

# Chemometric modelling of important biomarkers for online fluorescence monitoring of tumour-infiltrating lymphocyte cultivations

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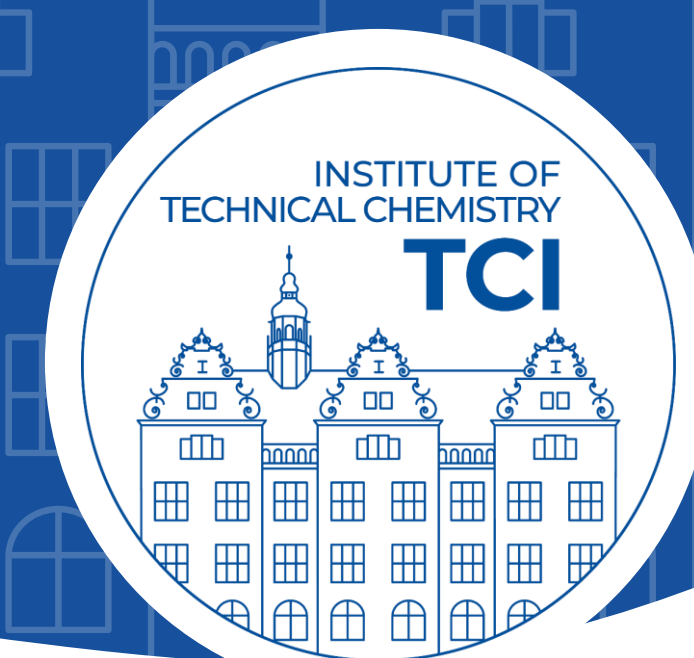
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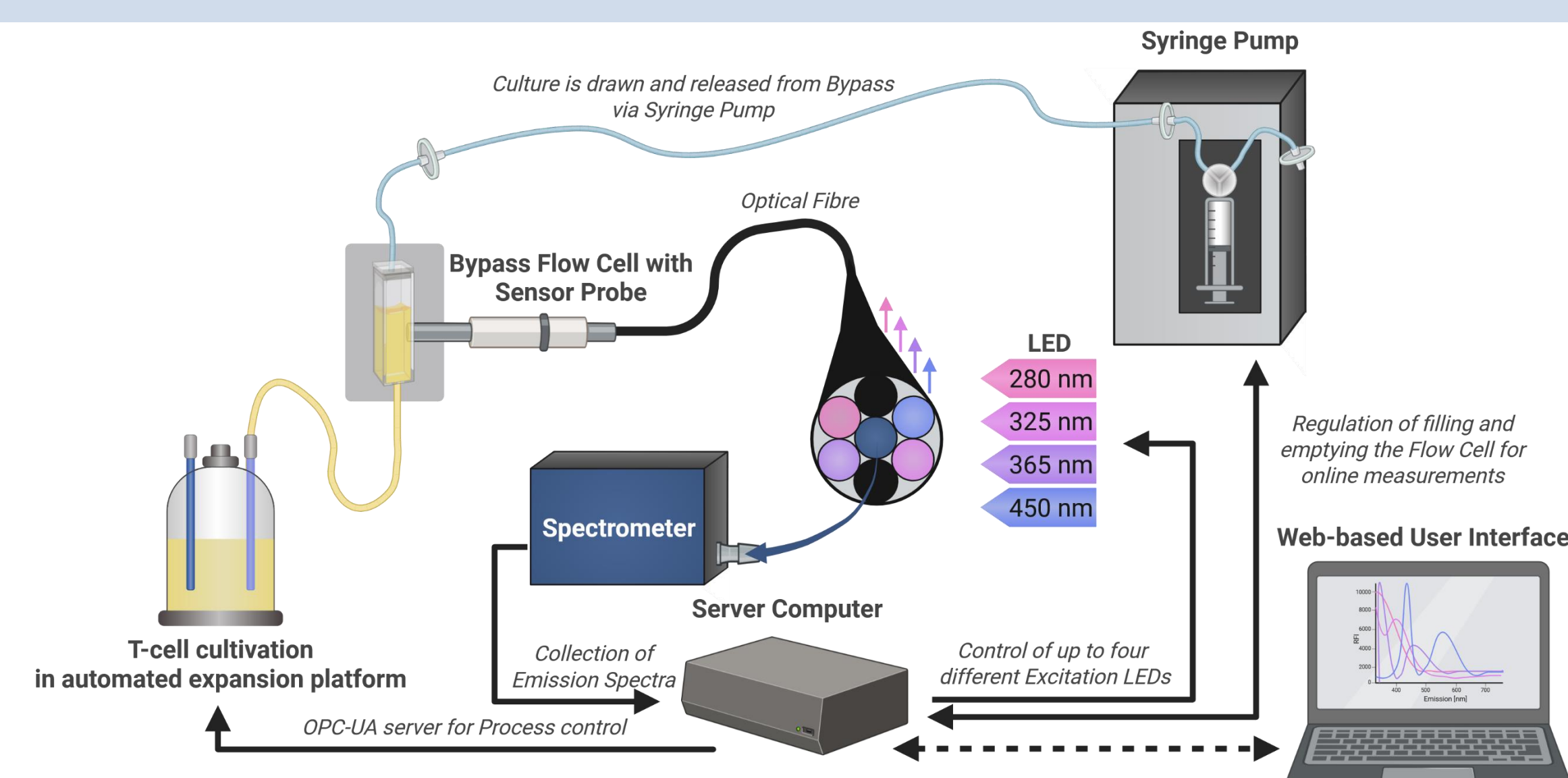
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## INTRODUCTION

