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Milk renneting: study of process factor influences by FT-NIR spectroscopy and chemometrics

--Manuscript Draft--

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Abstract:	The dairy industry is continuously developing new strategies to obtain healthier dairy products preserving expected properties. However, when modifying a food process, the reassessment of each parameters and their interaction should be considered as highly influencing the final quality. Among others, rennet process features are fundamental for both sensory properties and typical characteristics of a cheese. In this contest, the research addresses the development of a FT-NIR spectroscopic method, coupled with chemometrics, for the study of the effect of process variables on milk renneting. The effects of temperature (30 °C, 35 °C, 40 °C), milk fat concentration (0.1, 2.55, 5 g/100 mL), and pH (6.3, 6.5, 6.7) were investigated by means of a Box-Behnken experimental design. FT-NIR data collected along the 17 trials were explored by interval-PCA (i-PCA) and ANOVA–Simultaneous Component Analysis (ASCA). i-PCA revealed differences in the occurrence and trends of coagulation phases, related to the three considered factors. ASCA allowed the characterization of renneting evolution and the assessment of the factor role, demonstrating that main and interaction effects are significant for the process progress. The proposed approach demonstrated that i-PCA and ASCA on FT-NIR data, highlighting the effects of the operating factors, allow a rapid and accurate analysis of process modifications in cheese manufacturing.

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Title: **Milk renneting: study of process factor influences by FT-NIR spectroscopy and chemometrics**

Reviewer #1:

The manuscript "Milk renneting: study of process factor influences by FT-NIR spectroscopy and chemometrics" (FABT-D-19-00005) submitted to Food and Bioprocess Technology: An International Journal, reports the results of the study of the influence of temperature, milk fat concentration, and pH on milk renneting using FT-NIR spectroscopy and chemometrics. Examination of the manuscript showed that the following has to be addressed in order to improve the overall quality of the manuscript.

Firstly, we would like to thank the Reviewer for her/his valuable comments that have been addressed in the present document. The manuscript has been modified accordingly, reporting in red all the changes.

1. It is hypothesized that FT-NIR could be an approach for in-line monitoring in dairy industry (Lines 105-107, Page 5). Does this mean that FT-NIR spectroscopy has not yet been used to study dairy products? If not, previously conducted studies should be referred in Introduction. The chemometric tools presented in Introduction are well known and addressed well enough, while FT-NIR spectroscopy and its specific features and utilization as an analytical tool to study foods, especially dairy products, are presented insufficiently.

FT-NIR approach has been applied before for different purposes in the food sector. Actually, the implementation in the dairy sector has been previously studied for milk and cheese composition and quality attributes. However, there is still a lack of its implementation for real time assessment to determine how process and product respond under different conditions. Thus, as suggested by the Reviewer, a literature revision has been performed and references have been added to enrich the introduction (lines 71-79).

2. Section 3.1. cannot be reviewed properly since Fig. 2 to which is referred mostly in this section has to be redrawn in order to better differentiate the spectra. In the presented form it is not possible to examine the spectra depending on the factors considered. Probably, those portions of the spectra

which were submitted to the statistical analysis only should appear in Fig. 2, while the other parts not considered in the calculation are removed.

We agree with the reviewer. We followed her/his suggestion, but instead of removing some regions, we modified Figure 2 by adding an enlargement of the most significant spectral regions for each investigated factor. Please, see the new Figure 2 and integrated comments at lines 209-213, 217-2019 and 225-229.

3. Band assignment is important in the analysis of the spectral data. In this sense in Section 3.1 some more detailed discussion should be added regarding specific bands observed in the spectra which can give information on particular molecules, structural elements of molecules and milk constituents. Some bands are discussed in this section (Lines 202-206, Page 9), but not sufficient.

We are aware that it is important to perform an appropriate band assignment when dealing with spectral data, so we did enrich the text in Section 3.1 by adding peak assignments related to both chemical and physical milk nature (lines 230-259). However, it is difficult to get a perfect correlation between the absorption at a single wavelength and the concentration of each milk component by NIR absorptions. Indeed, this wavelength range is mainly composed by broad and overlapped bands.

4. Fig. 4 can be removed since it is discussed not much and the comments on Page 13 are sufficient.

Figure 4 has been removed as suggested and the text has been modified accordingly at lines 336-338.

Reviewer #2:

Dear Authors,

In my opinion the work is good. I will only suggest some changes that in my opinion will enrich your work.

Firstly, we would like to thank the Reviewer for her/his valuable comments. They have been addressed in the present document and the manuscript has been modified accordingly, reporting in red all the changes.

1) How the monitoring of the milk coagulation process in the production system is currently done. It is interesting to do this analysis so that a reader who is not from the area have the real vision of how the technique has improved the productive sector.

We agree with the Reviewer. We have added information about the techniques normally used in dairy industry for curd evaluation at lines 52-61.

2) Although the physical process of FT-NIR spectroscopy is well known, it is interesting to briefly describe it in the text since you have used the technique to monitor the coagulation of milk under the influence of different factors. There is no need to add more than one paragraph.

Following Reviewer suggestion and as requested also by Reviewer #1, we enriched the introduction of the manuscript with basic information about NIR spectroscopy theory and some examples of implementation in the dairy sector (lines 65-79).

3) Although all the chemometric analysis is well based, it would be interesting to explain better about the spectral changes of the FT-NIR spectrum that occur during the milk coagulation process. It seems that a lot of chemometrics is used without having the real mastery of physical information.

We would like to thank the Reviewer for her/his comment. Details about scattering effect in NIR response due to radiation redistribution linked to specific physical properties have been added in the Section 3.1 at lines 247-256.

Reviewer #3:

Very similar study was published on the book chapter by the group. "Strani, L., et al. "FT-NIR spectroscopy to monitor rennet coagulation in milk with different fat levels." Simposio Italiano di Spettroscopia NIR. Società Italiana di Spettroscopia NIR, 2018."
I mainly concern the novelty of the study. Please address. Also, the figure quality need to be improved.

We would like to thank the Reviewer for her/his comments. Actually, the cited book chapter is just an abstract of an oral presentation given during the Italian symposium of NIR spectroscopy. Besides, the work presented at the Symposium largely differs from the results presented in the present manuscript. Indeed, for the Symposium a Response Surface Methodology approach was used to evaluate factors' influence on the process. This approach, particularly appropriate for discrete data, resulted unsuitable for continuous data such as NIR spectra. Thus, a completely independent data

analysis was performed and presented in the manuscript submitted to FABT. Therefore, we guarantee the novelty of the presented results.

Concerning figures, we have changed Fig. 2 according to Reviewer #1 comment; however, we do believe the other figures fulfil the journal requirements when the full quality version is downloaded from the pdf generated for proof reading. In any case, any possible technical issues raised by the editorial office will be solved.

Milk renneting: study of process factor influences by FT-NIR spectroscopy and chemometrics

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Abstract

The dairy industry is continuously developing new strategies to obtain healthier dairy products preserving expected properties. However, when modifying a food process, the reassessment of each parameters and their interaction should be considered as highly influencing the final quality. Among others, rennet process features are fundamental for both sensory properties and typical characteristics of a cheese. In this contest, the research addresses the development of a FT-NIR spectroscopic method, coupled with chemometrics, for the study of the effect of process variables on milk renneting. The effects of temperature (30 °C, 35 °C, 40 °C), milk fat concentration (0.1, 2.55, 5 g/100 mL), and pH (6.3, 6.5, 6.7) were investigated by means of a Box-Behnken experimental design. FT-NIR data collected along the 17 trials were explored by interval-PCA (i-PCA) and ANOVA–Simultaneous Component Analysis (ASCA). i-PCA revealed differences in the occurrence and trends of coagulation phases, related to the three considered factors. ASCA allowed the characterization of renneting evolution and the assessment of the factor role, demonstrating that main and interaction effects are significant for the process progress. The proposed approach demonstrated that i-PCA and ASCA on FT-NIR data, highlighting the effects of the operating factors, allow a rapid and accurate analysis of process modifications in cheese manufacturing.

