

AUTOMATED CHEMICAL ANALYSIS

Off-line Reaction Monitoring Using Advanced Chemical Profiling Software

Example Use-Case: Enzymatic Hydrolysis of Lactose into Galactose and Glucose

Innovation with Integrity

Introduction

Monitoring chemical reactions is crucial for understanding, optimizing, and scaling up processes to ensure cost-efficiency and high yields, while maintaining the quality of the final product. This practice offers valuable insights into the mechanisms of chemical reactions, whether in traditional chemistry, bioproduction, or (bio)catalysis. By analyzing time course data, kinetic information can be extracted and used to build predictive models, optimize process conditions, and manage reaction-related risks.

Nuclear Magnetic Resonance (NMR) spectroscopy stands out as a powerful tool for providing the quantitative data needed for kinetic analyses. It not only delivers inherently quantitative information but also helps to elucidate the structures of products and by-products. With the Advanced Chemical Profiling (ACP) software, you can automatically extract quantitative data, such as the concentration of starting materials, reactants, catalysts, by-products, and products from NMR data in full automation.

This Application Note will guide you through the straightforward steps to set up a fully automated workflow for identification and quantification, using the example of lactase-driven enzymatic hydrolysis of lactose in milk into glucose and galactose. This workflow can be easily adapted to other reactions to gain insights into their kinetics.

The following paragraphs will detail the individual steps: setting up the database (Step 1), defining the reference standard (Step 2), and creating the method (Step 3).

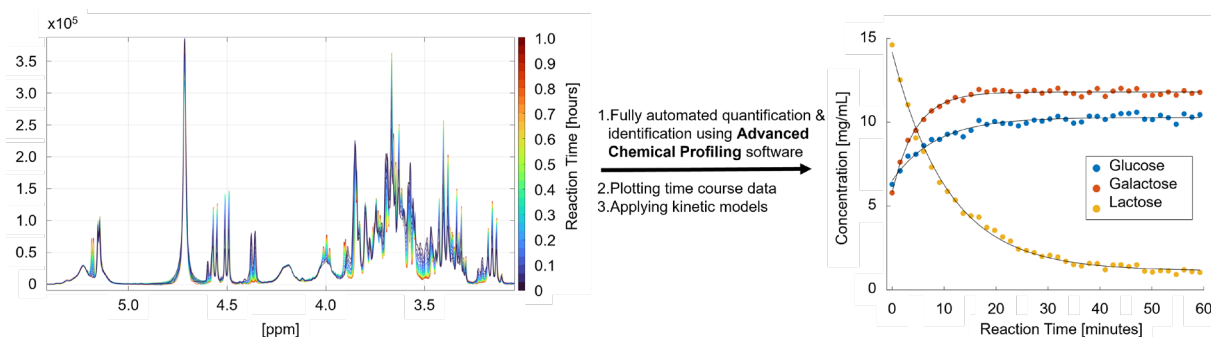


Figure 1: Time-course ¹H-NMR spectra of milk after the addition of lactase (left) and plot of the concentration of glucose (blue) galactose (orange) and lactose (yellow) over time (right).

Step 1: Setting up the database

Advanced Chemical Profiling uses a pattern recognition algorithm to identify and quantify substances in the sample. Therefore, the first step is to create a database with the patterns (¹H-NMR spectra) of the compounds of interest within the mixture. In the case of reaction monitoring, this can be starting materials, reactants, catalysts, products or by-products. Optimal performance is achieved when using the NMR spectra of pure compounds, as all signals are isolated and can be used for the fingerprinting routine. However, if distinct signals are present in the mixture spectra, those spectra can also be used to generate the individual entries in the database. The latter approach was used in the herein presented example: The ¹H-NMR spectrum (400 MHz) of pure milk was used to setup the database entry for lactose. For galactose and glucose, the spectra following the complete hydrolysis of lactose were utilized in the database. The β-protons of the sugars (colored protons in Figure 2) were used as input signals for the pattern recognition (fingerprinting) routine.

