J. Dairy Sci. 99:1–9
http://dx.doi.org/10.3168/jds.2016-10947

Rapid quantification of casein in skim milk using Fourier transform infrared spectroscopy, enzymatic perturbation, and multiway partial least squares: Monitoring chymosin at work

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ABSTRACT

In this study, we introduce enzymatic perturbation combined with Fourier transform infrared (FTIR) spectroscopy as a concept for quantifying casein in subcritical heated skim milk using chemometric multiway analysis. Chymosin is a protease that cleaves specifically caseins. As a result of hydrolysis, all casein proteins clot to form a creamy precipitate, and whey proteins remain in the supernatant. We monitored the cheese-clotting reaction in real time using FTIR and analyzed the resulting evolution profiles to establish calibration models using parallel factor analysis and multiway partial least squares regression. Because we observed casein-specific kinetic changes, the retrieved models were independent of the chemical background matrix and were therefore robust against possible covariance effects. We tested the robustness of the models by spiking the milk solutions with whey, calcium, and cream. This method can be used at different stages in the dairy production chain to ensure the quality of the delivered milk. In particular, the cheese-making industry can benefit from such methods to optimize production control.
Key words: multiway, parallel factor analysis, cheese, infrared spectroscopy

INTRODUCTION

Fourier transform infrared (**FTIR**) spectroscopy and multivariate data analysis represent a versatile partnership for rapid but affordable quantitative analysis of the constituents of (bio-)chemical systems. Nevertheless, quantitative chemometric calibrations may generate misleading results and must be handled with great care. Occasionally, calibration models are established for constituents that cannot be clearly distinguished from others in the sample matrix. Such models may not be robust and may appear valid only because of collinearities between the analyte and the interfering constituent(s). When measuring casein in milk, for example, the usual amount of casein is approximately 80% of the total protein content. Casein content can therefore be determined by FTIR measurement of total protein (Hewavitharana and van Brakel, 1997; Etzion et al., 2004). However, these calibrations break down when the casein:total protein ratio varies significantly (Venyaminov and Kalnin, 1990). Instead of measuring the analyte of interest, the test determines the degree of collinearity (or covariance) between the 2 constituents, and accuracy depends on that underlying relationship. Other studies have shown that prediction of individual fatty acids in pork backfat using Raman spectroscopy and partial least squares regressions led to misleading results because iodine was measured instead of fatty acids (Berhe et al., 2016). The established correlations were indirect and governed by underlying covariance effects. Similar problems were reported when quantifying individual fatty acids in bovine milk using FTIR and chemometric methods such as partial least squares regression (Eskildsen et al., 2014).

It is therefore relevant to develop ways of resolving the analyte of interest from the underlying matrix before performing chemometric analysis. Such methods may include any kind of chemical change to the system that enables the analyte to stand out from the remaining constituents, including the sample matrix. Such methods should also be tested for robustness against constituents that have previously shown collinear behavior toward the analyte.

One method was reported recently in which an enzymatic perturbation was introduced to resolve interfering analytes using FTIR spectroscopy by adding a substrate-specific kinetic dimension to the analysis. The

Received January 26, 2016.
Accepted April 21, 2016.
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resulting data indicated yet another (kinetic) mode and were analyzed using numerical tensor decomposition methods such as parallel factor analysis (**PARAFAC**), Tucker3, or multiway partial least squares (**NPLS**) regression (Baum et al., 2016). Another method employed excitation emission spectroscopy, using an enzymatic-induced change to discriminate between the collinear-behaving analytes tyrosine and levodopa in human plasma (Xie et al., 2015). The PARAFAC method was used to establish third-order calibration models, yielding extraordinary distinguishing power.

