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Study of Galactooligosaccharides production from dairy waste by FTIR and Chemometrics as Process Analytical Technology

--Manuscript Draft--

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Abstract:	Galactooligosaccharides (GOS) production from whey, a relevant by-product of dairy industry, answers to the Circular Economy principle of extending the life cycle of products. Indeed, it allows the reuse of dairy waste to produce prebiotics to be used in functional food preparations. For this purpose, the effective monitoring of GOS production should be performed in real time and by environmentally friendly techniques. Thus, FTIR spectroscopy, combined with different chemometric approaches, has been tested to assess a Process Analytical Technology to follow GOS production from cheese whey. Partial Least Square regression models were reliable for lactose, glucose and galactose determination (Root Mean Square Error of Prediction of 21.9, 11.1 and 12.4 mg mL-1, respectively). Furthermore, Multivariate Curve Resolution – Alternating Least Square models were proposed to describe trends of the reaction components along the process being an interesting alternative to chromatographic determinations. The real time implementation of the proposed approach will provide the dairy industry with a reliable and green Process Analytical Technology for dairy waste reallocation, avoiding sample pre-processing, large use of organic solvents and long times of analysis.
Suggested Reviewers:	Anna De Juan, Ph.D. Professor, University of Barcelona anna.dejuan@ub.edu She is one of the European main expert in Chemometrics, with high experience in MCR-ALS application for FTIR data.
	Antonia Montilla Spanish National Research Council a.montilla@csic.es She is an expert in the field of Biochemistry and Molecular Biology with high experience in oligosaccharides.
	Sally Gras Associate professor, University of Melbourne sgras@unimelb.edu.au Associate Professor Sally Gras is a Reader in the Department of Chemical and Biomolecular Engineering at The University of Melbourne and Director of The ARC Dairy Innovation Hub. She has great experience some of the major dairy research and technical challenges.
	Sylvie Roussel

	Ondulys sroussel@ondalys.fr Founder of Ondalys Company, France. She is expert in data analysis and optical sensors, with multidisciplinary skills in various sectors.
Response to Reviewers:	Editor and Reviewer comments: First of all, we want to deeply thank the comprehensive revision made by the three reviewers. It is always a pleasure to see that a reviewer takes so much time to review a manuscript and, moreover, makes such a nice and focused criticism. It is a pleasure, from a scientific point of view, to receive comments that make us learn and improve. Below are reported our responses. All the changes have been reported in red in the revised manuscript. Reviewer #1 The paper would be of interest to readers of FBP and could be published after the following comments have been incorporated. 1. Justification needs to be given on choice of FTIR. There are many other spectroscopic tools available - multiwavelength, NIR, Raman. Why FTIR? R/. We agree with the Reviewer, many spectroscopic tools are available, and all of them can lead to interesting results in such a kind of application. Among them FTIR has been widely used including organic synthesis, polymer science, petrochemical engineering, biological research, the pharmaceutical industry and analysis of food. Furthermore, portable FTIR spectrometers have also been researched and used for field analysis. From our expertise, FTIR fingerprint region gives the opportunity of differentiating sugars signals with reliable results as demonstrated by many colleagues and by a work of ours (Grassi, S., Amigo, J. M., Lyndgaard, C. B., Foschino, R., & Casiraghi, E. (2014). Assessment of the sugars and ethanol development in beer fermentation with FT-IR and multivariate curve resolution models. Food research international, 62, 602-608). Thus, we decided to limit the investigation to this technique, aware of the possibility of succeeding with other techniques. A brief description has been included in the Introduction to highlight what has been discussed above. 2. Section 3.1 - this section does not really contribute to the main focus of the paper which is to establish FTIR as a tool of choice for monitoring of Galactooligosaccharides. It fact it muddles it by taking us into the kinetics of the reaction. In my opinion, this needs to be removed from the paper. This is a method paper and it should focus on how the method performs against the chosen reference, HPLC. R/. According to the reviewer suggestion, the section has been reduced for Figure interpretation. However, authors consider that a point of comparison is needed since not relevant bibliography has been published combining the study of the specific reaction and FTIR. 3. Section 3.2 - It is unclear what is being used as the modeling dataset and what is being used as validation dataset. This needs to be clearly stated. R/. A sentence has been inserted to better explain the modelling steps: “The PLS models were calibrated using 64 averaged spectra and internally validated by Venetian Blind Cross-Validation. Furthermore, they were tested for prediction in an iterative way, i.e. by testing they prediction capability by a validation set consisting of 16 averaged spectra of one of the enzymatic reactions performed.” 4. When using spectroscopic methods a major problem is the lack of specificity. More needs to be done here to show that the proposed method has that. The best way to show is to plot the concentration of each Galactooligosaccharide separately such that the x axis shows the value measured by the FTIR and the y axis shows the value measured by the HPLC. These plots need to be generated for varying conditions to prove that the robustness is there. If the method works we should see the data points falling on a straight line passing through origin and at an angle of 45 deg. This one plot will replace the Figures 3, 1S and 2 S. Overall, I feel more data is required under varying conditions as other components may interfere with the spectra. R/. We would like to thank the reviewer for the comment. We are aware of the

possibility of representing the PLS results by regression line as suggested and represented in the graphical abstract. It is not common to use this representation for MCR-ALS when soft modelling is applied. Indeed, in this case no real concentration is used but relative proportion (expressed in arbitrary unit), thus it is difficult to set up a proper regression line. On the contrary concentration and spectral profiles graphs are mostly reported to show the achieved results as illustrated by the works of main experts of this technique (i.e. Professors de Juan and Tauler*). For these reasons we would like the Reviewer to consider the possibility of reproducing our results with the scheme proposed in Figure 3, 1S and 2S.
As concern the data numerosity, we believe that the experimental setup was not properly explain in M&M section, it has been amended in the revised manuscript. In our experience it covers the variability of the reactions under study. Indeed, two repetition of each enzymatic reaction were performed with the following scheme:
-5 different enzymatic combinations performed independently twice,
-8 sampling time per reaction,
-collection of two samples for each sampling point,
for a total of 80 measurements replicated twice, then averaged in the presented results. The dataset is then composed by 80 sampling points, divided in the PLS modelling in 64 samples (4*8*2) for the calibration set and 16 (1*8*2) for the test sets.
* Some of the reference works are listed below as an example of the proposed results representation.
de Juan, A., & Tauler, R. (2020). Multivariate Curve Resolution: 50 years addressing the mixture analysis problem–A review. Analytica Chimica Acta.
Benabou, S., Ruckebusch, C., Sliwa, M., Aviñó, A., Eritja, R., Gargallo, R., & de Juan, A. (2018). Study of light-induced formation of photodimers in the i-motif nucleic acid structure by rapid-scan FTIR difference spectroscopy and hybrid hard-and soft-modelling. Physical Chemistry Chemical Physics, 20(29), 19635-19646.
De Juan, A., & Tauler, R. (2016). Multivariate curve resolution-alternating least squares for spectroscopic data. In Data Handling in Science and Technology (Vol. 30, pp. 5-51). Elsevier.
de Juan, A., & Mas, S. (2013). Multivariate Curve Resolution Methods for Food Chemistry. In Data Handling in Science and Technology (Vol. 28, pp. 235-263). Elsevier.
Garrido, M., Rius, F. X., & Larrechi, M. S. (2008). Multivariate curve resolution–alternating least squares (MCR-ALS) applied to spectroscopic data from monitoring chemical reactions processes. Analytical and bioanalytical chemistry, 390(8), 2059-2066.
Blanchet, L., Ruckebusch, C., Huvenne, J. P., & de Juan, A. (2007). Hybrid hard-and soft-modeling applied to difference spectra. Chemometrics and Intelligent Laboratory Systems, 89(1), 26-35.

4. Recently I have seen a couple of papers published on similar use of spectroscopic tools for monitoring similar reactions. I would suggest the authors do a search and cite them and incorporate them in the discussion.

As suggested by the reviewer we integrated our literature survey. Actually, most of the recently published papers deal with the use of FTIR spectroscopy for structural characterization:
-“Native collagen, the enzyme β-galactosidase, modified supports, and the derivatives obtained were structurally characterized using […] infrared spectroscopy (IF)”. Gennari, A., Mobayed, F. H., Catto, A. L., Benvenutti, E. V., Volpato, G., & de Souza, C. F. V. (2019). Kluyveromyces lactis β-galactosidase immobilized on collagen: catalytic stability on batch and packed-bed reactor hydrolysis. Reaction Kinetics, Mechanisms and Catalysis, 127(2), 583-599.
-“The Fourier Transform Infrared Spectroscopy (FT-IR) spectra of glass bead before and after β-galactosidase immobilization were recorded at the 4000–400cm−1 region […]”. Eskandarloo, H., & Abbaspourrad, A. (2018). Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. Food chemistry, 251, 115-124.
-“The FTIR spectrum was recorded using a Thermo Nicolet 5700 spectrometer (Nicolet, Madison, WI, USA) with a resolution of 4 cm−1 using the KBr method.” Li, H., Cao, Y., Li, S., Jiang, Y., Chen, J., & Wu, Z. (2019). Optimization of a dual-functional biocatalytic system for continuous hydrolysis of lactose in milk. Journal of bioscience

and bioengineering, 127(1), 38-44.
-“FT-IR spectra of the dried bacterial cellulose was recorded using a Perkin Elmer FTIR spectrophotometer”. Kumar, V., Sharma, D. K., Sandhu, P. P., Jadaun, J., Sangwan, R. S., & Yadav, S. K. (2020). Sustainable process for the production of cellulose by an Acetobacter pasteurianus RSV-4 (MTCC 25117) on whey medium. Cellulose, 1-14.
-“Successful conjugation of amino and carboxyl functional groups on the surface of HPSNs is confirmed from the Fourier transforms infrared (FTIR) spectra of HPSNs-NH2 and HPSNs-COOH”. Misson, M., Du, X., Jin, B., & Zhang, H. (2016). Dendrimer-like nanoparticles based β-galactosidase assembly for enhancing its selectivity toward transgalactosylation. Enzyme and microbial technology, 84, 68-77.
Furthermore, few works report similar process monitoring by FTIR spectroscopy:

-Schiele, S. A., Meinhardt, R., Eder, C., & Briesen, H. (2020). ATR-FTIR spectroscopy for in-line anomer concentration measurements in solution: A case study of lactose. Food Control, 110, 107024.
-Romano, N., Santos, M., Mobili, P., Vega, R., & Gómez-Zavaglia, A. (2016). Effect of sucrose concentration on the composition of enzymatically synthesized short-chain fructo-oligosaccharides as determined by FTIR and multivariate analysis. Food chemistry, 202, 467-475.
-Cocciardi, R. A., Ismail, A. A., Van De Voort, F. R., & Sedman, J. (2004). Monitoring of lactose hydrolysis in milk by single-bounce attenuated total reflectance Fourier transform infrared spectroscopy. Milchwissenschaft, 59(7-8), 403-407.
The results of the literature survey were included in the introduction and in the discussion of the results, when appropriated.
Reviewer #2

This article is interesting, but it has some points that need be corrected. On the other hand, I am not an expert in chemometric methods or FTIR, so the article must be corrected by an expert.

Comments:
Highlights: They are not valid; they are not complete sentences with meaning
R/. Highlights were modified.

Introduction:
Page 3, line 2: "derived from galactose" change by "derived from lactose"
Page 3, line 17: Delete "mainly"
Page 3, line 54: Change by: "these methods require sample pre-processing (GC), large volumes of organic solvents (HPLC) and long analysis time per sample."
Page 3, line 60: Change by: "Fourier transform mid-infrared"
R/. The mention changes were modified accordingly in the Introduction section.

M&M:
A characterisation of cheese whey is needed.
R/. The amount of total lactose has been included in M&M section

2.2 Enzyme reactions
Is it possible to dissolve cheese whey at 370 mg/mL of lactose equivalent?
R./ In experimental sections, we assayed concentration of 400 mg/mL, nonetheless, solubility and viscosity did not allowed enzyme to interact with media at given conditions of temperature and pH. Thus, we reduced cheese whey concentration until we found an adequate relation of total solids and water to work with, this relation was 370 mg/mL. Although viscosity was still high, it was possible to perform reactions as planned.