Advanced Chemical Profiling

Bruker's Advanced Chemical Profiling (ACP) solution offers an automated, end-to-end workflow for identification and quantification of substances either in pure form or in mixtures from sample to report. Such automated workflows are particularly important in high-throughput environments such as production-related labs, analytical testing services, R&D centers, and pilot-upscaling support functions within centers of excellence. Advanced Chemical Profiling addresses the tasks of data processing, interpretation, and report filing for NMR data of chemical mixtures allowing identification and quantification of constituents in incoming goods, process intermediates, and final product formulations to improve both, quality, and efficiency in R&D lab-scale, pilot, and volume production processes. The database-driven solution is customizable by NMR scientists allowing for application in a wide range of use cases. The data processing methods within ACP can be directly linked to the acquisition parameters, allowing non-expert users to readily perform the analysis without any prior knowledge of NMR data interpretation required. This allows for the deployment of sophisticated NMR-based analysis in manufacturing and quality control environments.

Advanced Chemical Profiling is fully integrated into TopSpin, IconNMR and GoScan. The software can be downloaded from www.bruker.com/ACP, can be tested free-of-charge and comes with a detailed manual and step-by-step video tutorials that explain the use of the software.

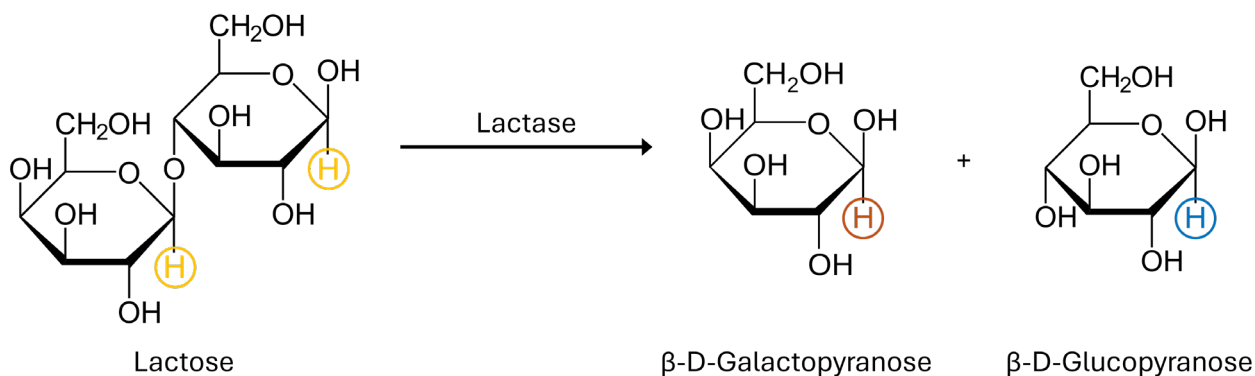


Figure 2: Reaction scheme of the enzymatic hydrolysis of lactose and indication of the protons used for the database setup (colored). For monosaccharides only the beta-form is shown.

Spectra can be easily imported via drag-and-drop into the ACP database setup wizard. Using the "Auto-Detect Region" feature, the software automatically detects signals in the spectra and assigns integrals. Both, the region and integral can be manually adjusted after the automatic detection. For compounds that have clear markers in their spectra, like the β -protons of the sugars, it is recommended to reduce the number of regions to these signals / multiplets. For identification and quantification purposes, the user is asked to provide the name and molar mass for each database entry. Additional information, such as the CAS number, IUPAC name, compound class, or other details can also be provided and will be included in the analysis report as well as the automatically generated xml result-file.