Keywords: milk renneting; dairy industry; near infrared spectroscopy; interval–PCA; ANOVA–Simultaneous Component Analysis; ASCA

1. Introduction

Recently, consumers' requests about healthier foodstuffs, such as reduced-fat dairy products, with similar properties (flavor, texture and firmness) to the traditional ones, have increased (Johnson et al. 2001). For this reason, dairy companies need efficient tools to control the fundamental steps of cheese making processes and to tune the process parameters to optimize the quality of the final product. Among dairy processes, coagulation is one of the most critical steps, making its monitoring a very important task for the dairy industry (Sbodio et al. 2002). Indeed, it is crucial to assess the optimal curd coagulation time and, in general, the behavior of milk during renneting, especially in case of recipe modification, as these parameters are fundamental for both sensory properties and typical characteristics of cheese (Martin et al. 1997). Normally, these properties are evaluated both visually, by expert cheesemakers breaking manually a little fraction of the curd, or by laboratory analyses. Formagraph is one of the most used equipment to assess milk coagulation properties, able to describe rheological changes during renneting, such as modifications of curd firmness (Visentin et al. 2015). Another largely used instrument is Optigraph, a single wavelength near infrared (NIR) benchtop instrument that can provide results comparable to those obtained by Formagraph (Cipolat-Gotet et al. 2012). However, these techniques are not applicable on-line, thus they cannot provide information useful for a real time control of the process. Real time monitoring will give the possibility to reduce the number of subjective and/or complex analyses and, further, it can ensure a persistent final product quality (Kondakci & Zhou 2017; Henihan et al. 2018). NIR is a technique that can satisfy these requirements because it is able to assess the principal compounds involved in the process (Woodcock et al. 2008; Shao & He 2009), and to assure an efficient control of every stage of the

process through the description of its trend (Grassi et al. 2014). Actually, NIR spectroscopy is a type of vibrational spectroscopy and, being fast, non-destructive, and non-invasive, it can be used for analyses on the production line. Nearly any molecule containing CH, NH, SH, or OH bonds can be detected, and several constituents can be measured simultaneously. However, because of the wide, overlapping peaks and weak absorbances, chemometric techniques are required to extract the useful information (Nelson 2018). It has been demonstrated that the use of this technique could be extremely convenient for dairy industry. Indeed, by NIR analyses it is possible to assess quickly and efficiently the composition and the desired characteristics of cheese products, such as dry matter (Wittrup & Nørgaard 1998), as well as crude protein and fat content (Čurda & Kukačková 2004). Even aging, sensory attributes (Downey et al. 2005), and shelf life (Cattaneo et al. 2005) can be assessed by NIR approaches. There are also several works in which NIR spectroscopy is used to evaluate features and composition of dairy raw materials, namely milk (Kasemsumran et al. 2007) and milk powder (Cama-Moncunill et al. 2016). Besides, the development of NIR fiber optic probes to be placed directly into the coagulation vats, eliminating the need of sample pretreatment, allows to obtain real time information (Laporte et al. 1998).

When modifying a food recipe, process variables should be reassessed considering their high influence on the final product quality. To this aim, experimental design techniques are excellent tools to determine how process and product respond under different conditions and to assess the best operating settings. Data collected from designed experiments are usually examined by multi-factor Analysis of Variance (ANOVA) in order to evaluate whether the effect of each factor (and of factor-factor interactions) on the observed experimental variability could be deemed significant or not (Kirk 1982).

Nevertheless, since ANOVA is a univariate method, it is not effective when applied on spectral data due to their inherent multivariate nature; in fact, the joint variability among different descriptors (covariance) must be considered to obtain comprehensive results. On the other hand, the systematic correlated variation in a multivariate dataset can be effectively captured and summarized by Principal Component Analysis (PCA), through the projection of the observations onto a reduced (parsimonious) subspace of latent variables (Jackson 1980). Furthermore, when the variance provided by small bands is covered by variance of larger bands, an efficient approach is the interval-PCA (i-PCA), which permits to analyze small spectral ranges and to highlight the variability due to bands of interest, independently on the variance of the whole spectrum. However, since in the analysis of data coming from designed measurements PCA does not consider the underlying experimental scheme in parameter estimation, its use in such problems would not be effective without the support of other methods. Accordingly, several approaches coupling ANOVA decomposition with a bilinear description of the partitioned variance, such as MANOVA (Ståhle and Wold 1990), PC-ANOVA (Bratchell 1989), ANOVA-Simultaneous Component Analysis (ASCA) (Smilde et al. 2005), ANOVA-PCA (Harrington et al. 2005), ANOVA-Target projection (Marini et al. 2015), Regularized MANOVA (rMANOVA) (Engel et al. 2015), have been proposed in the literature for the analysis of multivariate data coming from designed experiments. However, MANOVA has been criticized due to the incapacity of handle datasets with a number of variables larger than samples. Similarly, the addition of the residual matrix to the effect matrices before PCA may result in a not completely straightforward interpretation of ANOVA-PCA models. The other methods have been developed to overcome these problems, and they have been used in several works (Imram 1999;

Ullah and Jones 2015). In particular, ASCA allows to study the variance of data coming from an experimental design by splitting the variation and performing a Simultaneous Component Analysis (SCA), making possible to identify the most significant factors. First an ANOVA is carried out to obtain effect matrices from the response matrix of the design, and secondly a SCA is performed on the effect matrices (Jansen et al. 2005). Obviously, to properly apply this method to spectral data, it is fundamental to choose the appropriate preprocessing techniques in order to minimize the undesired variability (Grassi et al. 2017). The aim of this work is to study the effect of process variables on milk rennet coagulation by FT-NIR spectroscopy (FT-NIRs) coupled with i-PCA and ASCA methods. The proposed approach overcomes the existing PAT tools for quality assurance applied to the dairy industry (Woodcock et al. 2008; Henihan et al. 2018), thus filling a relevant knowledge gap in this field.

The hypothesis is that FT-NIRs can be a useful approach to provide dairy industry with an efficient methodology for process in-line monitoring and study of the operating condition contribution.

2. Materials and Methods

2.1. Design of Experiments

The study of milk renneting was carried out based on a 3-factor and 3-level Box-Behnken experimental design, including 12 trials and 5 replicates of the central point, performed in a random order to minimize the risk of systematic errors. The experimental factors and the levels taken into account were temperature (30 °C, 35 °C, 40 °C), milk fat content (0.1, 2.55, 5 g/100 mL) and pH (6.3, 6.5, 6.7). A schematic representation of the design is shown in Fig. 1.

2.2. Milk preparation and coagulation

Fresh skimmed milk and fresh cream were suitably combined to obtain milk with different fat concentrations. Skimmed milk had a fat content of 0.1 g/100 mL, while cream, obtained by centrifugation, had a fat level of 35 g/100 mL. Skimmed milk-cream mixtures (100 mL) were poured in a Pyrex glass flacon and placed in a cold store room under stirring conditions on a magnetic plate for 12 h in order to obtain a homogeneous sample. Afterwards, samples were conditioned at 20 °C and added with $CaCl_2$ (final concentration, 3 μM). Citric acid (5 M) was used for pH correction to the desired value (monitored through a previously calibrated 3510 pH-meter, Jenway, Dunmow, England). To maintain the selected design temperatures (30 °C, 35 °C, 40 °C), samples were introduced in a thermostatic bath (MR Hei-Standard, Heidolph Intruments GmbH, Schwabach, Germany). Then, 35 μL (175 IMCU/mL) of liquid rennet (Linea Rossa, Caseificio Clerici, Cadorago, Italy) composed of 75% chymosin and 25% bovine rennin, were added and coagulation was monitored for 40 min.

2.3. FT-NIR spectroscopy

Milk renneting was monitored by a FT-NIR spectrometer (MPA, Bruker Optics, Milan, Italy) through a fiber optic probe equipped with a transflectance adapter (1 mm pathlength) inserted directly in the sample. Spectra were collected every 60 s over the 12500 – 4000 cm^{-1} range, with 64 scans for both sample and background and a nominal resolution of 8 cm^{-1}. Instrument control was managed by using the OPUS software (v. 6.0 Bruker Optics, Milan, Italy).

2.4. Data analysis

Data preprocessing, PCA, i-PCA and ASCA models were performed with routines and toolboxes implemented in Matlab environment (the Mathworks Inc., Natick, MA, USA).

2.4.1. i-PCA

Data exploration was applied to extract useful information, linked to the tested experimental factors, about the behavior changes in the different coagulation phases. The spectra obtained from each trial were organized in as many datasets (40x2203); furthermore, a dataset containing the spectra of all trials (680x2203) was built. A PCA was performed to choose spectral ranges to be further considered. Lately, interval-PCA method was applied to extract relevant information from smaller and most significant parts of the spectrum and to exclude ranges that may contain noise and undesired signal. To this purpose, no matter the considered dataset, the FT-NIR spectra were divided in three different regions, each of them submitted to PCA. In particular, the regions between 7180 cm^{-1} and 6464 cm^{-1} and between 5823 cm^{-1} and 4000 cm^{-1} were discarded. The first range went from 12500 cm^{-1} to 9200 cm^{-1}, the second from 9199 cm^{-1} to 7181 cm^{-1} and the third from 6463 cm^{-1} to 5824 cm^{-1}. Prior to i-PCA, spectral ranges were pretreated by standard normal variate (SNV), smoothing (Savitzky-Golay method, filter width: 9 points; polynomial order: 1) and mean centering.