What is the final pH of reaction? b-galactosidase activity from K. lactis is very low at pH < 6.5.
R/. One of the advantages of working with Beta-galactosidase is that changes in pH are negligible because not REDOX reaction occurs. In this order, pH of the reaction was constant at 6.0 +/- 0.1. Optimum pH for Beta-galactosidase from K. lactis is in the range of 6.0 to 6.5. In this order, no problems are related to this condition.
Nonetheless, optimum pH for A. oryzae beta-galactosidase is in the range of 4.5 to 5.5. pH was adjusted at the described conditions, which is an intermediate. For this reason, we observed that the loss of enzymatic activity was bigger for K. lactis than the one for

	A. oryzae. Page 5, line 30-32: "For each duplicate of the enzymatic reaction a total of 8 spectra, at 0, 20, 40, 60, 120, 180, 240 and 300 min of reaction, were collected." Change by: "The same samples were analysed" R/. We apologies for the repetition, text has been modified accordingly. A concentration of 400 mg/mL is not "diluted samples" R/. This statement was given in order to specify that carbohydrate standards were commonly found in solid state, to have reproducible data points, dilutions from solid state to 400 mg/mL were performed to collect FTIR spectra. Text has been modified to: “Besides, samples of carbohydrate standards (400 mg/mL) were analysed following the above described procedure.” R&D: An explanation for utilisation of two combined galactosidases is needed. R./ A comment on this respect has been included in R&D section. Also, the first paragraph of introduction gives a wider explanation of the reasons to develop a Beta-galactosidase enzyme combination. Page 6, line 32-34: "During this reaction, substrate is mostly converted to GOS, whereas the remaining part is liberated as glucose and galactose" This idea is not correct. R./ A correction has been made to properly express the concept. Page 7, line 1-2: "notable changes due to enzyme source ratio modification" This expression is not correct. R./ A correction has been made to express de proper idea in the mentioned paragraph. Quality of figures is very low; it is difficult to difference the series. Codes throughout the text, Table 1: "Enz" change by Ao; "Hal" change by Kl Alias: WO change by Ao; W1/4 change by 3A/1K W1/2 change by 2A/2K W3/4 change by 1A/3K WK change by Kl R/. We apologies for the difficult coding used, it has been modified as suggested. For me it is very difficult to follow the explanation with the poor quality of the figures and codes used. R/. We apologies for the difficulties in the Figures interpretation. Figures were modified and a colour version was provided for revision process and online publication. Page 7, line 33-35: "This trend for W1/2 makes data obtained difficult to be process in further statistical data analysis." I do not understand; if data from W1/2 is not correct, please delete that series or repeat the reaction. Do duplicate samples give similar data? R/. No experimental error was attributed to this data, both repetitions lead to similar responses. The variation in the behaviour should be better explained by further studies, however all the analysis (HPLC, FTIR) confirmed that the experiment was well performed but the reactions have “peculiar” behaviour. Unfortunatelly, due to Covid-19 emergency, it is not possible to perform any other experiment in Italy nor in Colombia. In any case, the text has been modified accordingly. Fischer and Kleinschmitdh (2018) is not on the reference list. R/. We apologies for the lack, the reference has been implemented in the reference list. Figure 2: It is impossible to know what the different samples are. Figure 2D: The frames of the series are incorrect; the legend indicates that only lactose is a solid line, not dotted and in the 2D figure, only one line is dotted. R/. We apologies for difficulties in Figures interpretation. Figures were modified and a

colour version was provided for revision process and online publication.

Page 8, line 27-28: "in fact, cheese whey contains 70% of carbohydrates (with >98% of lactose (Grumezescu & Holban, 2019))."
I do not understand this reference. In addition, if the whey contains 70% carbohydrates, to prepare a solution of 370 g/L of lactose you have to dissolve 529 g/L of whey and I think this is impossible.
I am sorry, I can only evaluate point 3.1. I am not an expert in FTIR or MCR-ALS. However, I think it needs major changes. If possible, in the pdf of the article and in its later online version it would be convenient to use colors for the different samples. They should check the references.

R./ The paragraph has been modified and corrections have been included for a better understanding of the manuscript. Once again, we confirm that it was possible to have a solution of cheese whey at 370 mg of lactose/mL at our working conditions.

Reviewer #3
The monitoring of Galactooligosaccharides (GOS) production from dairy industry waste is an interesting topic. Also the implementation of Process Analytical Technology tools and real-time monitoring is important from the view of process understanding. However there are several major issue regarding the manuscript, thus I advise against publishing it in Food and Bioproduct Processing.

1, The authors states that real time monitoring of GOS production by FTIR spectroscopy was performed, however, there are no data supporting this. The calibration and the prediction dataset was acquired from off-line measurements. There are no information about real time measurement settings or real time data analysis.
R/. We thank the reviewer for the comment. We corrected the text in all the parts where this term was unproperly used:
1)The best model to predict in a non-destructive, fast and at line approach the amount of GOS (range 8.06 -151.62 mg/mL) […].
2)PLS regression models demonstrated to be reliable to assess lactose, glucose and galactose content in a non-destructive, fast and at line approach, which could be used for real time applications in the future.

2, Strange reaction pattern was observed (WO, W1/2 reaction) and no repetitions was made to investigate if the results are correct or there was an error during the experiments. Thus, no conclusions can be made on lactose consumption and GOS production using different enzymes and their mixtures.
R/. No experimental error was attributed to this data, both repetitions lead to similar responses. The variation in the behaviour should be better explained by further studies, however all the analysis (HPLC, FTIR) confirmed that the experiment was well performed but the reactions have “peculiar” behaviour. Unfortunatelly, due to Covid-19 emergency, it is not possible to perform any other experiment in Italy nor in Colombia.

3, Small dataset was used for multivariate data analysis compared to the complexity and variability of the enzymatic process. 2 out of 5 experiment was excluded during FTIR data analysis because of high measurements error.
As concern the data numerosity, we believe that the experimental setup was not properly explain in M&M section, it has been amended in the revised manuscript. In our experience it covers the variability of the reactions under study. Indeed, two repetition of each enzymatic reaction were performed with the following scheme:
-5 different enzymatic combinations performed independently twice,
-8 sampling time per reaction,
-collection of two samples for each sampling point,
for a total of 80 measurements replicated twice, then averaged in the presented results. The dataset is then composed by 80 sampling points, divided in the PLS modelling in 64 samples (4*8*2) for the calibration set and 16 (1*8*2) for the test sets. The measurement error could be negligible, as the two independent repetition resulted quite similar both by HPLC analysis and FTIR analysis. This was better reported in the text.

4, The results from multivariate curve resolution are not corresponding with experimental data. Also it gives no extra information about the galactooligosaccharide production (no inline measurements). The kinetic assessment can be performed based on the HPLC data.
R/. We apologies for the mistake, it seems that Figure S1 and S2 were exchanged, this mistake was amended in the new submission.
MCR-ALS models have been developed focusing more on the process kinetics rather than the prediction of each component. The obtained results are not optimal, but they lay the ground to a future kinetics monitoring of the process. The advantage of MCR-ALS over PLS is the possibility of predicting the process trend, rather than measuring the content of one or more specific compounds in the sample in a specific moment/time. We would like to reach the inline measurements by a pump transferring the reaction medium to the FTIR sample holder, but we are not able yet to perform further experiments. We apologies for that.
Surely, the kinetic assessment can be performed based on the HPLC data, but this would require that for any new reaction a sample preparation and analysis is performed, thus needing expert personnel, long time of analysis and sample destruction. The proposed method is oriented to substitute the routine measurements by HPLC.

5, The '3.5 GOS fitting analysis' section in 'results and discussion' has no experimental results, it contains only a discussion of literature data.
R/. Section 3.5 was removed, and the contents previously discussed here were moved to more appropriated sections.

Please see below my additional comments:
Page 3. Row 30. Substrate concentration also an important factor of GOS production. (add substrate concentration in the text at this line)
R/. The correction has been made in the document.

Page 3. Row 58. There are other examples in the literature about monitoring beta-galactosidase and sugars (oligosaccharides, glucose, lactose, galactose) that should be mentioned.
As suggested by the reviewer we integrated our literature survey. Actually, most of the recently published papers deal with the use of FTIR spectroscopy for structural characterization:
-“Native collagen, the enzyme β-galactosidase, modified supports, and the derivatives obtained were structurally characterized using […] infrared spectroscopy (IF)”. Gennari, A., Mobayed, F. H., Catto, A. L., Benvenutti, E. V., Volpato, G., & de Souza, C. F. V. (2019). Kluyveromyces lactis β-galactosidase immobilized on collagen: catalytic stability on batch and packed-bed reactor hydrolysis. Reaction Kinetics, Mechanisms and Catalysis, 127(2), 583-599.
-“The Fourier Transform Infrared Spectroscopy (FT-IR) spectra of glass bead before and after β-galactosidase immobilization were recorded at the 4000–400cm−1 region […]”. Eskandarloo, H., & Abbaspourrad, A. (2018). Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. Food chemistry, 251, 115-124.
-“The FTIR spectrum was recorded using a Thermo Nicolet 5700 spectrometer (Nicolet, Madison, WI, USA) with a resolution of 4 cm−1 using the KBr method.” Li, H., Cao, Y., Li, S., Jiang, Y., Chen, J., & Wu, Z. (2019). Optimization of a dual-functional biocatalytic system for continuous hydrolysis of lactose in milk. Journal of bioscience and bioengineering, 127(1), 38-44.
-“FT-IR spectra of the dried bacterial cellulose was recorded using a Perkin Elmer FTIR spectrophotometer”. Kumar, V., Sharma, D. K., Sandhu, P. P., Jadaun, J., Sangwan, R. S., & Yadav, S. K. (2020). Sustainable process for the production of cellulose by an Acetobacter pasteurianus RSV-4 (MTCC 25117) on whey medium. Cellulose, 1-14.
-“Successful conjugation of amino and carboxyl functional groups on the surface of HPSNs is confirmed from the Fourier transforms infrared (FTIR) spectra of HPSNs-NH2 and HPSNs-COOH”. Misson, M., Du, X., Jin, B., & Zhang, H. (2016). Dendrimer-like nanoparticles based β-galactosidase assembly for enhancing its selectivity toward transgalactosylation. Enzyme and microbial technology, 84, 68-77.
Furthermore, few works report process monitoring by FTIR spectroscopy:
-Schiele, S. A., Meinhardt, R., Eder, C., & Briesen, H. (2020). ATR-FTIR spectroscopy

for in-line anomer concentration measurements in solution: A case study of lactose. Food Control, 110, 107024.
-Romano, N., Santos, M., Mobili, P., Vega, R., & Gómez-Zavaglia, A. (2016). Effect of sucrose concentration on the composition of enzymatically synthesized short-chain fructo-oligosaccharides as determined by FTIR and multivariate analysis. Food chemistry, 202, 467-475.
-Cocciardi, R. A., Ismail, A. A., Van De Voort, F. R., & Sedman, J. (2004). Monitoring of lactose hydrolysis in milk by single-bounce attenuated total reflectance Fourier transform infrared spectroscopy. Milchwissenschaft, 59(7-8), 403-407.
The results of the literature survey were included in the introduction and in the discussion of the results, when appropriated.

Page 4. Row 58. How was the falcon tubes homogenized during reaction? Did you use a shaker?
R/. At the beginning of the reaction, sugar solutions were homogenized by stirring with an ultraturrax®. After this procedure, a magnetic bar was introduced into each falcon tube and permanently stirring at 800 rpm. This explanation was already included in the manuscript.

Page 5. Row 4. Did you verify the enzyme inactivation method? (5-10 min of heating are enough)
R/. Yes, in the experience of the research group, no time above 10 min is required to inactivate beta-galactosidase in boiling water. It is important to mention that K. lactis enzyme loses its activity above 50 °C, whereas A. oryzae enzyme loses it above 65°C.

Page 5. Row 26. What is the type of FTIR spectrometer? How did you perform inline measurement? Inline probe?
R/. The reference and specifications of the equipment were included. An ATR sample compartment was employed. In fact, it is an at line measurement which could be further change to online/inline measurement by a pump deviation to the sample compartment.

Page 5. Row 49. Did you apply real time monitoring and data processing? There is no information about it.
R/. We thank the reviewer for the comment. We corrected the text in all the parts where this term was unproperly used:
-The best model to predict in a non-destructive, fast and at line approach the amount of GOS (range 8.06 -151.62 mg/mL) […].
-PLS regression models demonstrated to be reliable to assess lactose, glucose and galactose content in a non-destructive, fast and at line approach, which could be used for real time applications in the future.

Page 5. Row 56. You should include how many data points did you use for calibration. Was there replicate measurements?
R/. As concern the data numerosity, we believe that the experimental setup was not properly explain in M&M section, it has been amended in the revised manuscript. However, in our experience it properly covers the variability of the reactions under study. Indeed, two repetition of each enzymatic reaction were performed
-5 different enzymatic combinations performed independently twice
-8 sampling time per reaction
-with collection of two samples for each sampling point,
For a total of 80 measurements replicated twice and thus averaged in the presented results. The dataset is then composed by 80 sampling points, divided in the PLS modelling in 64 samples (4*8*2) for the calibration set and 16 (1*8*2) for the test sets.