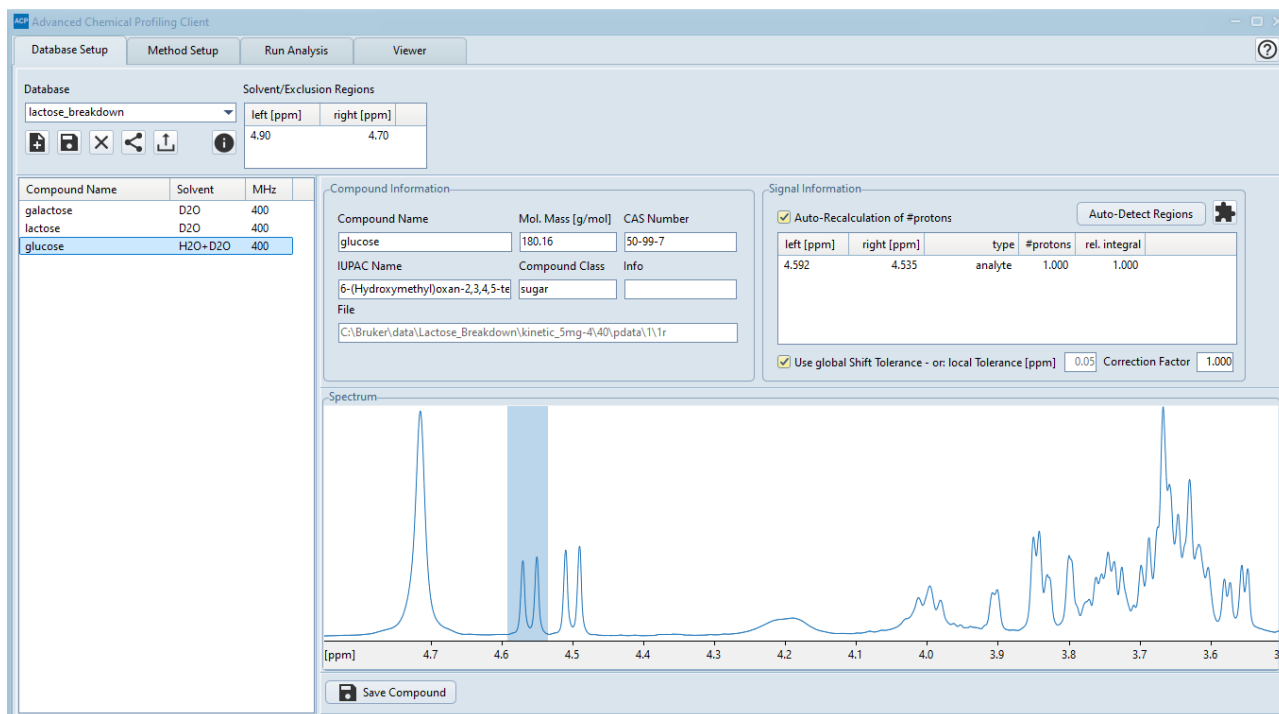


Figure 3: Database setup window within ACP.

Step 2: Defining the reference standard

While relative quantification works without the use of reference standards, absolute quantification with NMR, as used in the present example, requires the definition of a reference standard, which determines the instrument-dependent factor needed to convert signal areas into absolute concentrations of the corresponding compounds. ACP supports the use of internal and external standards depending on the user's preference and use-case specific requirements.

For the example presented in this application note, valine dissolved in D₂O was used as an external standard. Like the database setup, the spectrum can be added via drag-and-drop to the reference standard wizard. By using the "Add Region" feature, the range of signals to be used for quantification can be defined. The user is then asked to provide the number of protons for the selected signal, the molar mass of the reference material, the concentration of the sample, and, if necessary, a correction factor.

Conveniently, the external reference needs to be only set up once. Afterwards, the use of the reference standard can be added to a parameter set and run in full automation within Topspin, IconNMR or GoScan.