2.4.2. ASCA

ASCA (Jansen et al. 2005) was used to detect possible significant effects of the experimental factors and of their interactions on the FT-NIR spectral profiles. In

particular, to fully characterize the evolution of the coagulation and the role of the investigated factors (temperature, fat content and pH) across the process, time was included as the fourth design factor. Therefore, 10 different time levels, corresponding to a spectrum collected every 4 min, were considered, obtaining a final design matrix whose dimensions were 130x4.

In order to apply ASCA to a balanced set of measurements, for the five replicates of the central point five datasets were created, each containing one replicate along with the other 12 trials in the 12500 - 7181 cm^{-1} and 6463 - 5824 cm^{-1} ranges, i.e. merging the spectral ranges considered for i-PCA. The dataset for ASCA had thus the dimensions of 130x1545. ASCA was performed using the same pretreatments described in § 2.4.1 and the significance of the effects of each design term was tested by means of permutation tests with 1000 randomizations.

3. Results and Discussion

3.1. FT-NIR spectra

To better understand the influence of the three experimental factors (temperature, milk fat content, and pH) over the coagulation time, SNV pretreated spectra of the beginning (after 1 min), the transition phase (coagulum formation) and the end (40 min) of the coagulation averaged according to fat content, pH, or temperature for each considered time are shown in Fig. 2. The different lines refer to different acquisition times: solid lines represent the averaged factor level spectra, acquired 1 minute after the beginning of the coagulation trial; dashed lines are averaged factor level spectra collected during the formation of the coagulum (transition phase of coagulation process); the dotted lines represent the averaged factor level spectra that have been acquired at the end of the

process (40 min). Fig. 2 (a,b and c) show how temperature mostly affects the spectral ranges 12500-9000 cm^{-1} and 7000 - 5500 cm^{-1}, but mainly in a longitudinal fashion (i.e., across time). Indeed, the relative absorbance decreases with coagulation time in the range 12500-9000 cm^{-1} (Fig. 2b), whereas it increases in the region between 7000 and 5500 cm^{-1} (Fig. 2c). Furthermore, spectra collected after 40 min at 35 and 40 °C are almost identical, whereas the ones corresponding to 30 °C are slightly different in terms of absorbance. Differences among spectra acquired from skimmed milk (0.1 g/100 mL of fat) and milk samples with 2.55 g/100 mL and 5 g/100 mL of fat are highlighted in Fig. 2 (d, e and f). Large differences can be noticed between the coagulation trend of skimmed milk averaged spectra and samples with higher fat content in the region between 10800 and 9000 cm^{-1} (Fig 2e). The most relevant difference can be noticed examining the bands at 4332 and 4258 cm^{-1} (Fig. 2f) present in case of milk samples with fat concentration higher than 2.55 g/100 mL and directly linked to the fat absorbance (Brandao et al. 2010; Núñez-Sánchez et al. 2016). As far as pH is concerned, in Fig. 2 (g) it is possible to see that there are no visible differences between spectra collected at the beginning of the coagulation process at pH values of 6.5 and 6.7, whereas in the following times the differences are enhanced. In particular, relative absorbance decreases in the region 12500 – 15000 cm^{-1} along with coagulation progress for all the tested pH (Fig. 2h). Moreover, spectra collected at the beginning of the coagulation at pH 6.7 and at pH 6.5 show different spectral shape in correspondence of band with maxima at 5150 cm^{-1} (Fig.2 i).

Even if some differences are visible in the spectra obtained with different experimental conditions and over coagulation time, it is difficult to get a perfect correlation between the absorption at a single wavenumber and the concentration of each milk component.

Indeed, all the main milk components, such as fat, proteins, and water, absorb in the NIR region as they are constituted of C–H, N–H, O–H and C=O bonds, which arise bands between 12500 and 4000 cm^{-1} (Workman & Weyer 2007). Thus, molecule absorptions in the NIR region are overtones and combinations of fundamental vibrations, resulting in broad and overlapped bands. Some attempts of chemical band assignment have been reported in literature. Bands at 10400, 6900, and 5150 cm^{-1} can be ascribed to the O-H first overtone of water and O-H combination bands. Signals at 5700 cm^{-1} are linked to the presence of lactic acid and lactose (Workman and Weyer 2007; Wang et al. 2015). Other relevant bands were found at 10800 and 8600 cm^{-1}, ascribable to the lipid C-H bonds (Tsenkova et al. 2000). A review by Holroyd (2013) deeply investigated band assignments in liquid milk. From a broad literature survey, the author assigned the protein N-H absorption to the regions at 1100-9700 cm^{-1}, 5690-5800 cm^{-1}, 4550-4350 cm^{-1}, and around 4300 cm^{-1}, and the lipid O-H and N-H absorptions to around 4800 cm^{-1} and 4200 cm^{-1} (Holroyd 2013).

Moreover, NIR response is affected by light scattering that is a physical effect due to radiation redistribution inside a medium characterised by specific microstructural properties. It has been previously investigated how light scattering affects the whole NIR range (Cattaneo et al. 2009). Scattering phenomena in milk are largely due to size and number of suspended fat globules (Cabassi et al. 2013). To a smaller extent, casein micelles are responsible for the increase of the bulk scattering coefficient in NIR spectra (Aernouts et al. 2015). In particular, Aernouts et al. (2015) found for both content of fat globules and casein micelles a negative correlation with the water absorption band at 6900 cm^{-1}, while a positive correlation characterised the NIR range between 12500 and 9000 cm^{-1}.

Due to the complex nature of NIR signal, multivariate approaches are required to better evaluate the differences among spectra and to assess the influence of the experimental factors and of their interactions on the coagulation process.

3.2. Occurrence and trends of milk renneting phases by iPCA

Prior to i-PCA, PCA was performed on the whole spectral range to discard spectral regions irrelevant to monitor the coagulation process. Fat bands at 4332 and 4258 cm^{-1}, even if relevant to evaluate fat influence in the milk coagulation performance, were discarded because they covered the greatest part of data variance. Then, for each of the 17 trials, three different PCA were carried out, one for every spectral range considered. Successively, the scores resulting from each i-PCA model were normalized from 0 to 1 in order to make a comparison among them possible. In parallel, a PCA was made on the block-wise augmented dataset containing all the trials; also in this case, the scores were extracted and normalized. Fig. 3 shows the score trends obtained from the single i-PCA models of all the trials: PC1 scores obtained from the first range models (solid lines) can be selected to describe the liquid behavior of milk, linked to the first phase of the coagulation process when the coagulum has not yet been formed (Grassi et al. 2014). In fact, in all the trials it is possible to see a decrease of PC1 scores with time evolution, indicating a progressive decrement of the liquid phase and the beginning of the curd formation. Trends highlighted by the dashed lines, related to the PC2 scores obtained from the second range models, can be ascribed to the second phase of the coagulation process, when the clot begins to form. In particular, the peak of these profiles corresponds to the coagulation time.

Trends and phase occurrence times are strongly related to temperature, fat, and pH levels. High temperatures and low pH values allow to reach this point faster (Zoon et al.

1988; Sbodio et al. 2002), as confirmed by T7 (Fig. 3), even if this trial presents overlapped trends for the first and second spectral range. Moreover, the comparison of renneting trials carried out with the same milk fat content confirms that, at lower temperature and higher pH values, the transition phase is delayed; this can be observed, for instance, for the first four trials (T1-T4, fat level = 0.1 g/100 mL). Furthermore, the trials with longer coagulation times are T1 and T6, and this is possibly caused by the lowest temperature level (30 °C) combined with the higher pH values (6.5 and 6.7).

The PC1 scores of the i-PCA models calculated with the third spectral range look promising in evaluating the last phase of the coagulation process, when the curd eventually reaches the maximum of its consistency (dotted lines in Fig. 3). These scores present a reverse trend in comparison with the ones related to the first range, confirming that they describe the solid-like behavior of milk during renneting. Three of the four trials carried out with a milk fat content of 0.1 g/100 mL (T1, T2, and T3) show a decreasing trend after 20 min, when the coagulum is completely formed, and this could be explained by the disruption of the curd resulting in a decrease of the solid component. However, further analysis must be carried out to have a more reliable explanation of this phenomenon. Also fat content affects the trends behavior of these scores: indeed, the higher the fat content in milk samples, the higher the noise.

In the lower right part of Fig. 3, the PCA loadings related to one of the replicates of the central point (R3) are reported as an example. The band with the largest influence on the scores linked to the first spectral range is at 10400 cm^{-1}, assigned to the stretching of O-H bond in water. Concerning loadings of the second spectral range, the most important wavenumbers describing sample variability are between 7400 and 7181 cm^{-1}, connected to combination bands of C-H bonds of fatty acids and carbohydrates (Subramanian et al.