Process Analytical Technology (PAT) increasingly employs online sensor systems to support biopharmaceutical manufacturing by enabling real-time monitoring of critical process parameters. Fluorescence spectroscopy represents a highly sensitive analytical approach capable of detecting a wide range of endogenous fluorophores. Here, we report the development of the first-of-its-kind modular fluorescence sensor specifically designed for the co-cultivation of tumour-infiltrating lymphocytes (TILs) and dendritic cells (DCs). The sensor system was engineered with reduced excitation wavelengths to selectively detect four key biogenic fluorophores present in the cultivation medium: proteins, pyridoxine, nicotinamide adenine dinucleotide (NADH), and flavins. Based on this, chemometric models were developed to accurately predict changes in metabolite concentrations, enabling real-time monitoring of metabolite fluctuations. This technology was developed as part of a new manufacturing platform for autologous immunotherapies, developed by the EU-funded SMARTER consortium.

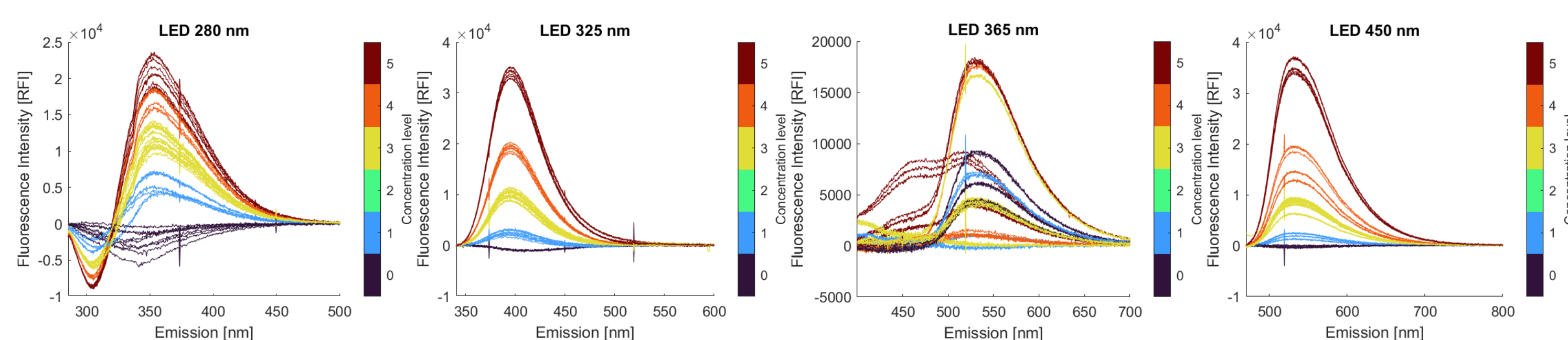


**FIG 1:** Modular fluorescence Fluoro4SMARTER sensor setup for online bioprocess monitoring via a Bypass system. The central server computer controls online measurements via Node-Red flows and hosts an OPC-server with for process control. The User Interface for software control can be accessed remotely via web-login. Created with BioRender.com