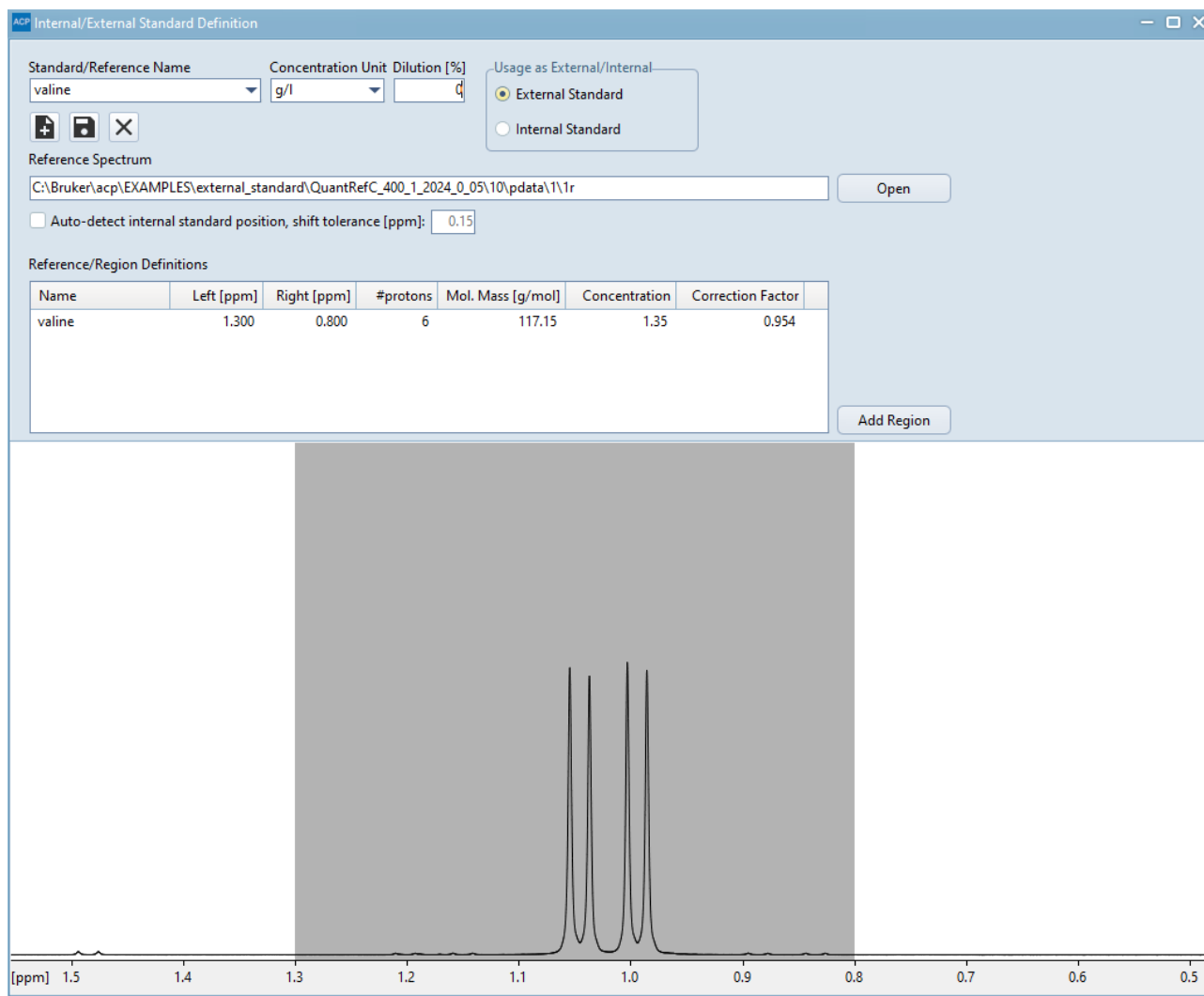


Figure 4: Setting up a reference standard in ACP.

Step 3: Creating the method

The method connects the database (Step 1) with the reference standard (Step 2) and further specifies the concentration unit (absolute units: mol/L, mmol/L, g/L, mg/mL, g/(100g sample); relative units: molar ratio, integral, integral/proton), the shift tolerance (i.e. the parameter which allows ACP to shift each multiplet by a certain value during identification and quantification and can be set either for individual multiplets or globally for all compounds), a concentration threshold and defines the information that will be included in the report.

In the presented example, the method "lactose_breakdown" was created combining the "lactose_breakdown" database and the external reference standard "valine". The concentration unit was defined as mg/mL with a lower threshold of 0.1 mg/mL.