Page 6. Row 37. It is really hard to follow the names of the reactions. It would be easier to read the text if you define what WK, W1/4, W1/2, W3/4, WO means at the beginning of 'results and discussion' also in the figure captions.
R/. We apologies for the difficult coding used, it has been modified as suggested by Reviewer 2.

Page 6 Row 52. Grammar should be corrected: "…in this reaction after 1 hour started GOS hydrolysis…"
Page 6 Row 56 Grammar should be corrected: "…hydrolysis is not as faster as…"

R/. Grammar mistakes have been corrected.

Figure 1. What is the standard deviation means on Figure 1? Based on the experimental section there was no replicate experiments. At least reaction W1/2 and WO should be investigated again to see if the results are correct. No conclusions can be made on lactose consumption and GOS production.
R/. For each reaction, two technological repetitions (two different assays) and two measurements (replicates of the measurements) were performed for each sampling point. In this order we could guarantee that enough number of samples is considered. Thus, standard deviation indicated in the figures refers to the deviation between the measurements (replicates of the measurement of each sample). For reactions W1/2 and WO, as well as for all the other conditions, the two independent assays gave superimposable results.

Page 9 Row 17 "the behaviour of both WO and W1/2 enzymes, that presented a lactose consumption and its conversion to GOS significantly different from the other three enzymes" - What is the reason for this? Are you sure the observed reaction pattern is not measurement error? You should perform a replicate measurement to investigate it. (this is the reason of section 3.5)
R/. As reported before, no experimental error was attributed to collected data, both repetitions lead to similar responses. The variation in the behaviour should be better explained by further studies, however all the analysis (HPLC, FTIR) confirmed that the experiment was well performed but the reactions have “peculiar” behaviour. Unfortunately, due to Covid-19 emergency, it is not possible to perform any other experiment in Italy nor in Colombia. Nonetheless, in section 3.1 and 3.2 we added some explanation to justify the obtained results.

Page 9 Row 23. 3*8 data points was used to calibrate PLS model, which is a small dataset compared to the complexity and variability of the enzymatic process.
R/. As concern the data numerosity, we believe that the experimental setup was not properly explain in M&M section, it has been amended in the revised manuscript. However, in our experience it properly covers the variability of the reactions under study. Indeed, two repetition of each enzymatic reaction were performed
-5 different enzymatic combinations performed independently twice
-8 sampling time per reaction
-with collection of two samples for each sampling point,
for a total of 80 measurements replicated twice and thus averaged in the presented results. The dataset is then composed by 80 sampling points, divided in the PLS modelling in 64 samples (4*8*2) for the calibration set and 16 (1*8*2) for the test sets.

Table 2. It is more correct to compare models for the same validation set. It can be seen from your data, that calibration model from W1/4 and W3/4 reaction had higher prediction error for WK reaction (test set from pure enzyme, calibration set from mixture) compared when the test data set and part of calibration set was from a reaction with enzyme mixture. Thus, the calibration data set should contain data points from similar reaction condition.
R/. We would like to thank the reviewer for the comment. The discussion of PLS results were modified accordingly and integrated by the comparison with other similar works.

Page 9. Row 52. RPD should be defined.
R/. RPD was defined in M&M section as “residual prediction deviation (RPD), i.e. ratio of standard error of performance to standard deviation, was calculated to compare the precision of the prediction with the average composition of all the samples, as stated by Camacho-Tamayo et al. (2014).”
For a better understanding the meaning of this parameter is also commented in the paragraph cited by the reviewer.

Figure 3. Figure S1 and Figure S2: MCR-ALS results are not corresponding with experimental data. Page 11. Row 14. "GOS results accurately predict in the first stage of production" - The interpretation of data should be corrected. The dataset used for MCR-ALS is small and also the inaccuracy can be due to rotation ambiguity. It occures, when the physicochemical constraints are not sufficiently strong to provide a unique resolution of the data matrix of the mixtures into spectra and concentration profiles of individual chemical components.

	R/. We apologies for the mistake, it seems that Figure S1 and S2 were exchanged, this mistake was amended in the new submission. The highlighted sentence has been removed. As far as concern rotation ambiguity, we are aware of its importance in heavily mixed data (such as kinetic process) and the need of the proper constrains to reduce it. Thus, we applied constrains considering chemical/physical meaning, interpretability, improved model reliability and reduction of rotational ambiguity. A systematic investigation on any constraint in spectral modes of data matrix has be monitored via calculation of feasible solutions and interpretation of obtained results, inspiring to the methods discussed by Golshan et al. (2016)*. We ended with the idea that non-negativity constraint, imposed on both concentration and spectral profiles, could solve MCR-ALS ambiguities present in our system. This was better explained in the Materials and Methods section. * Golshan, A., Abdollahi, H., Beyramysoltan, S., Maeder, M., Neymeyr, K., Rajkó, R., ... & Tauler, R. (2016). A review of recent methods for the determination of ranges of feasible solutions resulting from soft modelling analyses of multivariate data. Analytica Chimica Acta, 911, 1-13. Page 11. Row 20. "Fast decrease in GOS concentration is been previously associated to potassium ions from permeate salts in the solution and K. lactis enzyme source (Rico-Rodríguez et al., 2018; Rodrigues Mano et al., 2019)." - The experimental data did not show fast decrease, only the calculated values from MCR-ALS. R/. Actually, KI reaction followed by HPLC showed a reduction of GOS from its maximum of 110 mg/mL (after 60 min) to 57 mg/mL (after 300 min). This is reported in Figure 1B. This correspond to a drop from 100% to 50% of GOS content as shown in Figure 3B. This behaviour has been previously attributed to potassium salts dissolved in media. Nevertheless, with the experimental data collected, there is no possibility to evaluate any effect of minerals from cheese whey on the reaction. For this particular situation, further analysis must be developed. A better discussion of this behaviour has been reported in adequate sections by moving the content reported in pervious section '3.5 GOS fitting analysis'

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Milan, 12 December 2020

Dear Editor,

Please, on behalf of all co-authors, find here the revised version of the manuscript entitled:

Study of Galactooligosaccharides production from dairy waste by FTIR and Chemometrics as Process Analytical Technology

First of all, we want to deeply thank the comprehensive revision made by the three reviewers. It is always a pleasure to see that a reviewer takes so much time to review a manuscript and, moreover, makes such a nice and focused criticism.
It is a pleasure, from a scientific point of view, to receive comments that make us learn and improve.
Saying that, the comments made by the reviewers have been fully addressed in the "responses to the reviewers" letter and, moreover, the comments have generated a series of changes in the new version of the manuscript that have been properly highlighted in red.

Please, do not hesitate to contact me for any question or doubt that might arise from this letter.

Sincerely,

Silvia Grassi

Editor and Reviewer comments:

First of all, we want to deeply thank the comprehensive revision made by the three reviewers. It is always a pleasure to see that a reviewer takes so much time to review a manuscript and, moreover, makes such a nice and focused criticism. It is a pleasure, from a scientific point of view, to receive comments that make us learn and improve.

Below are reported our responses. All the changes have been reported in red in the revised manuscript.

Reviewer #1

The paper would be of interest to readers of FBP and could be published after the following comments have been incorporated.

1. Justification needs to be given on choice of FTIR. There are many other spectroscopic tools available - multiwavelength, NIR, Raman. Why FTIR?

R/. We agree with the Reviewer, many spectroscopic tools are available, and all of them can lead to interesting results in such a kind of application. Among them FTIR has been widely used including organic synthesis, polymer science, petrochemical engineering, biological research, the pharmaceutical industry and analysis of food. Furthermore, portable FTIR spectrometers have also been researched and used for field analysis.

From our expertise, FTIR fingerprint region gives the opportunity of differentiating sugars signals with reliable results as demonstrated by many colleagues and by a work of ours (Grassi, S., Amigo, J. M., Lyndgaard, C. B., Foschino, R., & Casiraghi, E. (2014). Assessment of the sugars and ethanol development in beer fermentation with FT-IR and multivariate curve resolution models. Food research international, 62, 602-608).

Thus, we decided to limit the investigation to this technique, aware of the possibility of succeeding with other techniques.

A brief description has been included in the Introduction to highlight what has been discussed above.

2. Section 3.1 - this section does not really contribute to the main focus of the paper which is to establish FTIR as a tool of choice for monitoring of Galactooligosaccharides. It fact it muddles it by taking us into the kinetics of the reaction. In my opinion, this needs to be removed from the paper. This is a method paper and it should focus on how the method performs against the chosen reference, HPLC.

R/. According to the reviewer suggestion, the section has been reduced for Figure interpretation. However, authors consider that a point of comparison is needed since not relevant bibliography has been published combining the study of the specific reaction and FTIR.

3. Section 3.2 - It is unclear what is being used as the modeling dataset and what is being used as validation dataset. This needs to be clearly stated.

R/. A sentence has been inserted to better explain the modelling steps: "*The PLS models were calibrated using 64 averaged spectra and internally validated by Venetian Blind Cross-Validation. Furthermore, they were tested for prediction in an iterative way, i.e. by testing they prediction capability by a validation set consisting of 16 averaged spectra of one of the enzymatic reactions performed*."

4. When using spectroscopic methods a major problem is the lack of specificity. More needs to be done here to show that the proposed method has that. The best way to show is to plot the concentration of each Galactooligosaccharide separately such that the x axis shows the value measured by the FTIR and the y axis shows the value measured by the HPLC. These plots need to be generated for varying conditions to prove that the robustness is there. If the method works we should see the data points falling on a straight line passing through origin and at an angle of 45 deg. This one plot will replace the Figures 3, 1S and 2 S. Overall, I feel more data is required under varying conditions as other components may interfere with the spectra.

R/. We would like to thank the reviewer for the comment. We are aware of the possibility of representing the PLS results by regression line as suggested and represented in the graphical abstract. It is not common to use this representation for MCR-ALS when soft modelling is applied. Indeed, in this case no real concentration is used but relative proportion (expressed in arbitrary unit), thus it is difficult to set up a proper regression line. On the contrary concentration and spectral profiles graphs are mostly reported to show the achieved results as illustrated by the works of main experts of this technique (i.e. Professors de Juan and Tauler*). For these reasons we would like the Reviewer to consider the possibility of reproducing our results with the scheme proposed in Figure 3, 1S and 2S.

As concern the data numerosity, we believe that the experimental setup was not properly explain in M&M section, it has been amended in the revised manuscript. In our experience it covers the variability of the reactions under study. Indeed, two repetition of each enzymatic reaction were performed with the following scheme:

- 5 different enzymatic combinations performed independently twice,
- 8 sampling time per reaction,
- collection of two samples for each sampling point,

for a total of 80 measurements replicated twice, then averaged in the presented results. The dataset is then composed by 80 sampling points, divided in the PLS modelling in 64 samples (4*8*2) for the calibration set and 16 (1*8*2) for the test sets.

* Some of the reference works are listed below as an example of the proposed results representation.

de Juan, A., & Tauler, R. (2020). Multivariate Curve Resolution: 50 years addressing the mixture analysis problem–A review. Analytica Chimica Acta.

Benabou, S., Ruckebusch, C., Sliwa, M., Aviñó, A., Eritja, R., Gargallo, R., & de Juan, A. (2018). Study of light-induced formation of photodimers in the i-motif nucleic acid structure by rapid-scan FTIR difference spectroscopy and hybrid hard-and soft-modelling. Physical Chemistry Chemical Physics, 20(29), 19635-19646.

De Juan, A., & Tauler, R. (2016). Multivariate curve resolution-alternating least squares for spectroscopic data. In Data Handling in Science and Technology (Vol. 30, pp. 5-51). Elsevier.

de Juan, A., & Mas, S. (2013). Multivariate Curve Resolution Methods for Food Chemistry. In Data Handling in Science and Technology (Vol. 28, pp. 235-263). Elsevier.

Garrido, M., Rius, F. X., & Larrechi, M. S. (2008). Multivariate curve resolution–alternating least squares (MCR-ALS) applied to spectroscopic data from monitoring chemical reactions processes. Analytical and bioanalytical chemistry, 390(8), 2059-2066.

Blanchet, L., Ruckebusch, C., Huvenne, J. P., & de Juan, A. (2007). Hybrid hard-and soft-modeling applied to difference spectra. Chemometrics and Intelligent Laboratory Systems, 89(1), 26-35.

4. Recently I have seen a couple of papers published on similar use of spectroscopic tools for monitoring similar reactions. I would suggest the authors do a search and cite them and incorporate them in the discussion.