In this paper, we introduce enzymatic perturbation as a tool for resolving interfering collinearities when measuring casein in skim milk using FTIR. Casein is a milk protein that is important for cheese-making. Because cheese yield depends mainly on casein concentration (Wedholm et al., 2006), it is important to develop robust determination methods as a way of optimizing the cheese-making process. Because the spectral fingerprints of casein and whey are hardly distinguishable (Susi and Byler, 1986; Kong and Yu, 2007), chymosin can be used to target the analysis by means of specific enzymatic hydrolysis. Chymosin is a protease found in rennet that catalyzes the conversion of κ-casein in milk to para-κ-casein by removing a glycopeptide from the soluble casein (Lucey and Fox, 1993; Langholm Jensen et al., 2013). As a result of this enzymatic conversion, all caseins coagulate to form a creamy precipitate combined with calcium, and whey proteins remain in the supernatant. The resulting cheese curd coagulation can be measured in situ using FTIR. To visualize the spectral changes, the initial spectrum (corresponding to the entire chemical background) can be subtracted from the entire time-resolved series to yield a spectral landscape that represents the enzyme-induced perturbation. We have used such visualizations in this paper and refer to them as evolution profiles (Baum et al., 2013b). Evolution profiles for 2 proteases are given in Figure 1. Both enzymes—chymosin and metalloprotease Prt1 from *Pectobacterium carotovorum*—hydrolyze proteins in milk, but they can be distinguished by the fact that chymosin cleaves solely caseins, whereas the metalloprotease is not protein-specific (Feng et al., 2014). The 2 profiles are clearly distinguishable, because parts of the spectral evolution are missing for chymosin, indicating its selectivity toward casein. In addition, the intensity of the spectral evolution stands in relationship with corresponding reaction rates and is, therefore, linked to underlying enzyme kinetics.

We hypothesized that targeted enzymatic perturbation, combined with in situ FTIR measurement and multiway analysis, would represent a powerful methodology for resolving spectral collinearities when establishing quantitative calibration models. Specific casein calibrations would rely on time-resolved measurement of milk coagulation induced by chymosin and would show robustness against varying whey, cream, and calcium levels.

MATERIALS AND METHODS

Materials

Lacprodan 80 (whey protein concentrate) was obtained from Arla Foods Ingredients P/S (Brabrand, Denmark). Calcium chloride and pectin extracted from citrus peel (galacturonic acid ≥74%) were purchased from Sigma Aldrich (St. Louis, MO). Fresh cream containing 38% fat was purchased in a local supermarket (Arla Foods Ingredients P/S). A commercial chymosin preparation, ChyMaxM1000, was obtained from Chr. Hansen (Hørsholm, Denmark).

Reagents

Calcium chloride was solubilized in deionized water at a concentration of 54 m*M*, and 9% (wt/vol) whey solution was prepared by slowly adding the protein to deionized water at 40°C. Magnetic stirring was applied for 30 min. Pectin was solubilized using the same procedure at a concentration of 1% (wt/vol). Chymosin was used undiluted in all experiments, corresponding to 8 international milk clotting units (**IMCU**).

Samples

Skim milk samples were obtained from Arla Foods Ingredients P/S. The samples were result of a process

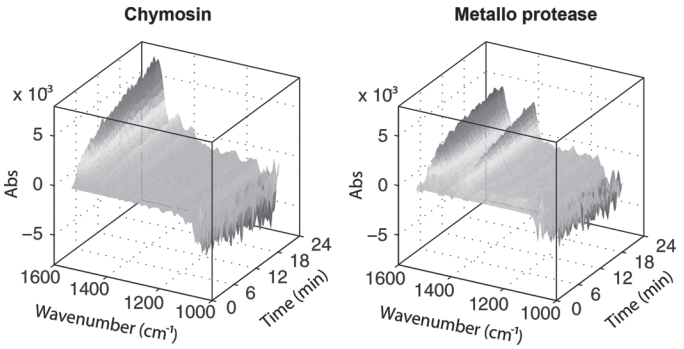

Figure 1. Fourier transform infrared spectra can be measured during the time-course of an enzymatic reaction resulting in a spectral evolution profile (utilizing difference spectra). The spectral evolution profiles for 2 proteases (commercial chymosin and metallo protease from *Pectobacterium carotovorum*) appear different. Such specific spectral landscapes can be used to distinguish between enzyme activities (including side activities). Color version available online.