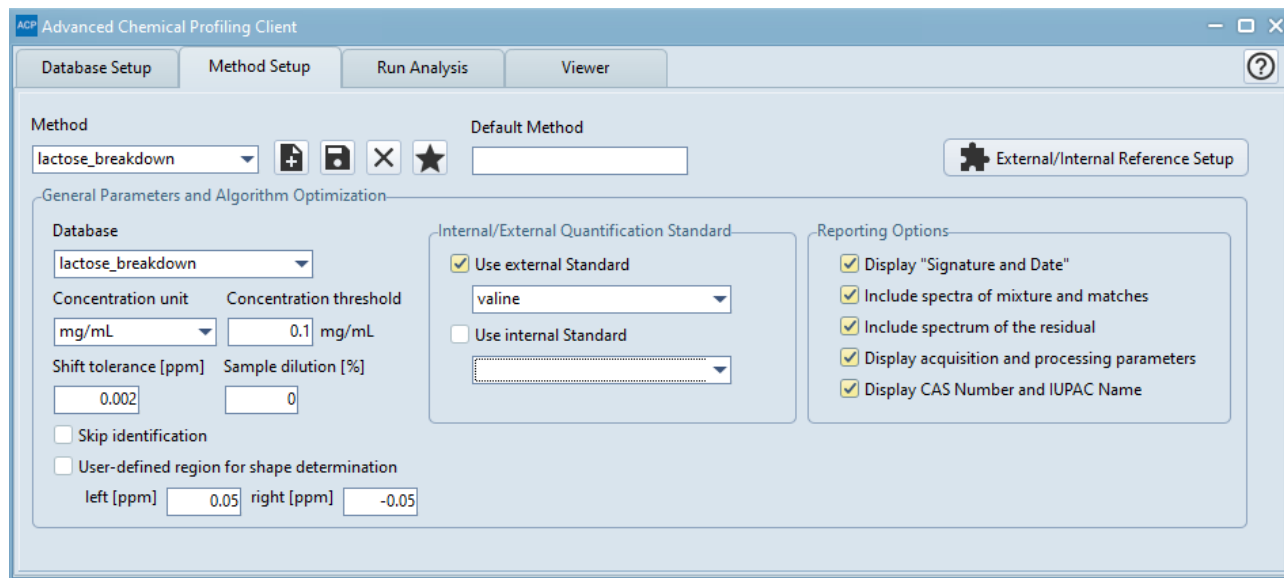


Figure 5: Method setup window in ACP.

Step 4: Identification and quantification

Option 1: Identification and quantification in full automation

Once the method is set up, it can be linked to an NMR acquisition parameter set. This allows for fully automated data acquisition, processing, and interpretation without any intervention from the user necessary. The user only needs to prepare the sample and select the appropriate parameter set in IconNMR or GoScan. After the measurement, a report (PDF and XML) with the mixture analysis performed by ACP will be automatically generated. The report provides information about the compounds identified, their respective concentrations, any information defined in the method setup (e.g. CAS, IUPAC name, etc.), a plot of the fitted spectra as well as a residual plot raising awareness if any unexpected compounds are present in the mixture or the database is missing an entry.

The setup of the database, reference and method requires moderate NMR expertise. However, once the method is linked to a parameter set, anyone with or without prior knowledge of NMR spectroscopy can execute the analysis as the complete workflow is hands-off after the sample is submitted to the instrument. The system will acquire the data and the Advanced Chemical Profiling software will take care of the data processing by matching the entries in the tailor-made database to the mixture and quantifying the components using the previously defined internal or external reference. Afterwards, the solution will automatically generate a PDF report with the identified mixture's constituents and their respective concentrations.