As suggested by the reviewer we integrated our literature survey. Actually, most of the recently published papers deal with the use of FTIR spectroscopy for structural characterization:

- "Native collagen, the enzyme β-galactosidase, modified supports, and the derivatives obtained were structurally characterized using […] infrared spectroscopy (IF)". Gennari, A., Mobayed, F. H., Catto, A. L., Benvenutti, E. V., Volpato, G., & de Souza, C. F. V. (2019). Kluyveromyces lactis β-galactosidase immobilized on collagen: catalytic stability on batch and packed-bed reactor hydrolysis. Reaction Kinetics, Mechanisms and Catalysis, 127(2), 583-599.
- "The Fourier Transform Infrared Spectroscopy (FT-IR) spectra of glass bead before and after β-galactosidase immobilization were recorded at the 4000–400 cm−1 region […]". Eskandarloo, H., & Abbaspourrad, A. (2018). Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. Food chemistry, 251, 115-124.
- "The FTIR spectrum was recorded using a Thermo Nicolet 5700 spectrometer (Nicolet, Madison, WI, USA) with a resolution of 4 cm−1 using the KBr method." Li, H., Cao, Y., Li, S., Jiang, Y., Chen, J., & Wu, Z. (2019). Optimization of a dual-functional biocatalytic system for continuous hydrolysis of lactose in milk. Journal of bioscience and bioengineering, 127(1), 38-44.
- "FT-IR spectra of the dried bacterial cellulose was recorded using a Perkin Elmer FTIR spectrophotometer". Kumar, V., Sharma, D. K., Sandhu, P. P., Jadaun, J., Sangwan, R. S., & Yadav, S. K. (2020). Sustainable process for the production of cellulose by an Acetobacter pasteurianus RSV-4 (MTCC 25117) on whey medium. Cellulose, 1-14.
- "Successful conjugation of amino and carboxyl functional groups on the surface of HPSNs is confirmed from the Fourier transforms infrared (FTIR) spectra of HPSNs-NH2 and HPSNs-COOH". Misson, M., Du, X., Jin, B., & Zhang, H. (2016). Dendrimer-like nanoparticles based β-galactosidase assembly for enhancing its selectivity toward transgalactosylation. Enzyme and microbial technology, 84, 68-77.

Furthermore, few works report similar process monitoring by FTIR spectroscopy:

- Schiele, S. A., Meinhardt, R., Eder, C., & Briesen, H. (2020). ATR-FTIR spectroscopy for in-line anomer concentration measurements in solution: A case study of lactose. Food Control, 110, 107024.
- Romano, N., Santos, M., Mobili, P., Vega, R., & Gómez-Zavaglia, A. (2016). Effect of sucrose concentration on the composition of enzymatically synthesized short-chain fructo-oligosaccharides as determined by FTIR and multivariate analysis. Food chemistry, 202, 467-475.
- Cocciardi, R. A., Ismail, A. A., Van De Voort, F. R., & Sedman, J. (2004). Monitoring of lactose hydrolysis in milk by single-bounce attenuated total reflectance Fourier transform infrared spectroscopy. Milchwissenschaft, 59(7-8), 403-407.

The results of the literature survey were included in the introduction and in the discussion of the results, when appropriated.

Reviewer #2

This article is interesting, but it has some points that need be corrected. On the other hand, I am not an expert in chemometric methods or FTIR, so the article must be corrected by an expert.

Comments:
Highlights: They are not valid; they are not complete sentences with meaning

R/. Highlights were modified.

Introduction:
Page 3, line 2: "derived from galactose" change by "derived from lactose"
Page 3, line 17: Delete "mainly"
Page 3, line 54: Change by: "these methods require sample pre-processing (GC), large volumes of organic solvents (HPLC) and long analysis time per sample."
Page 3, line 60: Change by: "Fourier transform mid-infrared"

R/. The mention changes were modified accordingly in the Introduction section.

M&M:
A characterisation of cheese whey is needed.

R/. The amount of total lactose has been included in M&M section

2.2 Enzyme reactions
Is it possible to dissolve cheese whey at 370 mg/mL of lactose equivalent?

R./ In experimental sections, we assayed concentration of 400 mg/mL, nonetheless, solubility and viscosity did not allowed enzyme to interact with media at given conditions of temperature and pH. Thus, we reduced cheese whey concentration until we found an adequate relation of total solids and water to work with, this relation was 370 mg/mL. Although viscosity was still high, it was possible to perform reactions as planned.

What is the final pH of reaction? b-galactosidase activity from K. lactis is very low at pH < 6.5.

R/. One of the advantages of working with Beta-galactosidase is that changes in pH are negligible because not REDOX reaction occurs. In this order, pH of the reaction was constant at 6.0 +/- 0.1. Optimum pH for Beta-galactosidase from *K. lactis* is in the range of 6.0 to 6.5. In this order, no problems are related to this condition. Nonetheless, optimum pH for *A. oryzae* beta-galactosidase is in the range of 4.5 to 5.5. pH was adjusted at the described conditions, which is an intermediate. For this reason, we observed that the loss of enzymatic activity was bigger for *K. lactis* than the one for *A. oryzae*.

Page 5, line 30-32: "For each duplicate of the enzymatic reaction a total of 8 spectra, at 0, 20, 40, 60, 120, 180, 240 and 300 min of reaction, were collected." Change by: "The same samples were analysed"

R/. We apologies for the repetition, text has been modified accordingly.

A concentration of 400 mg/mL is not "diluted samples"

R/. This statement was given in order to specify that carbohydrate standards were commonly found in solid state, to have reproducible data points, dilutions from solid state to 400 mg/mL were performed to collect FTIR spectra. Text has been modified to: “Besides, samples of carbohydrate standards (400 mg/mL) were analysed following the above described procedure.”

R&D:
An explanation for utilisation of two combined galactosidases is needed.

R./ A comment on this respect has been included in R&D section. Also, the first paragraph of introduction gives a wider explanation of the reasons to develop a Beta-galactosidase enzyme combination.

Page 6, line 32-34: "During this reaction, substrate is mostly converted to GOS, whereas the remaining part is liberated as glucose and galactose"
This idea is not correct.

R./ A correction has been made to properly express the concept.

Page 7, line 1-2: "notable changes due to enzyme source ratio modification"
This expression is not correct.

R./ A correction has been made to express de proper idea in the mentioned paragraph.

Quality of figures is very low; it is difficult to difference the series.

Codes throughout the text, Table 1: "Enz" change by Ao; "Hal" change by Kl
Alias: WO change by Ao;
W1/4 change by 3A/1K
W1/2 change by 2A/2K
W3/4 change by 1A/3K
WK change by Kl

R/. We apologies for the difficult coding used, it has been modified as suggested.

For me it is very difficult to follow the explanation with the poor quality of the figures and codes used.

R/. We apologies for the difficulties in the Figures interpretation. Figures were modified and a colour version was provided for revision process and online publication.

Page 7, line 33-35: "This trend for W1/2 makes data obtained difficult to be process in further statistical data analysis."
I do not understand; if data from W1/2 is not correct, please delete that series or repeat the reaction. Do duplicate samples give similar data?

R/. No experimental error was attributed to this data, both repetitions lead to similar responses. The variation in the behaviour should be better explained by further studies, however all the analysis (HPLC, FTIR) confirmed that the experiment was well performed but the reactions have “peculiar” behaviour. Unfortunatelly, due to Covid-19 emergency, it is not possible to perform any other experiment in Italy nor in Colombia. In any case, the text has been modified accordingly.

Fischer and Kleinschmitdh (2018) is not on the reference list.

R/. We apologies for the lack, the reference has been implemented in the reference list.

Figure 2: It is impossible to know what the different samples are.
Figure 2D: The frames of the series are incorrect; the legend indicates that only lactose is a solid line, not dotted and in the 2D figure, only one line is dotted.

R/. We apologies for difficulties in Figures interpretation. Figures were modified and a colour version was provided for revision process and online publication.

Page 8, line 27-28: "in fact, cheese whey contains 70% of carbohydrates (with >98% of lactose (Grumezescu & Holban, 2019))."
I do not understand this reference. In addition, if the whey contains 70% carbohydrates, to prepare a solution of 370 g/L of lactose you have to dissolve 529 g/L of whey and I think this is impossible.
I am sorry, I can only evaluate point 3.1. I am not an expert in FTIR or MCR-ALS. However, I think it needs major changes. If possible, in the pdf of the article and in its later online version it would be convenient to use colors for the different samples.
They should check the references.

R./ The paragraph has been modified and corrections have been included for a better understanding of the manuscript. Once again, we confirm that it was possible to have a solution of cheese whey at 370 mg of lactose/mL at our working conditions.

Reviewer #3

The monitoring of Galactooligosaccharides (GOS) production from dairy industry waste is an interesting topic. Also the implementation of Process Analytical Technology tools and real-time monitoring is important from the view of process understanding. However there are several major issue regarding the manuscript, thus I advise against publishing it in Food and Bioproduct Processing.

1, The authors states that real time monitoring of GOS production by FTIR spectroscopy was performed, however, there are no data supporting this. The calibration and the prediction dataset was acquired from off-line measurements. There are no information about real time measurement settings or real time data analysis.

R/. We thank the reviewer for the comment. We corrected the text in all the parts where this term was unproperly used:

1) The best model to predict in a non-destructive, fast and at line approach the amount of GOS (range 8.06 -151.62 mg/mL) […].
2) PLS regression models demonstrated to be reliable to assess lactose, glucose and galactose content in a non-destructive, fast and at line approach, which could be used for real time applications in the future.

2, Strange reaction pattern was observed (WO, W1/2 reaction) and no repetitions was made to investigate if the results are correct or there was an error during the experiments. Thus, no conclusions can be made on lactose consumption and GOS production using different enzymes and their mixtures.

R/. No experimental error was attributed to this data, both repetitions lead to similar responses. The variation in the behaviour should be better explained by further studies, however all the analysis (HPLC, FTIR) confirmed that the experiment was well performed but the reactions have “peculiar” behaviour. Unfortunatelly, due to Covid-19 emergency, it is not possible to perform any other experiment in Italy nor in Colombia.

3, Small dataset was used for multivariate data analysis compared to the complexity and variability of the enzymatic process. 2 out of 5 experiment was excluded during FTIR data analysis because of high measurements error.

As concern the data numerosity, we believe that the experimental setup was not properly explain in M&M section, it has been amended in the revised manuscript. In our experience it covers the variability of the reactions under study. Indeed, two repetition of each enzymatic reaction were performed with the following scheme:

- 5 different enzymatic combinations performed independently twice,
- 8 sampling time per reaction,
- collection of two samples for each sampling point,

for a total of 80 measurements replicated twice, then averaged in the presented results. The dataset is then composed by 80 sampling points, divided in the PLS modelling in 64 samples (4*8*2) for the calibration set and 16 (1*8*2) for the test sets.

The measurement error could be negligible, as the two independent repetition resulted quite similar both by HPLC analysis and FTIR analysis. This was better reported in the text.

4, The results from multivariate curve resolution are not corresponding with experimental data. Also

it gives no extra information about the galactooligosaccharide production (no inline measurements). The kinetic assessment can be performed based on the HPLC data.

R/. We apologies for the mistake, it seems that Figure S1 and S2 were exchanged, this mistake was amended in the new submission.

MCR-ALS models have been developed focusing more on the process kinetics rather than the prediction of each component. The obtained results are not optimal, but they lay the ground to a future kinetics monitoring of the process. The advantage of MCR-ALS over PLS is the possibility of predicting the process trend, rather than measuring the content of one or more specific compounds in the sample in a specific moment/time. We would like to reach the inline measurements by a pump transferring the reaction medium to the FTIR sample holder, but we are not able yet to perform further experiments. We apologies for that.

Surely, the kinetic assessment can be performed based on the HPLC data, but this would require that for any new reaction a sample preparation and analysis is performed, thus needing expert personnel, long time of analysis and sample destruction. The proposed method is oriented to substitute the routine measurements by HPLC.

5, The '3.5 GOS fitting analysis' section in 'results and discussion' has no experimental results, it contains only a discussion of literature data.

R/. Section 3.5 was removed, and the contents previously discussed here were moved to more appropriated sections.

Please see below my additional comments:

Page 3. Row 30. Substrate concentration also an important factor of GOS production. (add substrate concentration in the text at this line)

R/. The correction has been made in the document.

Page 3. Row 58. There are other examples in the literature about monitoring beta-galactosidase and sugars (oligosaccharides, glucose, lactose, galactose) that should be mentioned.