2011). Lastly, scores of the third spectral range models are mostly influenced by the region between 6100 – 5824 cm^{-1}, ascribed to the presence of lactic acid and lactose (Wang et al. 2015).

Scores of the models obtained from the i-PCA performed on the block-wise augmented dataset containing all the trials were studied in order to assess if a single model is able to give the same results of the models based on separated trials. Similar results were obtained for the first and the third coagulation phases (using the PC1 scores of the first and third range, respectively), but PC2 scores of the second spectral range model could not be used to describe the transition phase (results not shown).

These results reveal that i-PCA on FT-NIRs data was able to discriminate the three different phases of the rennet coagulation process already described by Grassi et al. (2014) during lactic acid fermentation of yoghurt. Thus, i-PCA can be used to efficiently describe and control milk renneting under different operating conditions.

3.3 Investigation of the effect of experimental factors by ASCA

ASCA was performed with the aim of verifying if factors considered in the experimental design and their interactions have a significant influence on milk spectral profiles during renneting. Since five datasets, one for each of the different replicates of the central point, were investigated with ASCA, only the results of one of them are reported and commented in this work because the results obtained from the other datasets were extremely comparable.

The first step of ASCA is the decomposition of the total data variability into the individual contributions of as many effect matrices as the number of terms in the design. In particular, since time was also included as experimental factor (see § 2.4.2),

variability in the spectral dataset was split into 16 arrays: 4 accounting for the main effect of the experimental factors, 6 corresponding to the effect of all possible two-way interactions, 4 describing the effect of three-way interactions, 1 for the effect of the only possible four-way interaction, and 1 for the residuals. However, based on the aim of the present work, the successive stages of the investigation were limited only to the contribution of the main effects and the two-way interactions. First of all, the significance of the effects of experimental factors and their interactions was assessed. Permutation tests were performed to compare the experimental sum of squares for the effect matrices of the four main factors as well as "time x temperature" and "time x fat" interactions with their corresponding distributions under the null hypothesis. Results concerning the other interactions are not reported, because they show the same pattern, meaning that all the effects are significant for the spectral profile trend description.

Interpretation of the effects of the significant terms on the multivariate spectral profiles was accomplished through a simultaneous component analysis performed on each factor (or interaction) matrix. The histograms accounting for the score distribution on SC1 (explaining more than 93% of the total variability) for the three Box-Behnken experimental factors (temperature, fat content, and pH) after back projection of the residuals are represented in Figs. 4a, 4c, and 4e, whereas Figs. 4b, 4d, and 4f show the corresponding loadings together with their 95% confidence interval. The clear separation of the score distribution in the histograms is a further confirmation of the significant differences between the different levels of each experimental factor. Particularly, scores related to milk with 0.1 g/100 mL of fat are very far from the distributions of the other two levels, suggesting a relevant difference among those

samples. The relevant effect of fat globules on the rheological properties of the rennet gels has been reported also by Logan et al. (2014). For each design term investigated, in order to evaluate which are the regions of the FT-NIR spectrum which are mostly affected by a particular factor or interaction, the corresponding loadings were inspected. To this purpose, for each SCA model, the 95% confidence interval around each loading vector was calculated by a nonparametric bootstrap procedure, as reported by De Luca et al. (2016). In Figs. 4b, 4d, and 4f, the statistically significant spectral regions are represented by a solid line, whereas a dotted line indicates the parts of the signal associated to loadings statistically indistinguishable from zero. As far as the models associated to the effects of milk fat content and pH, the whole spectral range is significant, and the loading profiles are almost equal (Figs. 4d and 4f). On the contrary, the important ranges for temperature are 6250 - 6450 cm^{-1}, 7200 - 8800 cm^{-1}, and 10400 - 11600 cm^{-1}; they can be mainly ascribed to the absorption of proteins, fat, and water. Obviously, their variation is influenced by the aggregation degree of casein micelles. Temperature loadings have a reverse trend if compared to the fat and pH ones (Fig. 4b, 4d and 4f), suggesting that this factor has an opposite influence on the process with respect to the other two.

Time was evaluated projecting scores on SC1 (96.69%) and SC2 (2.95%) with the corresponding residuals, as shown in Fig. 5a. Spectra of samples acquired at the beginning of coagulation highly differ from the ones collected at the end, in agreement with the results obtained by i-PCA. Moreover, scores on SC1 show a continuous decrease over time with a clear slowing down after 20 min, suggesting that the major changes occur at the beginning of the process, as confirmed by Fig. 5b. This trend is similar to the one obtained from scores on PC1 of first range i-PCA models (Fig. 3),

which described the liquid behavior of milk during the coagulation process. Besides, the SC2 score pattern resembles the trend of PC2 scores obtained from models related to the second range of the i-PCA. Indeed, they show a maximum value in correspondence to the transition time; then, they start to decrease again until reacquiring negative values. Fig. 5c and 5d show the loading plots for SC1 and SC2, respectively, with the corresponding 95% confidence intervals. Also in this case, for both the SCs, the whole spectral region results statistically significant in describing the effect of time on milk renneting, thus confirming that the selected ranges (12500 - 9200 cm^{-1}; 9199 - 7181 cm^{-1} and 6463 - 5824 cm^{-1}) are the ones to be considered for process monitoring.

4. Conclusions

In the present study the possibility of assessing the influence on milk renneting of different process conditions, i.e. temperature, milk fat content, and pH, was addressed by coupling chemometric techniques with FT-NIRs. Interval-PCA confirmed the ability of FT-NIRs in discriminating the three different phases of the renneting process. Indeed, it was possible to model the phase before the coagulum formation by PC1 scores obtained from the first spectral range (12500 - 9200 cm^{-1}); the trends of PC2 scores of the second range (9199 - 7181 cm^{-1}) well modelled the clotting beginning; the PC1 scores of the third spectral range (6463 - 5824 cm^{-1}) looked promising to describe the last phase of the coagulation process. Moreover, a strong effect of temperature, fat, and pH levels was highlighted by i-PCA trends and times of phase occurrence.

ASCA applied to the spectral data assessed that the effects of experimental factors and their interactions were statistically significant. In particular, the simultaneous component analysis clearly demonstrated that milk samples with the lowest fat content

(0.1 g/100 mL) had a coagulation behavior significantly different from that of the other samples. Furthermore, the loadings evaluation, after a nonparametric bootstrap procedure, confirmed that the spectral ranges selected for i-PCAs are the strategic ones for milk renneting monitoring.

As assumed in the initial hypothesis, FT-NIRs, coupled with i-PCA and ASCA methods, demonstrated to be a valid approach to study the different phases of the renneting process and to assess the effect of temperature, fat content and pH. This study does the groundwork for the assessment of process parameter effects, thus giving to the dairy industry the opportunity of monitoring and studying the coagulation process when developing new strategies to obtain healthier dairy products.

Conflict of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Figure Captions for printed version

Fig. 1 Schematic representation of the Box-Behnken experimental design. The gray dot in the middle of the cube represents the five replicates (R) of the central point (temperature 40°C, milk fat content 2.55 g/100 mL, pH 6.5); experimental trials are indicated by the letter T followed by followed by an identification number

Fig. 2 SNV pretreated FT-NIR spectra of the beginning (solid lines), transition phase (dashed lines) and end (dotted lines) of milk renneting, averaged according to temperature (a, b and c), fat content (d, e and f), and pH (g, h and i). Different colors refer to a different level of the considered factor: black lines, lowest level; light gray lines, medium level; dark grey lines, highest level

Fig. 3 Score profiles obtained from the i-PCA models calculated for each milk renneting trial. Solid lines, PC1 scores of the 12500 to 9200 cm^{-1}range; dashed lines, PC2 scores of the 9199 to 7181 cm^{-1} range; dotted lines, PC1 scores of the 6463 to 5824 cm^{-1}range. The lowest right block shows the loadings of the third central point replicate (R3) for each of the three i-PCA models: solid lines, PC1 loadings related to first range model; dashed lines, PC2 loadings related to second range models; dotted lines, PC1 loadings related to third range models

Fig. 4 Histograms of ASCA score frequency with projected residuals along SC1 for the different levels of experimental factors considered in the study of milk renneting: temperature (a), fat content (c), pH (e). The corresponding loadings are shown in panels

b, d, and f (black lines) with 95% confidence interval (dashed gray lines). Statistically non-significant regions are represented with a black dotted line in the loading plots

Fig. 5 a) Score plot for the effect of renneting time (filled symbols) with projected residuals (empty symbols); b) SC1 (black line) and SC2 (gray line) score profiles along renneting time; c) loadings for SC1 (black line) with 95% confidence interval (dashed gray lines); d) loadings for SC2 (black line) with 95% confidence interval (dashed gray lines). Statistically non-significant regions are indicated with a black dotted line in the loading plots