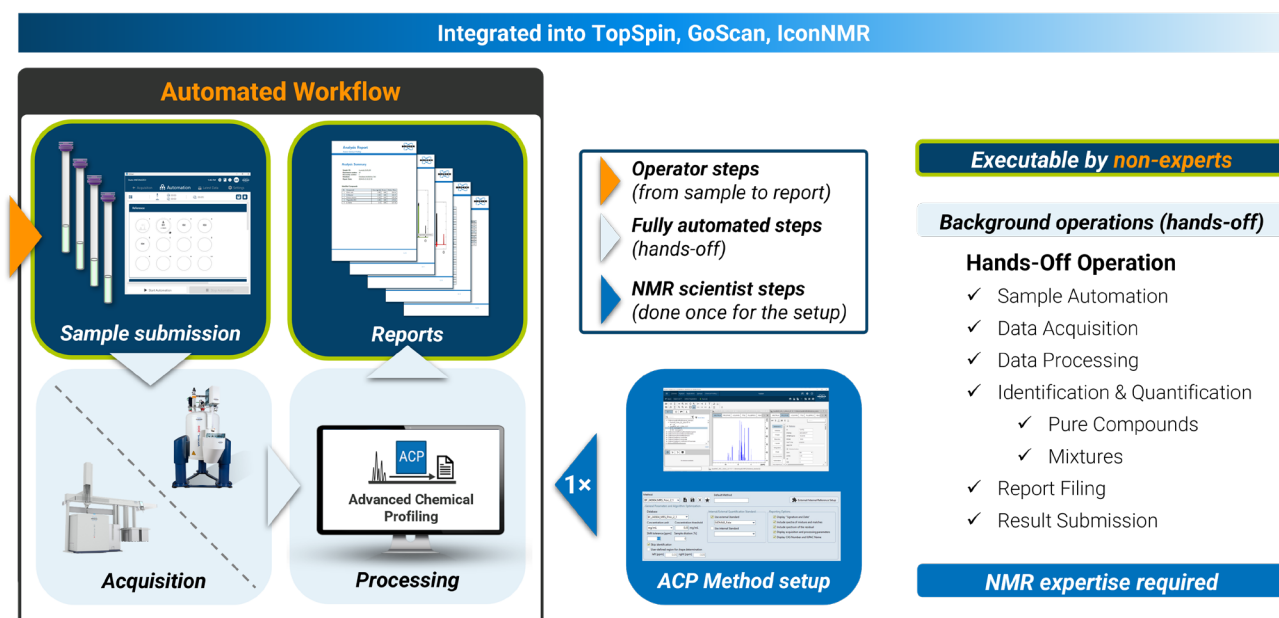


Figure 6: High-level workflow for fully automated analysis and reporting.

Option 2: Identification and quantification in manual-mode

In addition to the previously described analysis in full automation, ACP offers the option to re-process already existing data or refine analyses after the measurement either for individual spectra or for a set of several spectra in batch mode. This is particularly useful if one compound present in the mixture is missing in the database and should be added afterwards. However, any other modification of the database or method is possible in the manual mode as well. In the manual analysis, ACP offers an interactive plot with overlaid mixture spectra, fitted spectra and the residual for a deep dive into the data.

For the presented example, the time course spectra were analyzed in batch mode and results were exported via the "Export to Excel" function. After assigning the reaction time information to the exported data, the reaction kinetics can be modelled.

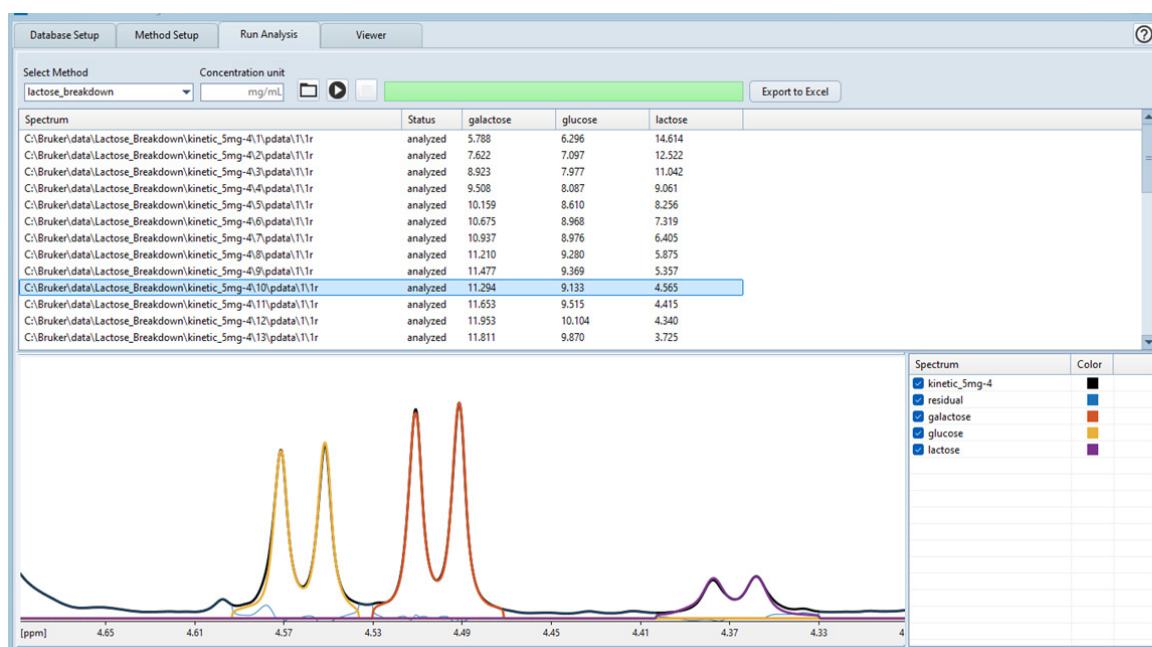


Figure 7: Manual analysis with ACP in batch mode.

Conclusion

The individual steps highlighted in this Application Note show the few and simple steps required to setup a database, reference standard, and method in Advanced Chemical Profiling for NMR-based offline reaction monitoring. The fully-automated identification and quantification workflow with ACP can be used to deduce time-dependent data of the enzymatic degradation of lactose in milk. This approach can be readily translated to any other chemical reaction to monitor its kinetic profile.

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