As suggested by the reviewer we integrated our literature survey. Actually, most of the recently published papers deal with the use of FTIR spectroscopy for structural characterization:

- “Native collagen, the enzyme β-galactosidase, modified supports, and the derivatives obtained were structurally characterized using […] infrared spectroscopy (IF)”. Gennari, A., Mobayed, F. H., Catto, A. L., Benvenutti, E. V., Volpato, G., & de Souza, C. F. V. (2019). Kluyveromyces lactis β-galactosidase immobilized on collagen: catalytic stability on batch and packed-bed reactor hydrolysis. Reaction Kinetics, Mechanisms and Catalysis, 127(2), 583-599.
- “The Fourier Transform Infrared Spectroscopy (FT-IR) spectra of glass bead before and after β-galactosidase immobilization were recorded at the 4000–400 cm−1 region […]”. Eskandarloo, H., & Abbaspourrad, A. (2018). Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. Food chemistry, 251, 115-124.
- “The FTIR spectrum was recorded using a Thermo Nicolet 5700 spectrometer (Nicolet, Madison, WI, USA) with a resolution of 4 cm−1 using the KBr method.” Li, H., Cao, Y., Li, S., Jiang, Y., Chen, J., & Wu, Z. (2019). Optimization of a dual-functional biocatalytic system for continuous hydrolysis of lactose in milk. Journal of bioscience and bioengineering, 127(1), 38-44.

- “FT-IR spectra of the dried bacterial cellulose was recorded using a Perkin Elmer FTIR spectrophotometer”. Kumar, V., Sharma, D. K., Sandhu, P. P., Jadaun, J., Sangwan, R. S., & Yadav, S. K. (2020). Sustainable process for the production of cellulose by an Acetobacter pasteurianus RSV-4 (MTCC 25117) on whey medium. Cellulose, 1-14.
- “Successful conjugation of amino and carboxyl functional groups on the surface of HPSNs is confirmed from the Fourier transforms infrared (FTIR) spectra of HPSNs-NH2 and HPSNs-COOH”. Misson, M., Du, X., Jin, B., & Zhang, H. (2016). Dendrimer-like nanoparticles based β-galactosidase assembly for enhancing its selectivity toward transgalactosylation. Enzyme and microbial technology, 84, 68-77.

Furthermore, few works report process monitoring by FTIR spectroscopy:

- Schiele, S. A., Meinhardt, R., Eder, C., & Briesen, H. (2020). ATR-FTIR spectroscopy for in-line anomer concentration measurements in solution: A case study of lactose. Food Control, 110, 107024.
- Romano, N., Santos, M., Mobili, P., Vega, R., & Gómez-Zavaglia, A. (2016). Effect of sucrose concentration on the composition of enzymatically synthesized short-chain fructo-oligosaccharides as determined by FTIR and multivariate analysis. Food chemistry, 202, 467-475.
- Cocciardi, R. A., Ismail, A. A., Van De Voort, F. R., & Sedman, J. (2004). Monitoring of lactose hydrolysis in milk by single-bounce attenuated total reflectance Fourier transform infrared spectroscopy. Milchwissenschaft, 59(7-8), 403-407.

The results of the literature survey were included in the introduction and in the discussion of the results, when appropriated.

Page 4. Row 58. How was the falcon tubes homogenized during reaction? Did you use a shaker?

R/. At the beginning of the reaction, sugar solutions were homogenized by stirring with an ultraturrax®. After this procedure, a magnetic bar was introduced into each falcon tube and permanently stirring at 800 rpm. This explanation was already included in the manuscript.

Page 5. Row 4. Did you verify the enzyme inactivation method? (5-10 min of heating are enough)

R/. Yes, in the experience of the research group, no time above 10 min is required to inactivate beta-galactosidase in boiling water. It is important to mention that *K. lactis* enzyme loses its activity above 50 °C, whereas *A. oryzae* enzyme loses it above 65°C.

Page 5. Row 26. What is the type of FTIR spectrometer? How did you perform inline measurement? Inline probe?

R/. The reference and specifications of the equipment were included. An ATR sample compartment was employed. In fact, it is an at line measurement which could be further change to online/inline measurement by a pump deviation to the sample compartment.

Page 5. Row 49. Did you apply real time monitoring and data processing? There is no information about it.

R/. We thank the reviewer for the comment. We corrected the text in all the parts where this term was unproperly used:

- The best model to predict in a non-destructive, fast and at line approach the amount of GOS (range 8.06 -151.62 mg/mL) […].

- PLS regression models demonstrated to be reliable to assess lactose, glucose and galactose content in a non-destructive, fast and at line approach, which could be used for real time applications in the future.

Page 5. Row 56. You should include how many data points did you use for calibration. Was there replicate measurements?

R/. As concern the data numerosity, we believe that the experimental setup was not properly explain in M&M section, it has been amended in the revised manuscript. However, in our experience it properly covers the variability of the reactions under study. Indeed, two repetition of each enzymatic reaction were performed

- 5 different enzymatic combinations performed independently twice
- 8 sampling time per reaction
- with collection of two samples for each sampling point,

For a total of 80 measurements replicated twice and thus averaged in the presented results. The dataset is then composed by 80 sampling points, divided in the PLS modelling in 64 samples (4*8*2) for the calibration set and 16 (1*8*2) for the test sets.

Page 6. Row 37. It is really hard to follow the names of the reactions. It would be easier to read the text if you define what WK, W1/4, W1/2, W3/4, WO means at the beginning of 'results and discussion' also in the figure captions.

R/. We apologies for the difficult coding used, it has been modified as suggested by Reviewer 2.

Page 6 Row 52. Grammar should be corrected: "…in this reaction after 1 hour started GOS hydrolysis…"
Page 6 Row 56 Grammar should be corrected: "…hydrolysis is not as faster as…"

R/. Grammar mistakes have been corrected.

Figure 1. What is the standard deviation means on Figure 1? Based on the experimental section there was no replicate experiments. At least reaction W1/2 and WO should be investigated again to see if the results are correct. No conclusions can be made on lactose consumption and GOS production.

R/. For each reaction, two technological repetitions (two different assays) and two measurements (replicates of the measurements) were performed for each sampling point. In this order we could guarantee that enough number of samples is considered. Thus, standard deviation indicated in the figures refers to the deviation between the measurements (replicates of the measurement of each sample). For reactions W1/2 and WO, as well as for all the other conditions, the two independent assays gave superimposable results.

Page 9 Row 17 "the behaviour of both WO and W1/2 enzymes, that presented a lactose consumption and its conversion to GOS significantly different from the other three enzymes" - What is the reason for this? Are you sure the observed reaction pattern is not measurement error? You should perform a replicate measurement to investigate it. (this is the reason of section 3.5)

R/. As reported before, no experimental error was attributed to collected data, both repetitions lead to similar responses. The variation in the behaviour should be better explained by further studies, however all the analysis (HPLC, FTIR) confirmed that the experiment was well performed but the

reactions have “peculiar” behaviour. Unfortunately, due to Covid-19 emergency, it is not possible to perform any other experiment in Italy nor in Colombia. Nonetheless, in section 3.1 and 3.2 we added some explanation to justify the obtained results.

Page 9 Row 23. 3*8 data points was used to calibrate PLS model, which is a small dataset compared to the complexity and variability of the enzymatic process.

R/. As concern the data numerosity, we believe that the experimental setup was not properly explain in M&M section, it has been amended in the revised manuscript. However, in our experience it properly covers the variability of the reactions under study. Indeed, two repetition of each enzymatic reaction were performed

- 5 different enzymatic combinations performed independently twice
- 8 sampling time per reaction
- with collection of two samples for each sampling point,

for a total of 80 measurements replicated twice and thus averaged in the presented results. The dataset is then composed by 80 sampling points, divided in the PLS modelling in 64 samples (4*8*2) for the calibration set and 16 (1*8*2) for the test sets.

Table 2. It is more correct to compare models for the same validation set. It can be seen from your data, that calibration model from W1/4 and W3/4 reaction had higher prediction error for WK reaction (test set from pure enzyme, calibration set from mixture) compared when the test data set and part of calibration set was from a reaction with enzyme mixture. Thus, the calibration data set should contain data points from similar reaction condition.

R/. We would like to thank the reviewer for the comment. The discussion of PLS results were modified accordingly and integrated by the comparison with other similar works.

Page 9. Row 52. RPD should be defined.

R/. RPD was defined in M&M section as “residual prediction deviation (RPD), i.e. ratio of standard error of performance to standard deviation, was calculated to compare the precision of the prediction with the average composition of all the samples, as stated by Camacho-Tamayo et al. (2014).”

For a better understanding the meaning of this parameter is also commented in the paragraph cited by the reviewer.

Figure 3. Figure S1 and Figure S2: MCR-ALS results are not corresponding with experimental data. Page 11. Row 14. "GOS results accurately predict in the first stage of production" - The interpretation of data should be corrected. The dataset used for MCR-ALS is small and also the inaccuracy can be due to rotation ambiguity. It occures, when the physicochemical constraints are not sufficiently strong to provide a unique resolution of the data matrix of the mixtures into spectra and concentration profiles of individual chemical components.

R/. We apologies for the mistake, it seems that Figure S1 and S2 were exchanged, this mistake was amended in the new submission. The highlighted sentence has been removed.

As far as concern rotation ambiguity, we are aware of its importance in heavily mixed data (such as kinetic process) and the need of the proper constrains to reduce it. Thus, we applied constrains considering chemical/physical meaning, interpretability, improved model reliability and reduction of rotational ambiguity.

A systematic investigation on any constraint in spectral modes of data matrix has be monitored via calculation of feasible solutions and interpretation of obtained results, inspiring to the methods discussed by Golshan et al. (2016)*. We ended with the idea that non-negativity constraint, imposed on both concentration and spectral profiles, could solve MCR-ALS ambiguities present in our system.

This was better explained in the Materials and Methods section.

* Golshan, A., Abdollahi, H., Beyramysoltan, S., Maeder, M., Neymeyr, K., Rajkó, R., ... & Tauler, R. (2016). A review of recent methods for the determination of ranges of feasible solutions resulting from soft modelling analyses of multivariate data. Analytica Chimica Acta, 911, 1-13.

Page 11. Row 20. "Fast decrease in GOS concentration is been previously associated to potassium ions from permeate salts in the solution and K. lactis enzyme source (Rico-Rodríguez et al., 2018; Rodrigues Mano et al., 2019)." - The experimental data did not show fast decrease, only the calculated values from MCR-ALS.

R/. Actually, KI reaction followed by HPLC showed a reduction of GOS from its maximum of 110 mg/mL (after 60 min) to 57 mg/mL (after 300 min). This is reported in Figure 1B. This correspond to a drop from 100% to 50% of GOS content as shown in Figure 3B.

This behaviour has been previously attributed to potassium salts dissolved in media. Nevertheless, with the experimental data collected, there is no possibility to evaluate any effect of minerals from cheese whey on the reaction. For this particular situation, further analysis must be developed. A better discussion of this behaviour has been reported in adequate sections by moving the content reported in pervious section '3.5 GOS fitting analysis'

HIGHLIGHTS

- Galactooligosaccharides (GOS) were synthesized by different enzymatic combinations
- GOS production was monitored by HPLC, FTIR spectroscopy and Chemometrics
- Partial Least Square models enabled the quantification of relevant compounds
- Multivariate Curve Resolution models enabled process kinetics assessment