## RESULTS

### 1. Fluoro4SMARTER offline Calibration

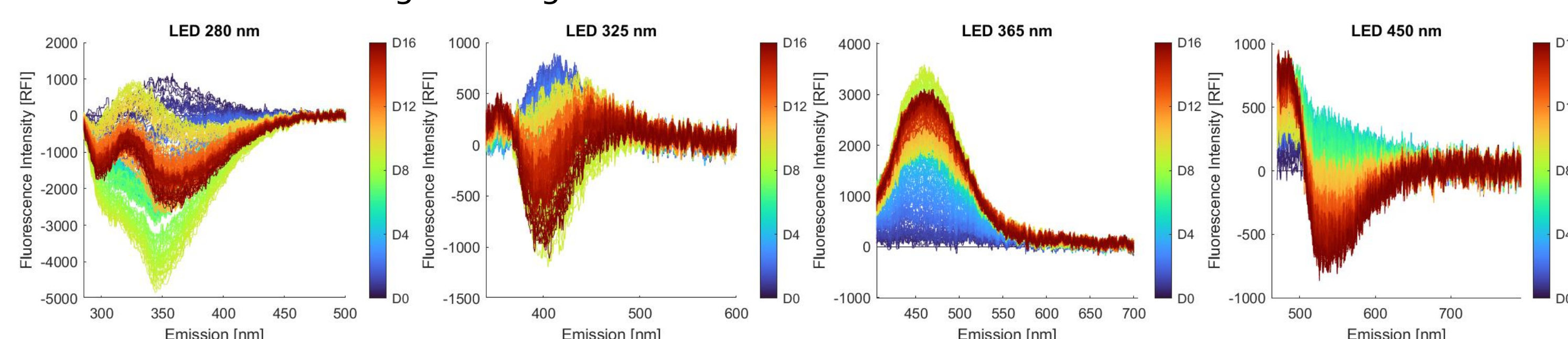
For the model calibration, a multilevel approach with 19 linear independent mixtures of the four analytes Tryptophan, Pyridoxine, NADH, and Riboflavin dissolved in cultivation medium was applied. High selectivity and specificity was shown, with little interference from the concentrations of the other analytes. Only for NADH, which shows an inner filter effect with Riboflavin, no clear correlation was observed. Overall, this demonstrates the systems ability to resolve specific signals under varying conditions.



**FIG 2:** Fluoro4SMARTER multilevel calibrations emission spectra of the four analytes Tryptophan (280 nm), Pyridoxine (325 nm), NADH (365 nm) and Riboflavin (450 nm) with background spectrum of medium subtracted.

### 2. Normalisation of online spectra

Subsequent cultivation spectra measured in the bypass were normalised against the initial measurement in order to correct background signal contributions from the medium and the sensor setup. Distinct signal changes were observed for all four wavelengths over the 16-day cultivation period, including a reseeding step at day 9, which results in a notable jump of the fluorescence signal in figure 3.



**FIG 3:** Normalised emission spectra of a TIL cultivation over the period of 16 days. Tryptophan (280 nm), Pyridoxine (325 nm), NADH (365 nm) and Riboflavin (450 nm).

## CONCLUSION

The modular fluorescence sensor Fluoro4SMARTER was developed as part of the SMARTER project and its high specificity and selectivity for four relevant analytes in TIL cultivations was shown by calibration measurements. By integrating a syringe pump controlled bypass system, online measurements became possible. The chemometric models reflected both metabolic processes, as well as external interventions such as feeds or re-seeding. These

results demonstrate that chemometric modelling can support online fluorescence based monitoring by offering insights into dynamic changes during cultivations. The simultaneous integration of a OPC server provides these modelled data in real time to the control software of the process.



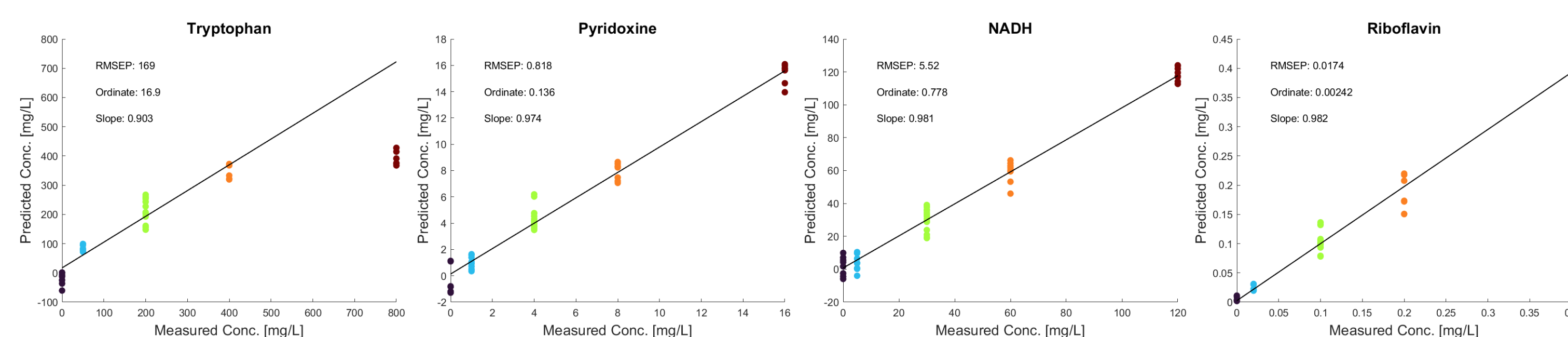
This project has received funding from the European Union's Horizon Europe research and innovation programme under grant number 101071054. Views and opinions expressed are those of the author(s) only and do not necessarily reflect those of the European Union or the European Innovation Council. Neither the European Union nor the granting authority can be held responsible for them.