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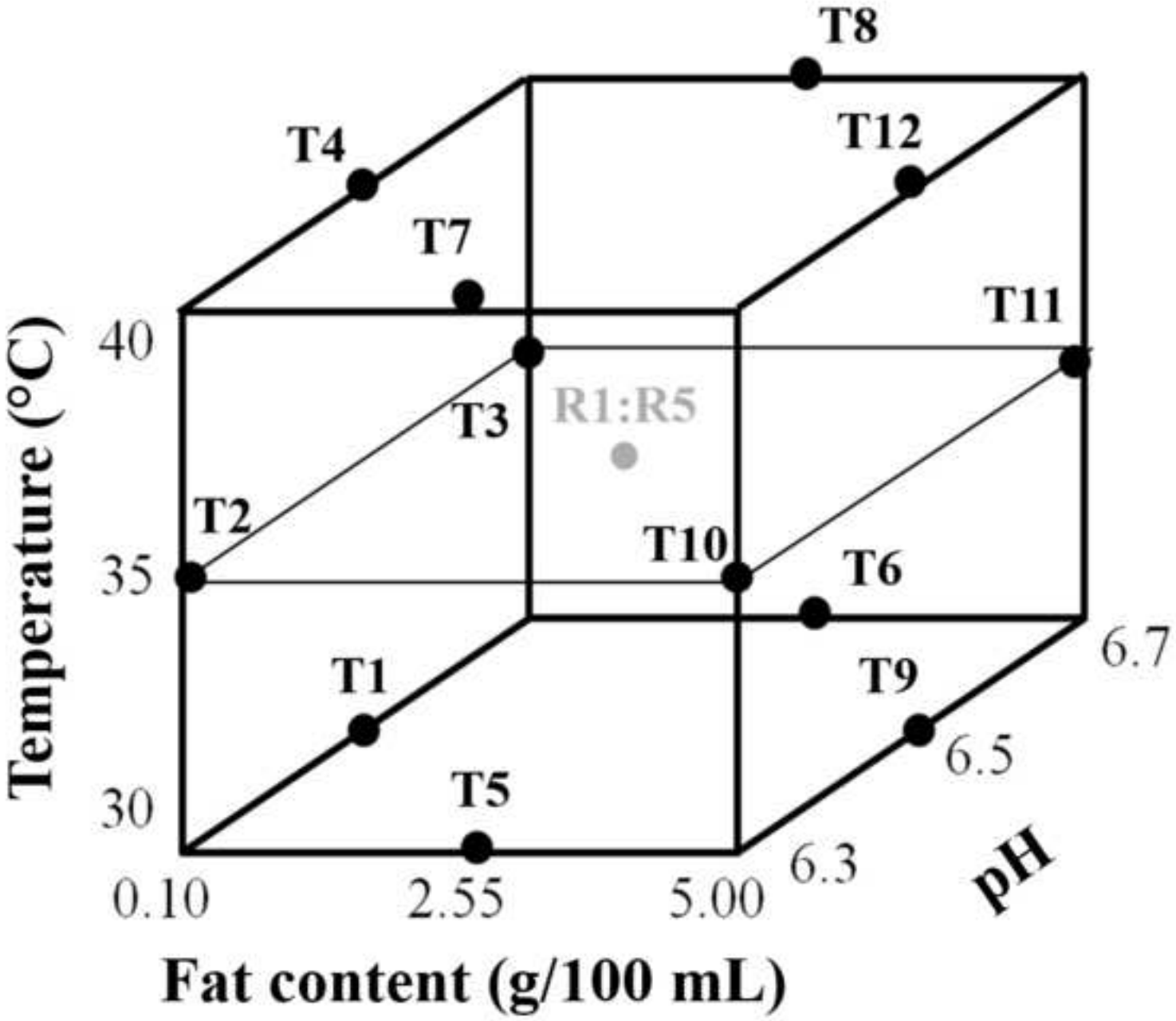
Fig. 5 a) Score plot for the effect of renneting time (filled symbols) with projected residuals (empty symbols); b) SC1 (blue line) and SC2 (red line) score profiles along renneting time; c) loadings for SC1 (green line) with 95% confidence interval (dashed red lines); d) loadings for SC2 (green line) with 95% confidence interval (dashed red lines). Statistically non-significant regions are indicated with a blue dotted line in the loading plots



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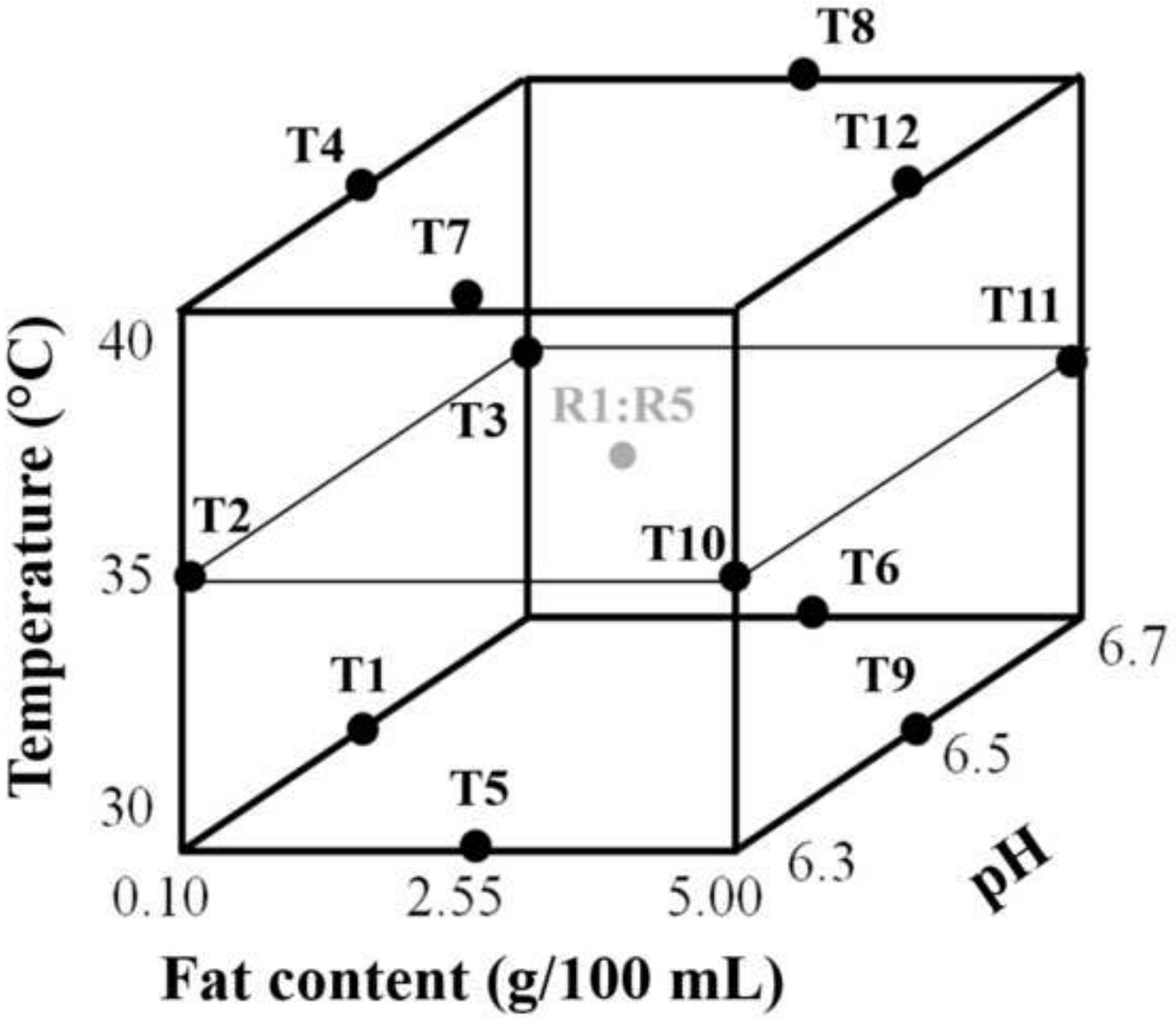