Graphical Abstract

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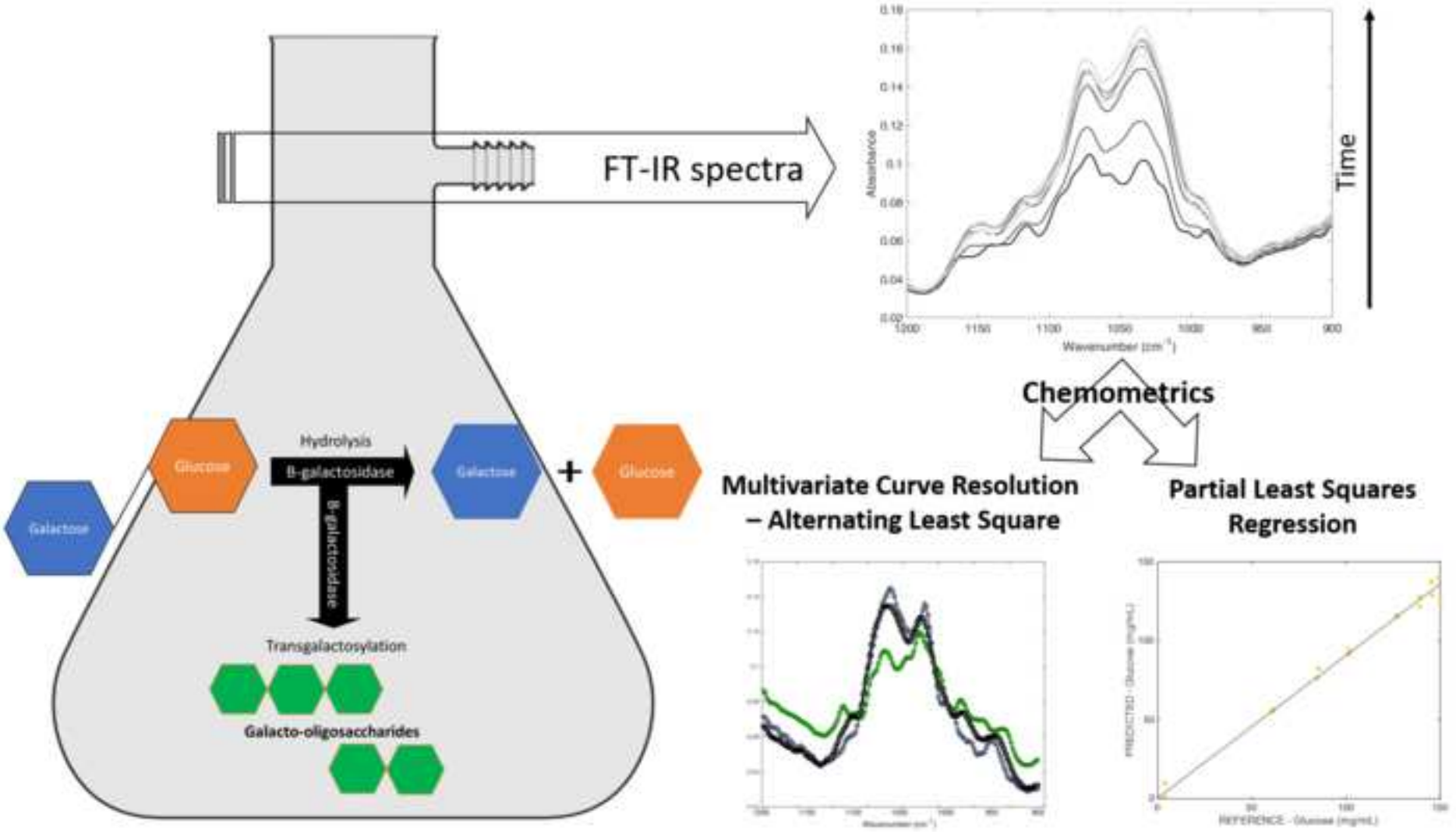

Study of Galactooligosaccharides production from dairy waste by FTIR and Chemometrics as Process Analytical Technology

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Abstract

Galactooligosaccharides (GOS) production from whey, a relevant by-product of dairy industry, answers to the Circular Economy principle of extending the life cycle of products. Indeed, it allows the reuse of dairy waste to produce prebiotics to be used in functional food preparations. For this purpose, the effective monitoring of GOS production should be performed in real time and by environmentally friendly techniques. Thus, FTIR spectroscopy, combined with different chemometric approaches, has been tested to assess a Process Analytical Technology to follow GOS production from cheese whey. Partial Least Square regression models were reliable for lactose, glucose and galactose determination (Root Mean Square Error of Prediction of 21.9, 11.1 and 12.4 mg mL^{-1}, respectively). Furthermore, Multivariate Curve Resolution – Alternating Least Square models were proposed to describe trends of the reaction components along the process being an interesting alternative to chromatographic determinations. The real time implementation of the proposed approach will provide the dairy industry with a reliable and green Process Analytical Technology for dairy waste reallocation, avoiding sample pre-processing, large use of organic solvents and long times of analysis.

Keywords: Prebiotic synthesis, cheese by-products, Infrared spectroscopy, Process Analytical Technology, Chemometrics

1. Introduction

Galactooligosaccharides (GOS) comprise a group of oligomers, derived from lactose, possessing functional properties since they act as prebiotics and promote benefits in microbial gut and human health (Rodrigues Mano et al., 2019). Indeed, GOS are one of the main prebiotics used for functional food preparations. Among their functional properties, it is possible to mention their ability to increase beneficial gut microbiota, immune system modulation and/or antipathogenic effect (Byfield et al., 2010; Sangwan et al., 2011). Besides their functional characteristics, GOS possess desired technological properties which allows them to be used on different food matrixes like dairy, bakery or beverages, among others (Sangwan et al., 2011).

GOS are produced by lactose transgalactosylation. This reaction is catalysed by β-galactosidase (βgal) enzymes, obtained from a wide variety of microorganisms: bacteria, yeast and/or fungi (Byfield et al., 2010). Two of the most used βgal in industry are obtained from *Kluyveromyces lactis* and *Aspergillus oryzae* (Fischer and Kleinschmidt, 2015). As βgal possesses double activity (hydrolytic and transgalactosylation), it is necessary to control the thermodynamics of the reaction by increasing lactose concentration in the media, which favours GOS formation over monosaccharide release (González-Delgado et al., 2016). GOS production depends on many factors like substrate and initial sugar concentration, pH and temperature of reaction, presence of enzyme inhibitors and source of enzyme (Chockchaisawasdee et al., 2004).

It has been suggested that a variation in the GOS degree of polymerisation and linkages plays an important role on the effect of gut microbiota (Akiyama et al., 2015). Different assays have shown that GOS produced by βgal from *K. lactis* and *A. oryzae* differs in yields and composition. Whilst *K. lactis* GOS are mainly composed of di- and tri-saccharides, *A. oryzae* βgal produces GOS from di- to hexa-saccharides (Gosling et al., 2010). However, yields for lactose transgalactosylation with *K. lactis* βgal are around 45 – 50% of total products (Rico-Rodríguez et al., 2018). Nonetheless, with *A. oryzae* βgal this value is lower than 35% (Otieno, 2010).

On the other hand, GOS quantification is generally performed by high performance liquid chromatography (HPLC) (Hernández-Hernández et al., 2012) or gas chromatography (GC) (Ruiz-Matute et al., 2012), two of the most reliable analytical techniques for the purpose. However, these methods require sample pre-processing (GC), large volumes of organic solvents (HPLC) and long analysis time per sample.

Nowadays, the research for quick-environmentally friendly analytical techniques has focused on vibrational spectroscopic technologies, such as mid-infrared, near-infrared and Raman spectroscopy (Moros et al., 2010).

Fourier transformed mid-infrared (FTIR) has been used in enzymatic studies for structural characterization from β-galactosidase immobilization, encapsulation and conjugation (Gennari et al., 2019; Li et al., 2019; Eskandarloo & Abbaspourrad, 2018; Misson et al., 2016) to derivatives examination (Kumar et al., 2020). However, little investigation has been proposed for process monitoring in this field, mainly represented by the in-line anomer concentration measurements in solution proposed by Schiele et al. (2020). Furthermore, Romano et al. (2016) evaluated, by FTIR and Partial Least Squares (PLS) regression, the effect of sucrose concentration on the composition of enzymatically synthesized short-chain fructo-oligosaccharides; similarly, the lactose hydrolysis in milk has been studied by Cocciardi et al. (2004). On the other hand, FTIR proved to be reliable to control processes in a fast, real time and non-destructive way (Grassi et al., 2014), or as a tool for evaluating quality parameters in dairy products (Mohamed et al., 2020) among others.

Despite this, FTIR technique has few drawbacks. Among them, the difficult interpretation of the signals, which are often composed of overlapping spectral bands resulting from absorption of multiple compounds in the sample. In this context, chemometric techniques can help to overcome interpretation problems and they allow to extract relevant information. Spectral data can be modelled by both hard- and soft-modelling methods. Among hard modelling methods, Partial Least Square (PLS) regression is one of the most used for predicting quality parameters from a single spectrum after a proper model calibration, thanks to its ability to handle highly overlapping and colinear data (Martens and Næs, 1989). Instead, Multivariate Curve Resolution – Alternating Least Square (MCR-ALS) (De Juan and Tauler, 2006), a soft-modelling method, is useful to describe trends of the reaction components along the process.

This work aims to develop an analytical technology process based on FTIR spectroscopy, coupled with multivariate analysis, for at-line monitoring of GOS production from whey.

2. Material and methods

2.1. Reagents

Cheese whey was purchased from CIMPA SAS (Bogotá, Colombia) with lactose content of 71.12 % and <1% of monosaccharides. Commercial β-galactosidase Enzeco fungal lactase from *A. oryzae* (Ao) was provided by Enzyme Development Corporation (New York, USA); HA-Lactase5200 from *K. lactis* (Kl) was provided by CHR Hansen (Bogotá, Colombia). Enzymes had a total protein content,

determined by Bradford method (Bradford, 1976), of 38.9 ± 0.1% (Ao) and 4.8 ± 0.1% (Kl). Enzyme activities were measured as reported by Rico-Rodríguez et al. (2018) giving enzyme activity of 15045 U/g (Ao) and 5172 U/g (Kl). Bovine serum albumin (BSA), sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, and sodium hydroxide were purchased from Sigma–Aldrich (St. Louis, MO). Glucose (Glu), galactose (Gal), lactose (Lac) and raffinose (DP3), were purchased from Panreac® (Barcelona, Spain).

2.2. Enzyme reactions

All assays were carried out in 50 mL falcon tubes with an effective volume of 20 mL (370 mg mL^{-1} of lactose equivalent) in sodium phosphate buffer solution 0.01 M at pH 6.0 and temperature of 42 ± 1 °C. Enzyme doses for each assay are reported in Table 1. Sampling was done by duplicate after 0, 20, 40, 60, 120, 180, 240, 300 min of reaction under continuous stirring at 800 rpm. Samples were immersed in boiling water (5 min) for enzyme inactivation; afterwards, samples were cooled and stored at -20 °C until carbohydrate quantification.

Table 1 - Reaction arrays and dose for each assay

Assay	Alias	Hal Dose (U)	Enz Dose (U)
Enz:Hal (100:0)	WO	37.8	0
Enz:Hal (75:25)	W1/4	28.4	0.9
Enz:Hal (50:50)	W1/2	18.9	1.9
Enz:Hal (25:75)	W3/4	9.9	2.8
Enz:Hal (0:100)	WK	0	3.8

2.3. Carbohydrate quantification

Substrates and products of enzyme reactions were analysed in an Ultimate 3000 HPLC instrument (Themo Fisher Scientific, USA). For GOS3, GOS4, GOS5, lactose and monosaccharides a BP-100 Ca^{++} (300 mm x 7.8 mm – Benson polymerics, USA) was set at 80°C and 0.5 mL/min of deionised water flowing rate. GOS2 were separated from lactose with a Spherisorb S5-NH_2 (250 mm x 4.0 mm – Waters, USA) with acetonitrile:water (75:25) as eluent at 30°C and 0.75 mL/min flowing rate. Both measurements were done in a Transgenomic® RI detector. Calibration curves were performed with appropriate sugar standards in the linear range 0.05 mg mL^{-1} to 5 mg mL^{-1}.

2.4. FTIR measurements

IR spectra, from different enzyme mixture reaction samples, were collected in transmission mode, with a FT-IR spectrometer (IR-Prestige21, Shimatsu Corporation, Japan) equipped with an ATR cell. The spectra information was collected in the range 4000 – 400 cm^{-1}, with a resolution of 4 cm^{-1} and 25 scans for both background and samples. For each duplicate of the enzymatic rection, the same samples were analysed twice; the total number of collected spectra was 160. Besides, samples of carbohydrate standards (400 mg mL^{-1}) were analysed following the same procedure.

2.5. Data processing

The whole wavenumber range (4000 – 400 cm^{-1}) was reduced to the fingerprint region (1200 – 900 cm^{-1}) and spectra were averaged on sample base. Before applying multivariate analysis methods, several pre-treatments were performed in order to assess which one could efficiently correct scattering effects and reduce noise: Standard Normal Variate (SNV), first and second derivatives, baseline correction (automatic weighted least squares) and smoothing (Savitzky–Golay, filter width 7 points; polynomial order 1).

To predict the amounts of lactose, GOS, glucose and galactose in whey samples, PLS regression was performed by using the PLS Toolbox software (Eigenvector Research, Inc., Wenatchee, Washington, USA) implemented in Matlab (MathWorks, Natick, MA, USA). For each evaluated parameter, data were partitioned in a calibration (including 64 averaged spectra) and a validation set (including 16 averaged spectra). The validation sets were constructed on reaction bases. This means that one out of five enzymatic reactions (16 averaged spectra) was kept out from the calibration set in an iterative way, thus developing five different models, each of them tested by one different reaction test set. Models were also internally validated by Venetian Blind Cross-Validation, with 10 splits and 2 samples per split, being sure to keep duplicates together. To evaluate model reliability, Root Mean Square Error of Prediction (RMSEP) was compared with the Root Mean Square Error of Laboratory (RMSEL). Furthermore, residual prediction deviation (RPD), i.e. ratio of standard error of performance to standard deviation, was calculated to compare the precision of the prediction with the average composition of all the samples, as stated by Camacho-Tamayo et al. (2014).

