reveals that phosphorylated glycans attached to an endoprotease have no to minor impact on the digestion speed and specificity. In **Chapter 7**, a complete characterization of an endo-xylanase at the structural and functional level is performed. The different nature of the proteoforms requires a combination of multiple native liquid chromatography (LC) modes for their characterization, including size exclusion chromatography (SEC) to separate size variants, AEX to resolve differently charged proteoforms, and boronate affinity chromatography (BAC) to enrich glycated proteoforms. After separation, the activity of all separated proteoforms is measured showing that some modifications do not impact the function (i.e., N-terminal truncation), while others negatively influence the enzyme functionality (i.e., glycation). **Chapter 8** focuses further on the influence of glycation on the activity of an industrial lipase. The combination of BAC with MS detection and activity assays shows that upon temperature stress the lipase can contain up to four sugar moieties. Opposite to the endo-xylanase, the functionality is not negatively impacted by the presence of this PTM.

Finally, current challenges and future perspectives of approaches to structurally and functionally characterize proteoforms are discussed in **Chapter 9**. The key parameters are described to maintain protein nativity during analysis as well as the importance to find the balance between methodological improvement and preservation of protein functionality is highlighted. The last part of this chapter evaluates the applicability of BAC for the enrichment of glycated proteins. The focus is specifically on the development of new stationary phases, the use of proper detection methods, and novel application fields.