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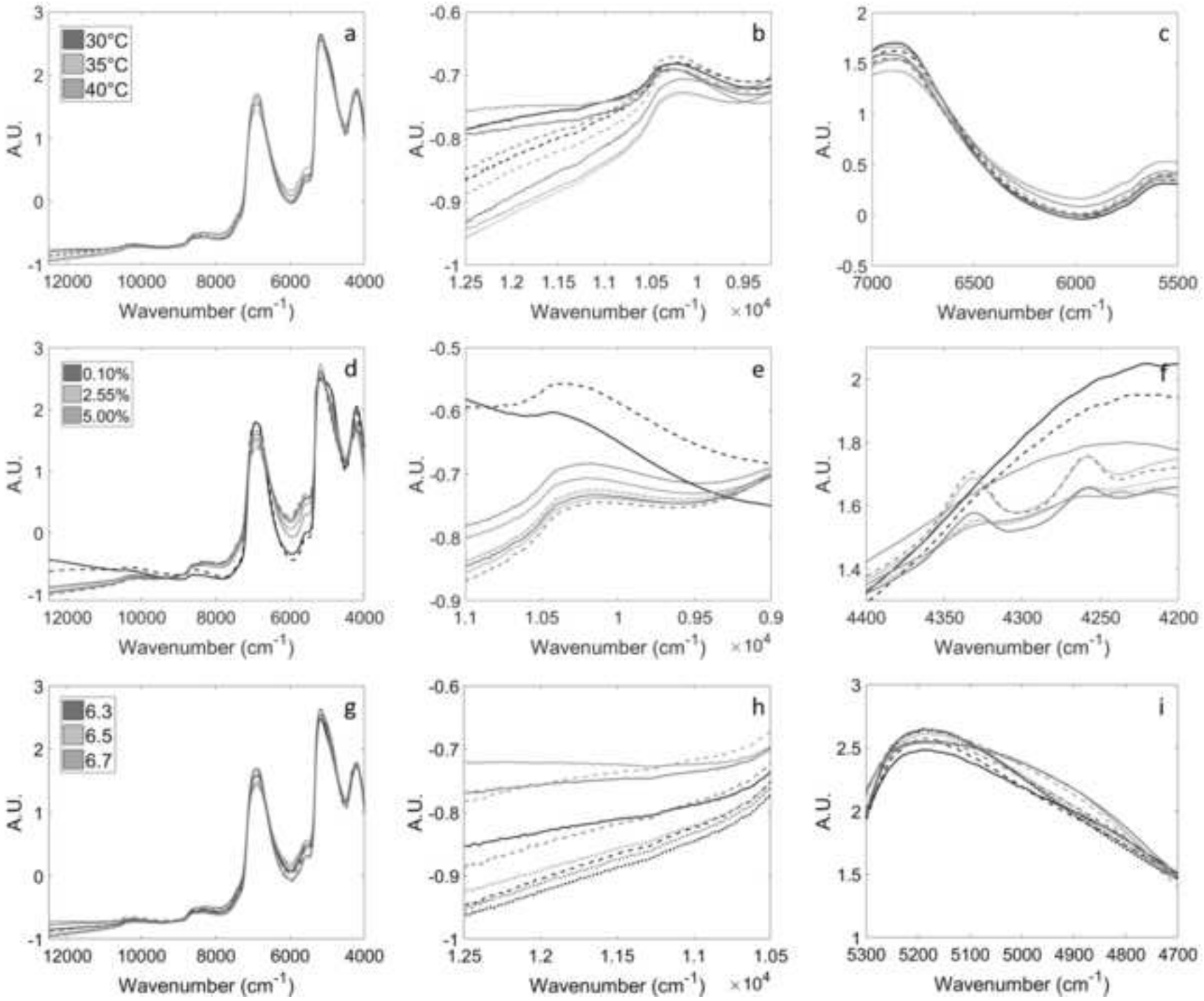

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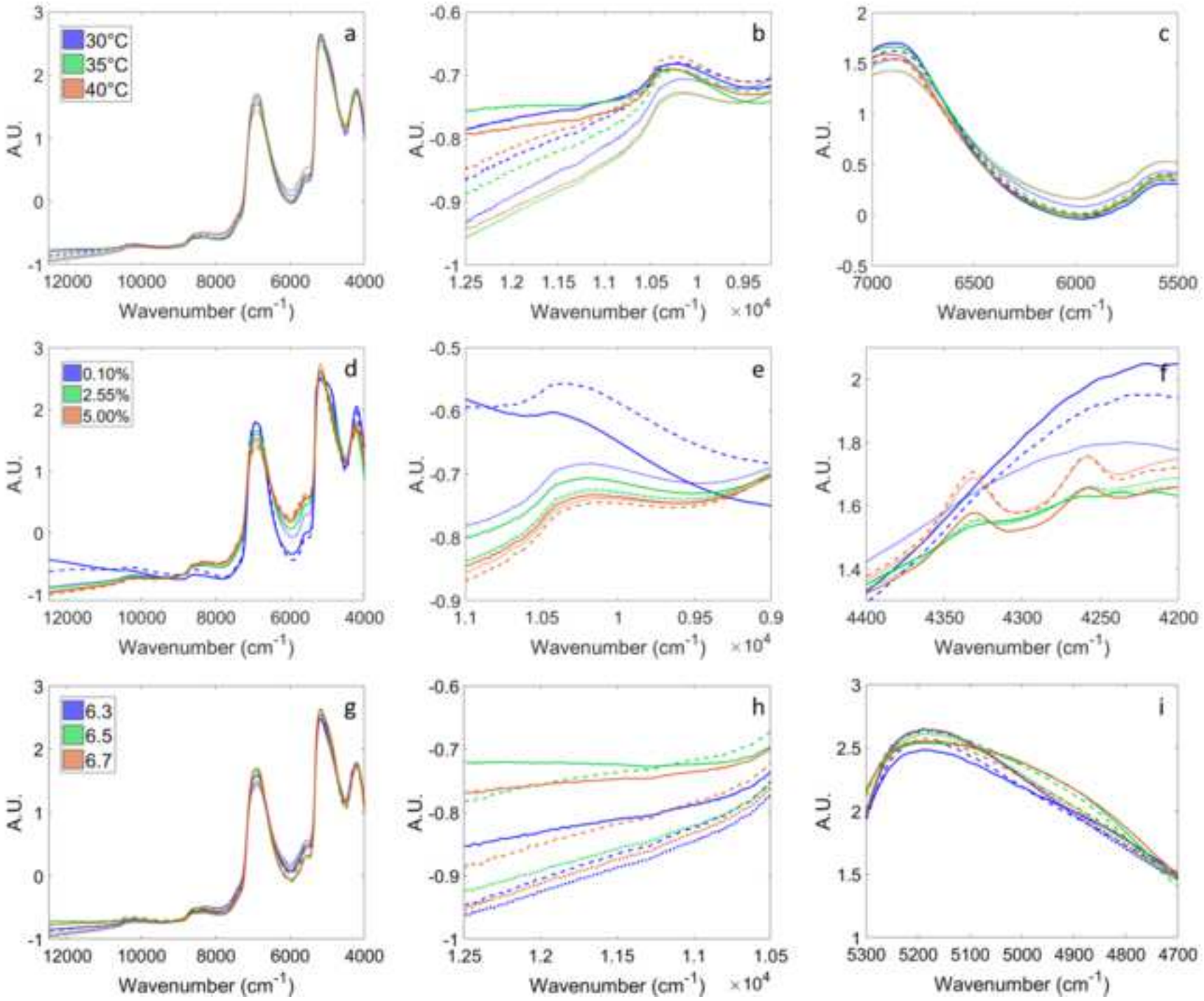