MCR-ALS method was applied on spectra using MCR-ALS 2.0 Toolbox (Jaumot et al., 2015) implemented in Matlab (MathWorks, Natick, MA, USA). Spectral data were arranged in five matrices, one for each enzymatic reaction considering together the duplicates, and analysed separately. For each model the number of components was selected by Pure Variable detection method. Model optimization was achieved by iterative implementation of ALS algorithm, i.e. until the Lack of Fit (LOF) difference in two consecutive iterative cycles was lower than 0.1%. A

systematic investigation on any constraint in spectral modes of data matrix has be performed via calculation of feasible solutions and interpretation of obtained results. Then, non-negativity constraint was imposed on both concentration and spectral profiles to solve MCR-ALS ambiguities. For further details about the MCR-ALS procedure and optimization the reader can refer to Grassi et al. (2019).

3. Results and discussion

3.1. Kinetics of GOS production by HPLC

Transgalactosylation reactions by βgal from two sources (*K. lactis* and *A. oryzae*) and their mixtures followed the characteristic lactose consumption (Fig. 1). Lactose conversion for βgal from the yeast *Kluyveromyces lactis* results in values over 90% with a GOS production above 40% - mainly disaccharides and trisaccharides - and few tetrasaccharides (<20% of the total GOS). However, combination with βgal from *A. oryzae* could lead to better profiles of oligomers present in the final mixture. In Fig. 1A it is observed that lactose consumption is highly affected by βgal mixture, i.e. by the enzyme source. When *K. lactis* enzyme is predominant in the mixture, Kl and 1A/3K trials, lactose concentration dropped faster than in other cases to values below 50 mg mL^{-1}. Nonetheless, kinetic behaviour of sample 2A/2K, which had equal dose of both enzymes, showed an unusual trend. Lactose consumed (50% of initial lactose) by 2A/2K reaction was lower than Ao (63% of initial lactose), which corresponded to *A. oryzae* pure enzyme.

In this set of reactions, monosaccharides (Fig. 1C and 1D) had one of the most significant variations. Though in all reactions there was a typical increase in the concentration of monosaccharides (glucose and galactose), there was also, in each treatment, a significant variation in their amount.. *K. lactis* enzyme is known by its poor ability to include glucose in GOS molecule, hence, the concentration of this monosaccharide is above 40% of total carbohydrates present in media (Jenab et al., 2017; Rico-Rodríguez et al., 2018). Moreover, galactose release depends on the ability of the enzyme to transform lactose into GOS at defined conditions of reaction as the later are mainly composed of this monosaccharide. In this case, it is possible to observe that a gradually decreasing in final concentration of those monosaccharides occurs with the reduction in the concentration of initial *K. lactis* enzyme or with the increase of *A. oryzae* enzyme source.

Fig. 1B shows GOS kinetic concentration for each reaction. Samples Ao, 2A/2K and 3A/1K show a similar kinetics with GOS concentration increasing over the 5 hours of reaction. A typical trend is shown by Kl; in this reaction, GOS hydrolysis started after 1 hour. For 1A/3K the reaction exhibits similar trend, nonetheless, maximum concentration was found at 120 min and GOS hydrolysis is not as fast as Kl. Highest concentration of GOS was 36% of total carbohydrates and it was reached after

60 min for 1A/3K and after 300 min for 2A/2K. In Fig. 1B it is notable a clear differentiation in the kinetics of the pure enzyme *K. lactis*, if compared to all enzyme combinations.

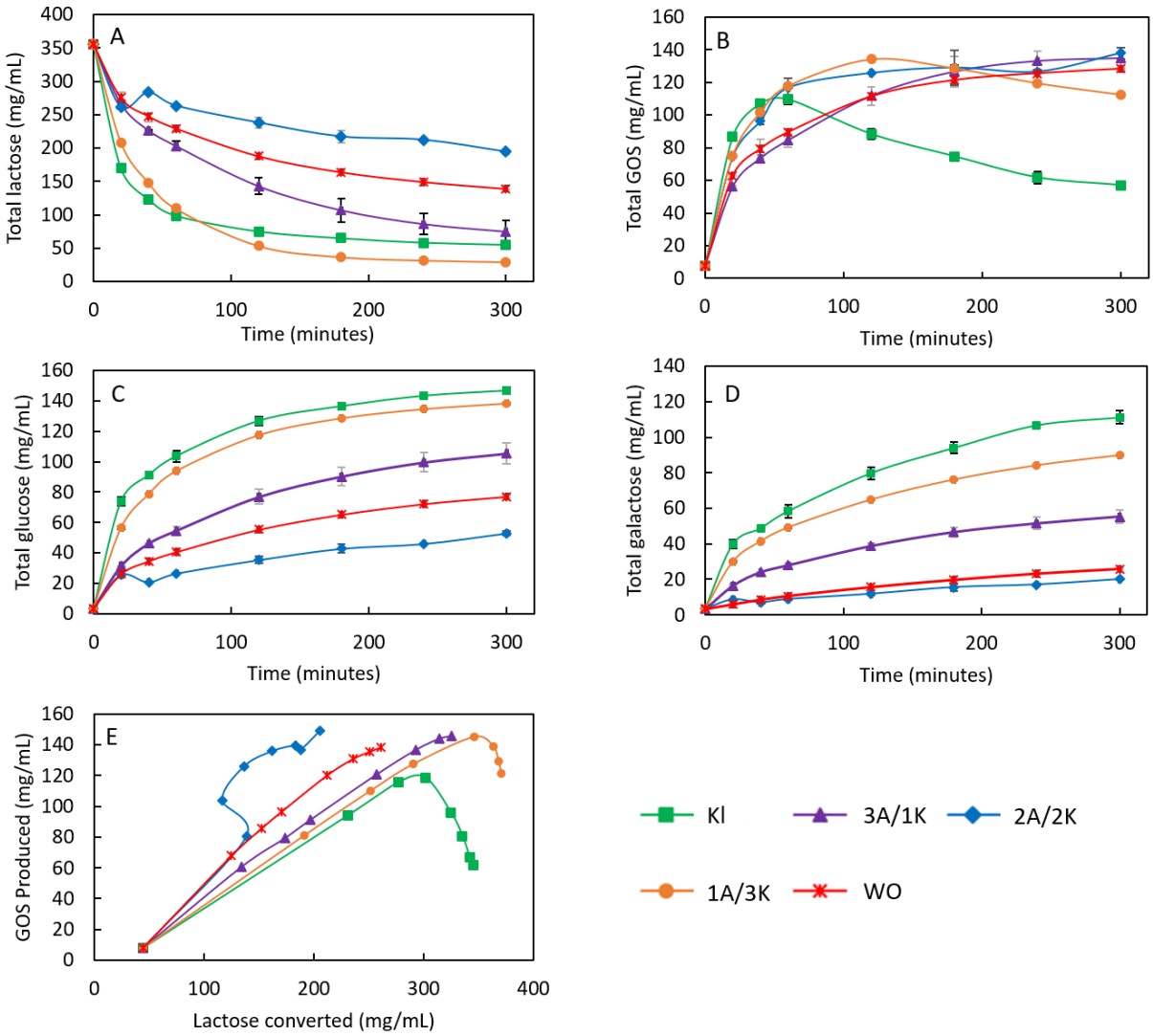

Fig. 1 - Lactose transgalactosylation in presence of β-galactosidase mixture: (A) Lactose consumption; (B) GOS production; (C) Glucose production; (D) Galactose production; (E) Lactose conversion to GOS. Error bars represent the standard deviations between

On the other hand, a graph of lactose conversion to GOS is presented in Fig. 1E. This graph shows the ability of βgal to transform lactose to GOS in reaction media. Although in Fig. 1E the conversion of lactose is well differentiated for pure enzymes and their mixtures, 2A/2K has a bulging trend, an unexpected behaviour possibly linked to the interaction between the enzymes.

Results suggest that βgal from *K. lactis* consumes high amounts of lactose (73%) to reach 30% of GOS in media with initial lactose of 370 mg mL^{-1}. However, the amount of required lactose changes gradually when enzyme doses change in different reactions. Reaction Ao, in which only *A. oryzae*

enzyme is present, required the lowest amount of lactose to produce the maximum concentration of GOS when initial lactose was 370 mg mL^{-1}. Fischer and Fischer and Kleinschmidt (2018) performed a similar assay, however, they evaluated time of enzyme addition instead of enzyme concentration. The yield they found was significantly lower than those obtained in the present work, even for pure enzymes. Another difference, very important for this kind of reactions, is the source of lactose employed in both works. While Fischer and Fischer and Kleinschmidt (2018) evaluated pure lactose as reaction media, our work assayed cheese whey, which as reported by same authors, might interfere significantly with enzyme transgalactosylation ability (Fischer and Kleinschmidt, 2015). Same results were found in a previous work from our group (Rico-Rodríguez et al., 2020).

3.2. FTIR spectroscopy

FTIR data obtained from enzymatic reactions followed a similar trend. As the composition of total carbohydrates in the media changed with time, so FTIR spectra changed. In Fig. 2 it is possible to observe data collected for treatments Kl (Fig. 2A), 1A/3K (Fig. 2B) and 3A/1K (Fig. 2C) as well as spectra of standards of lactose, glucose, galactose and GOS (370 mg mL^{-1}) (Fig. 2D).

Changes in spectra profiles of GOS reactions are more evident in the first minutes of the reactions, especially for Kl and 3A/2K reactions. These changes are linked to the fast drop of lactose concentration after 40 minutes of reaction and to the rise in glucose, galactose and GOS signals.

Indeed, the FTIR spectra of cheese whey (beginning of the reactions) are characterised by a broad absorption band from 1200 to 964 cm^{-1}, with two maxima at 1074 and 1034 cm^{-1} and a shoulder at 995 cm^{-1}. Those signals are mainly related to lactose presence (the main carbohydrate present in cheese whey - > 98% of total carbohydrates). Pure lactose (Fig. 2D) showed a profile characterised by a band with a maximum at 1157 cm^{-1}, followed by a broad band between 1110 and 970 cm^{-1} with two maxima with similar absorbance at 1074 cm^{-1} and 1034 cm^{-1} and a shoulder at 995 cm^{-1}.

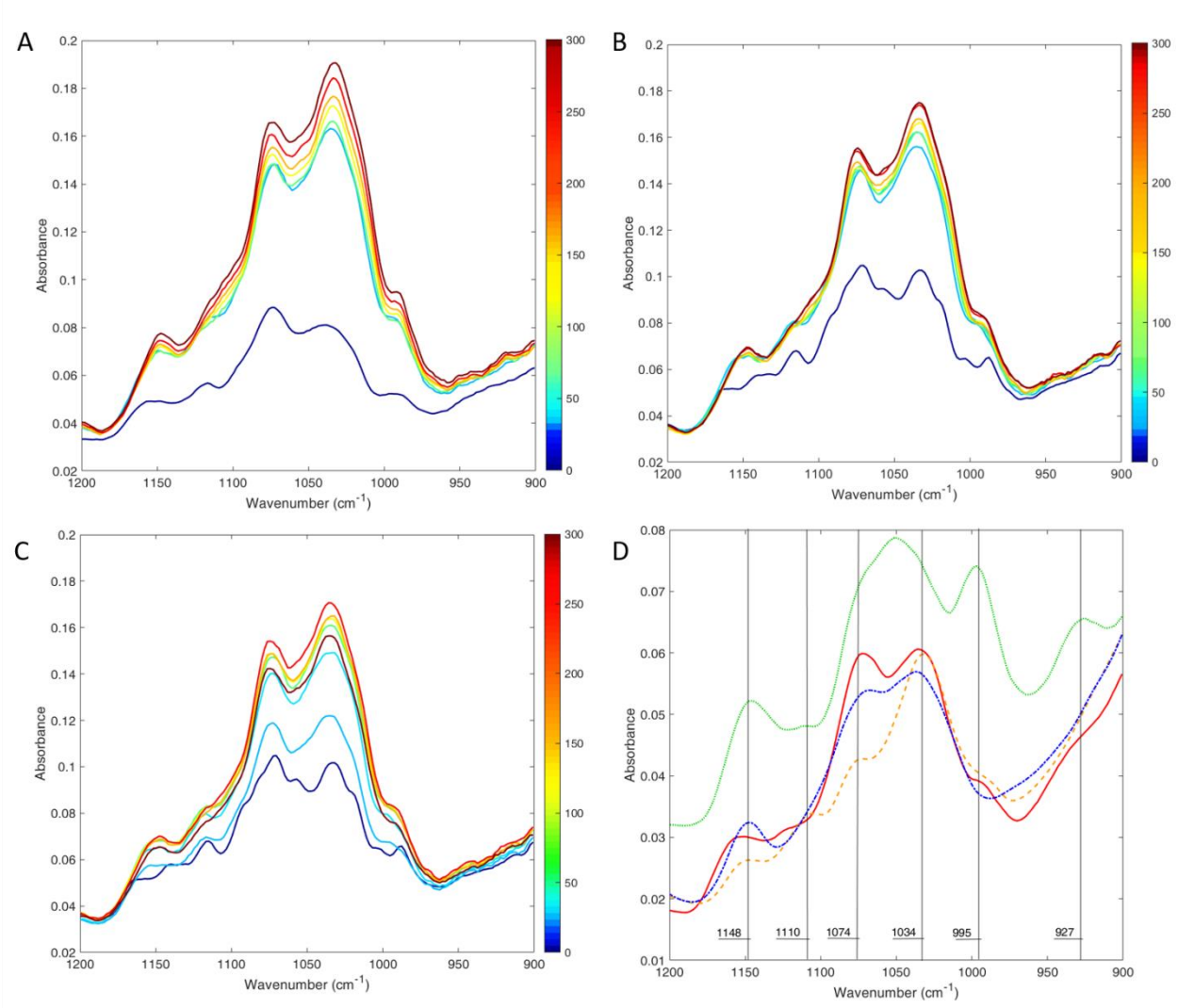

Fig. 2 - FTIR spectra in the region 1200–900 cm^{-1} collected from different enzymatic reactions (A, Kl; B, 3A/1K; C, 1A/3K) and pure components spectra (D). In A, B and C spectra are coloured according to reaction time (min). In D spectra correspond to lactose (red, -), glucose (orange, ---), galactose (blue, -.-.) and GOS (green, ⋯).