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### 3. Chemometric modelling

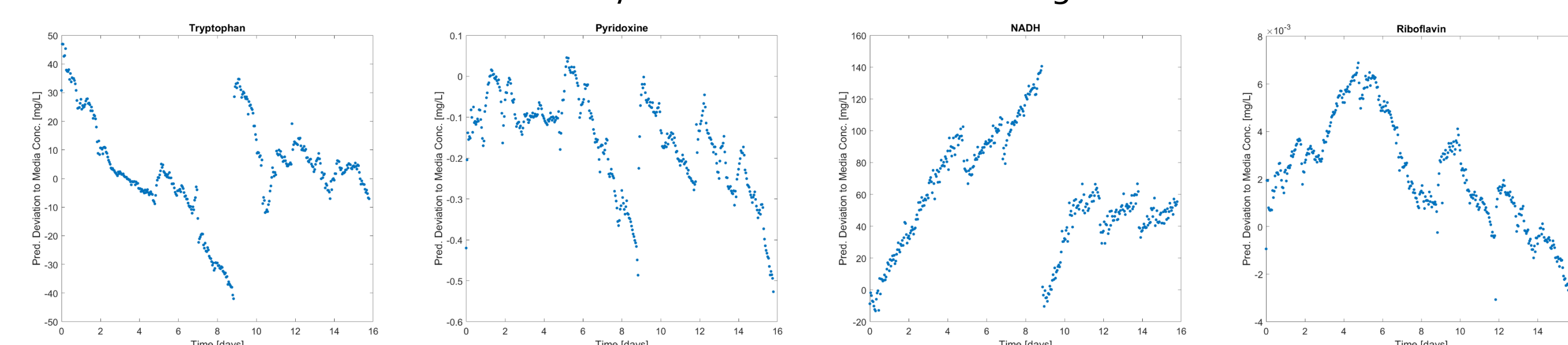
Based on the multilevel offline measurements, Partial-Least-Square (PLS) regression models were established to predict the concentrations of the individual analytes from the corresponding fluorescence data. At high Tryptophan concentrations, signal saturation was observed. The remaining three analytes showed a good fit with regression slopes  $\geq 0.974$ , indicating a high prediction quality. However, the NADH model required 7 principal components (PCs) due to the inner filter effect with riboflavin.



**FIG 3:** Comparison of measured and model-predicted concentrations of Tryptophan (2 PCs), Pyridoxine (1 PC), NADH (7 PCs), Riboflavin (1 PC).

### 4. Application of the chemometric model to online data

The final chemometric model was applied to online fluorescence data to predict changes in the concentrations of the four analytes. The results reveal decreasing levels of Tryptophan, Pyridoxine and Riboflavin, while NADH, as an intracellular parameter increased over time with rising cell concentration. Spikes in the calculated data were caused by feed additions, which either introduced fresh analytes or diluted them through media addition.



**FIG 4:** Predicted deviation to medium concentration in mg/L over the entire time of 16 days of a TIL cultivation. Reseeding step on day 9.

## OUTLOOK

Future work towards establishing the final SMARTER biomanufacturing platform will focus on validating the developed models through offline LC-MS quantification of the four analytes. This will enable not only the quantification of deviations in media composition, but also the determination of total concentrations through complementary fluorescence measurements. To enhance predictive accuracy and ensure robustness, the models will be further refined by incorporating larger and more diverse online datasets. In addition, the correlation between fluorescence signals and cell concentrations will be investigated with the aim of enabling real-time cell density prediction during cultivation. To broaden applicability, the current hardware setup is being expanded into a multiplex configuration, enabling online monitoring to be scaled from one to up to four vessels in parallel.