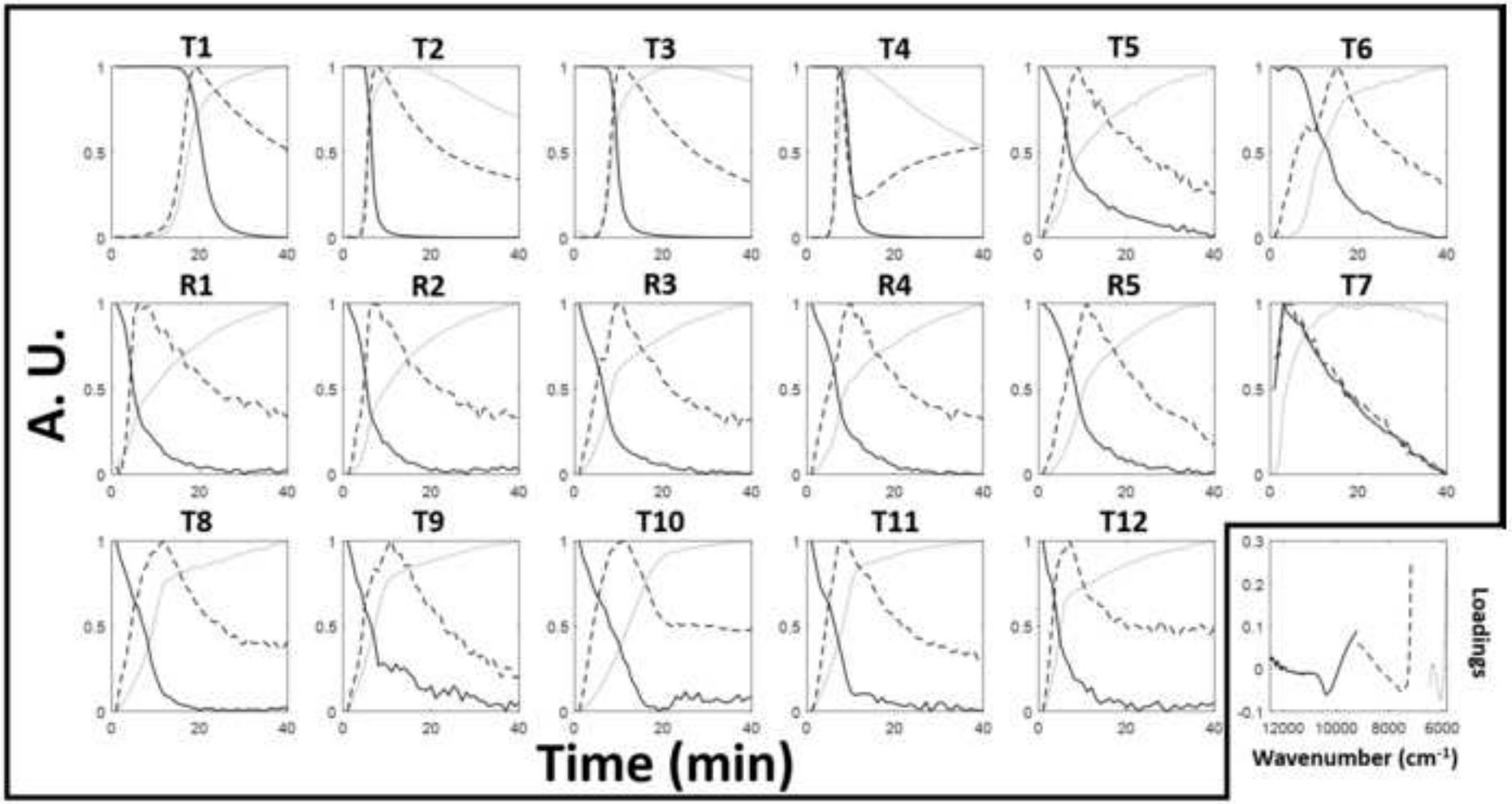

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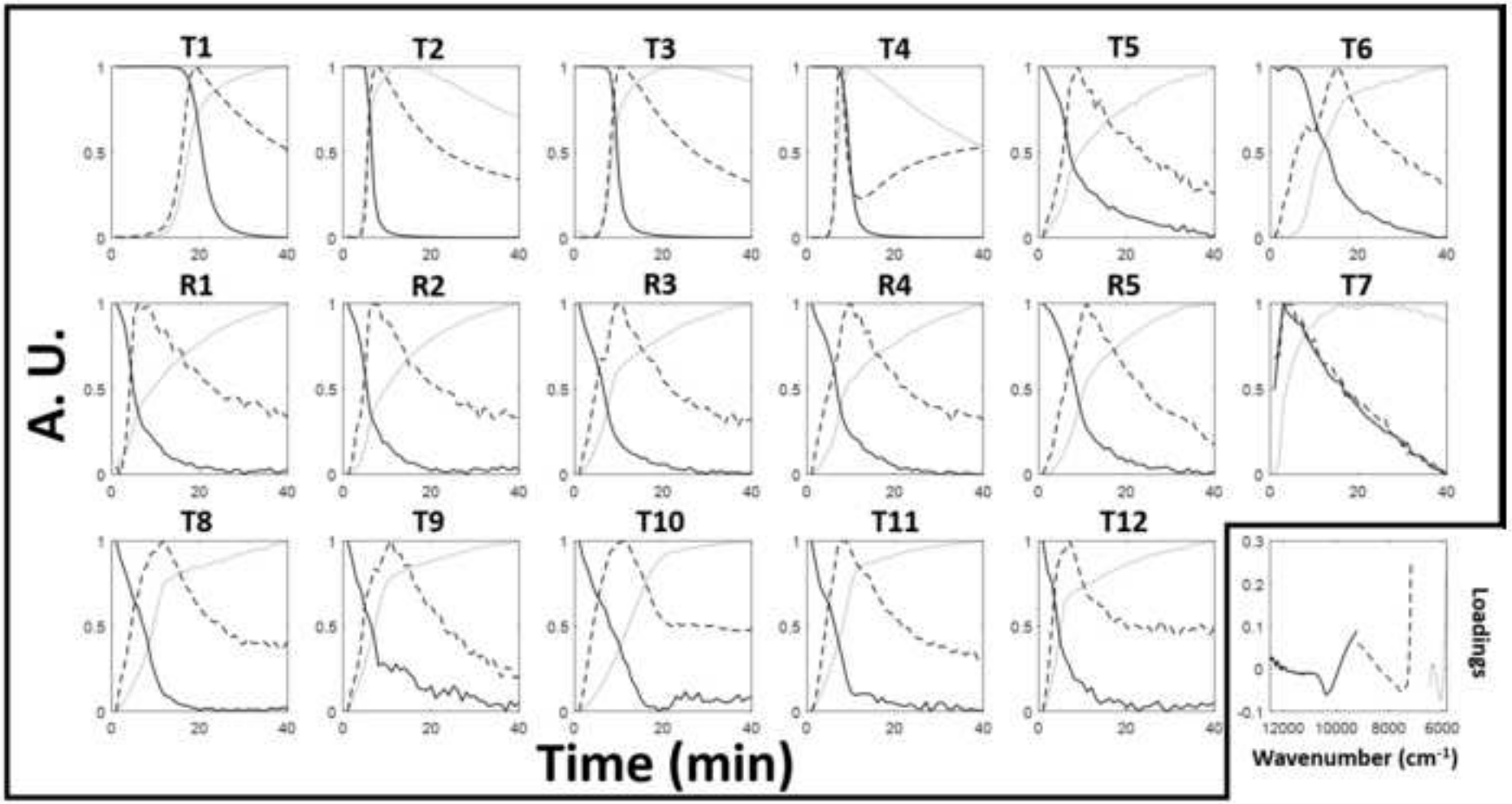

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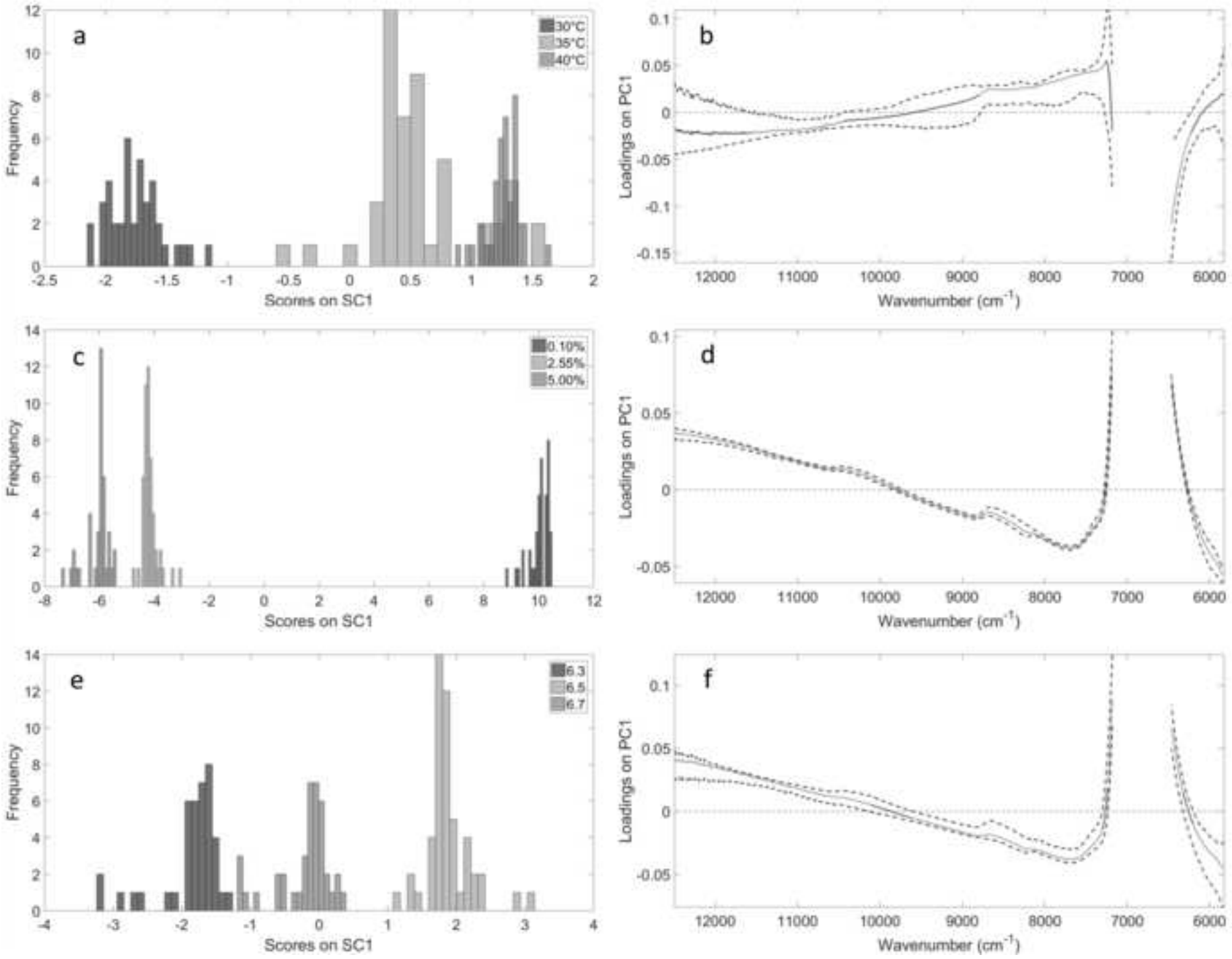

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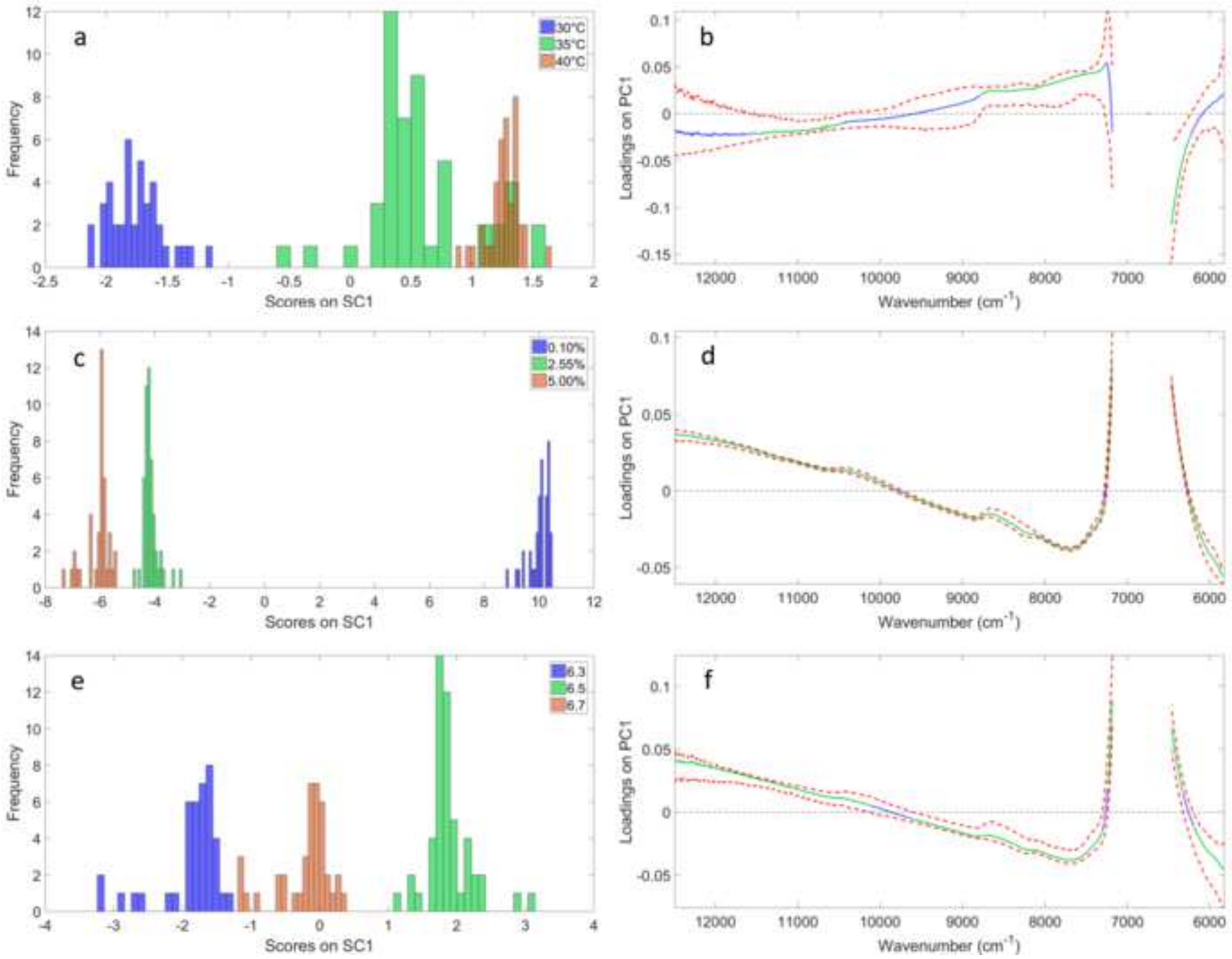

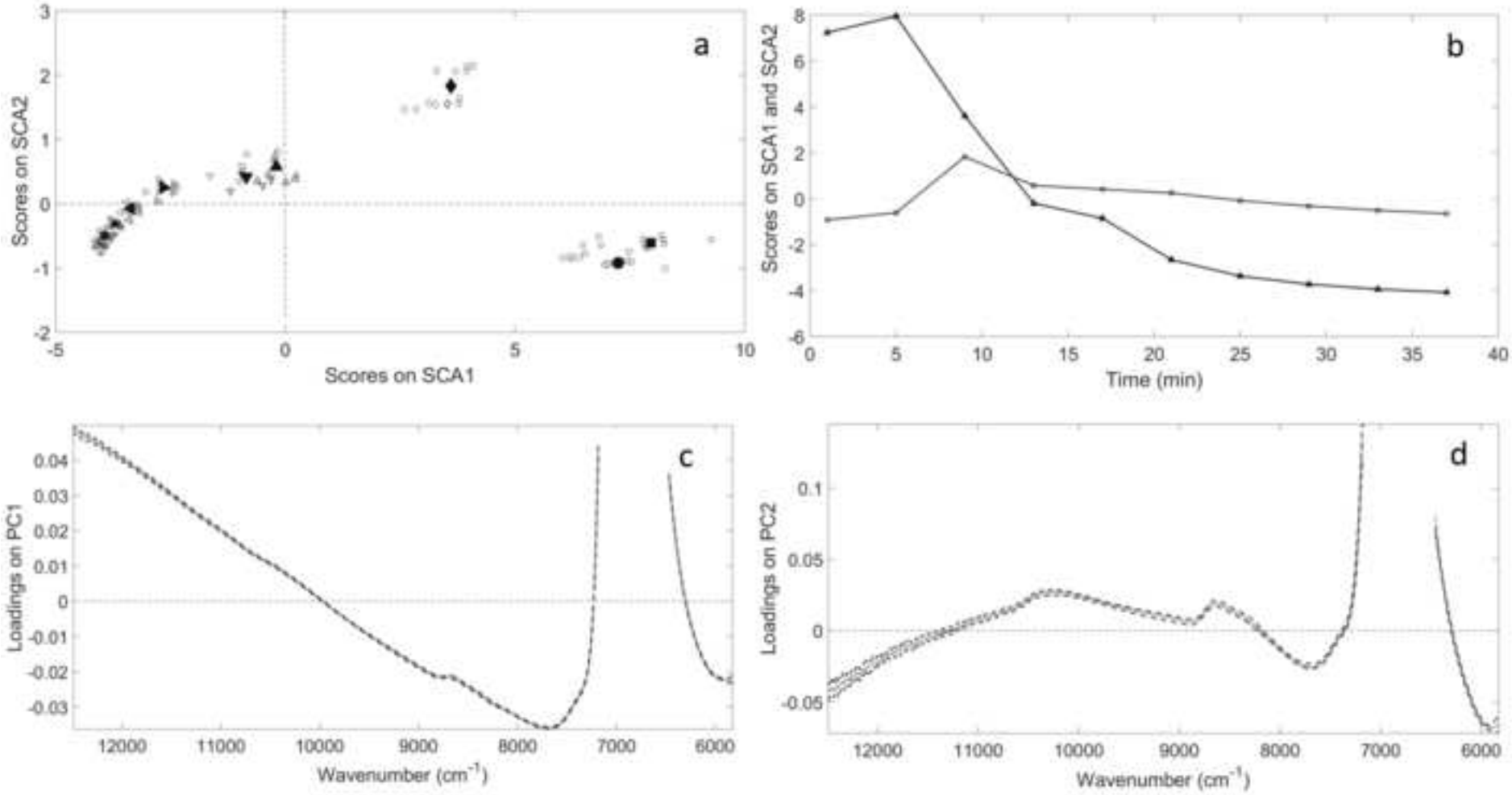

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