Spectra collected along reactions showed slightly different profiles from those collected at the beginning. Particularly, with the progress of time, the peak at 1100 cm^{-1} disappears, the difference between the absorbance at 1074 and 1034 cm^{-1} increases and a small signal becomes visible at 1050 cm^{-1}. Those changes can be attributed to the consumption of lactose and the increase of glucose, galactose and GOS. Indeed, pure glucose and galactose FTIR spectra (Fig. 2D) showed a spectral profile slightly different from the one of lactose. Even if the maxima are similar, glucose FTIR profile differs from the other pure components due to the presence of a shoulder at 1110 cm^{-1} and a high difference between the maxima at 1074 and 1034 cm^{-1}; whereas the spectra collected for pure galactose is characterised by a higher absorbance at 1148 cm^{-1} and the absence of the shoulder at 995 cm^{-1}. For pure GOS spectra (Fig. 2D) the band between 1110 and 970 cm^{-1} does not show double

maxima but a higher absorbance at 1050 cm^{-1}. Moreover, the shoulder observed at 995 cm^{-1} for lactose and glucose is a distinct peak followed by a peak at 927 cm^{-1}.

Even if the contribution of the pure compounds can be inferred by visual inspection, the complexity of FTIR data could be better managed by multivariate approaches such as PLS and MCR-ALS, with the aim of monitoring GOS formation.

3.3. PLS regression

The PLS models were calibrated using 64 averaged spectra and internally validated by Venetian Blind Cross-Validation. Furthermore, they were tested for prediction in an iterative way, i.e. by testing they prediction capability by a validation set consisting of 16 averaged spectra of one of the enzymatic reactions performed. In Table 2 are reported the best results obtained applying PLS regression on pre-treated spectral data, they were obtained when the validation set was constructed by Kl, 3A/1K and 1A/3K data. Less promising results (data not shown) were obtained when Ao and 2A/2K enzymes were used for validation. These enzymes showed an unexpected behaviour, more specifically the reactions presented a lactose consumption and its conversion to GOS significantly different from the other three enzymes (Fig. 1A and 1C).

Considering reactions with Kl, 3A/1K and 1A/3K enzymes PLS results were good both in terms of RMSEP and R^2_P. In particular, lactose models (range 31.50 to 384.44 mg mL^{-1}) presented very high R^2 values in prediction (R^2_P > 0.96) and a RMSEP value approximately lower than 3 times the RMSEL, an acceptable error for a NIR application (Shenk and Westerhaus, 1996). Similarly, Cocciardi et al. (2004) predicted lactose during hydrolysis reaction in milk. They obtained a reliable PLS models (SEP of 0.20% w/v) considering a lactose variability between 0 and 5 % w/v. Our results, converted into percentage, are comparable being our lowest RMSEP for lactose prediction 6.2% (i.e. 21.9 mg/mL), that is comparable in magnitude with Cocciardi et al. (2004) SEP expressed in percentage (i.e. 4%).

Furthermore, in Table 2 are reported the models to predict in a non-destructive, fast and at-line approach the amount of GOS (range 8.06 -151.62 mg mL^{-1}). The model validated with 3A/1K spectra, pre-processed with the baseline correction, using 7 LV gave a RMSEP of 16.0 mg mL^{-1} and a R^2_P of 0.88. With the same validation set, good prediction ability was obtained for both glucose (range 3.90 to 160.25 mg mL^{-1}) and galactose (range 3.60 to 122.97 mg mL^{-1}). In these cases, RMSEP values are extremely close to RMSEL values, indicating an excellent estimation of these parameters (Shenk and Westerhaus, 1996).

Table 2 - PLS regression models for lactose, GOS, glucose and galactose content prediction from FTIR spectra.

	Test set	LV	Pre-treatment	RMSEL (mg mL^{-1})	RMSEC (mg mL^{-1})	RMSECV (mg mL^{-1})	RMSEP (mg mL^{-1})	R^2_{Cal}	R^2_{CV}	R^2_P
Lactose	**Kl**	**6**	**Baseline correction**	8.2	**14.9**	**19.2**	**21.9**	**0.98**	**0.97**	**0.98**
	3A/1K	3	1st Derivative		24.7	28.3	28.4	0.94	0.92	0.98
	1A/3K	5	Baseline correction		16.6	20.3	22.3	0.98	0.97	0.96
GOS	Kl	7	SNV	3.2	9.4	17.9	18.7	0.95	0.84	0.76
	3A/1K	**7**	**Baseline correction**		**10.2**	**14.3**	**16.0**	**0.94**	**0.89**	**0.88**
	1A/3K	7	1st Derivative		11.7	17.3	19.5	0.92	0.84	0.92
Glucose	Kl	2	1st Derivative	8.0	12.6	13.7	14.5	0.91	0.89	0.96
	3A/1K	**6**	**Raw**		**8.1**	**10.6**	**11.1**	**0.97**	**0.94**	**0.99**
	1A/3K	5	Baseline correction		8.7	10.3	12.1	0.97	0.96	0.93
Galactose	Kl	2	1st Derivative	8.1	11.9	12.9	23.6	0.8	0.76	0.95
	3A/1K	**6**	**SNV**		**9.6**	**11.5**	**12.4**	**0.91**	**0.88**	**0.97**
	1A/3K	5	Baseline correction		10.1	12.5	12.6	0.92	0.88	0.75

LV, latent variables; SNV, Standard Normal Variate; RMSEL, Root Mean Square Error of Laboratory; RMSEC, Root Mean Square Error of Calibration; RMSECV, Root Mean Square Error of Cross-Validation; RMSEP, Root Mean Square Error of Prediction; R^2, coefficient of determination in calibration (Cal), cross-validation (CV) and prediction (P).

Referring to the work by Cocciardi et al. (2004), minimum SEP values of 0.13% w/v and 0.075% w/v for glucose and galactose prediction were reached, respectively. However, the authors performed a leave-one-out cross-validation within each enzymatic reaction, thus considering a small inter-enzyme variability and a small range of sugar content variability (0 - 2.5% w/v). Another relevant work in the field, by Romano et al. (2016), focused on the development of PLS models to predict the effect of sucrose concentration on fructo-oligosaccharides (FOS). They reached optimal results when predicting glucose production by a PLS model with a R^2_P of 0.98 and an RMSEP of 2.028 g of glucose/ 100 g of sucrose. Being their variability in a range of 0 – 56 g of glucose/ 100 g of sucrose, the %RMSEP will correspond to 3.6, which is lower than our (i.e. %RMSEP = 7.1).

Furthermore, our model performances were evaluated in terms of precision of the prediction with the average composition of all the samples, by means of residual prediction deviation (RPD). GOS and galactose models have an RPD of 2.8 and 2.7 respectively, suggesting a good predictive performance of the models, whereas lactose and glucose presented an RPD > 3 (5.9 and 4.9, respectively), indicating an excellent predictive performance (Saeys et al., 2005).

3.4. MCR-ALS models

The promising results obtained by PLS regression, suggested the possibility of building MCR-ALS models to follow the enzymatic reaction in vision of a Process Analytical Technology able to predict the process kinetics. Thus, an MCR-ALS model for the duplicates of each enzymatic combination has been developed.

The application of ALS procedure to the FTIR spectra collected allowed the resolution of both spectral and concentration profiles by four-component models, retaining at least 99.9% of the total variance, and a LOF lower than 0.44 %.

Three out of four MCR-ALS profiles were assigned to lactose, GOS and a combination of Glu and Gal; whereas the fourth profile was assumed to be an interference used to isolate possible noise in the process as reported by Ahmadi, Tauler, & Abdollahi (2015). This is in agreement with previous works, reporting that the quantification of monosaccharides, Glu and Gal, as one group (Palai et al., 2012) or as a ratio (González-Delgado et al., 2016) can ease the analysis of their effect on the kinetic model for GOS prediction. According to these works, it is reasonable that one of the MCR resulting profiles is associated to the sum of the monosaccharides.

The profile identity was confirmed by comparing the spectral features of the pure compounds (Fig. 2D) and their kinetics measured by HPLC (Fig. 1). Fig. 3 reports the comparison between MCR-ALS components and the measured trends for Kl. Lactose MCR-ALS spectral profile (Fig. 3D) showed

the characteristics observed for pure lactose FTIR spectra (Fig. 2D) with a broad band between 1110 and 970 cm^{-1} with two maxima at similar absorbances (1074 cm^{-1} and 1034 cm^{-1}) and a shoulder at 995 cm^{-1}. The related MCR-ALS concentration profile described a fast decrease in lactose within the 40 minutes followed by a plateau until the end of the process monitoring. This behaviour is similar to the lactose content quantified by HPLC analysis, even though by the chromatography determination lactose reached the plateau less fast, being the concentration close to 0% after 120 min (Fig. 3A). The MCR-ALS profile attributed to GOS showed a peak at 1148 cm^{-1}, a broad band between 1110 and 970 cm^{-1}, a distinct peak at 950 cm^{-1} and a small feature around 927 cm^{-1} (Fig. 3D). All these characteristics were observed for GOS pure spectra reported in Fig. 2D. Even if there was a spectral correspondence, the GOS concentration profile failed in describing the GOS behaviour as observed by HPLC analysis. Indeed, even if the GOS concentration profile described a fast increase in relative concentration within the first 20 minutes, its drop has been much higher than the one recorded by HPLC (Fig. 3B).

Different reasons could explain the failure in kinetic prediction. First of all, it should be considered that in our approach GOS were evaluated as a unique product from the transgalactosylation reaction. However, the biochemical reactions for GOS production are rather a complex mechanism in which lactose is converted to one of a wide group of oligosaccharides derived from lactose (Gosling et al., 2010). Indeed, GOS are a carbohydrate-based molecule group, thus, they can be considered as a class, but the different proportion of di-, tri-, tetra-, penta- and hexa- saccharides might affect the kinetic modelling results. For this reason, trying to explain the reaction through a mathematical or a statistical model is not as simple as one can expect if the total amount of GOS is considered. Several attempts to model the kinetics have been assayed (Jenab et al., 2017; Palai et al., 2012; Warmerdam et al., 2014; Yin et al., 2017), nonetheless, uncertainness is always present.

Furthermore, the considered reaction media, i.e. cheese whey, is very complex. Indeed, it contains salts and whey proteins, as well as a mixture of sugars of different size and linkages; all of them leading to bands overlapping in the FTIR fingerprint region (1200 – 950 cm^{-1}). Even more, the total content of saccharides does not change over time, but saccharides are transformed by the transgalactosylation reaction; so, FTIR measurements could be less reliable in detecting changes in GOS production. Moreover, to improve the efficacy of this technique, it would be necessary to test standards of every possible carbohydrate formed during the reaction.

Even if the MCR-ALS models failed in assessing GOS production, model reliability was confirmed for monosaccharides released by the reaction, i.e. glucose and galactose. In fact, the spectral profile (Fig. 3D) exhibits features resulting from the combination of pure glucose and pure galactose FTIR

spectra (Fig. 2D). In this case, high correspondence was found between the MCR-ALS concentration profile and the monosaccharides concentrations measured by the chromatographic method (Fig. 3C).

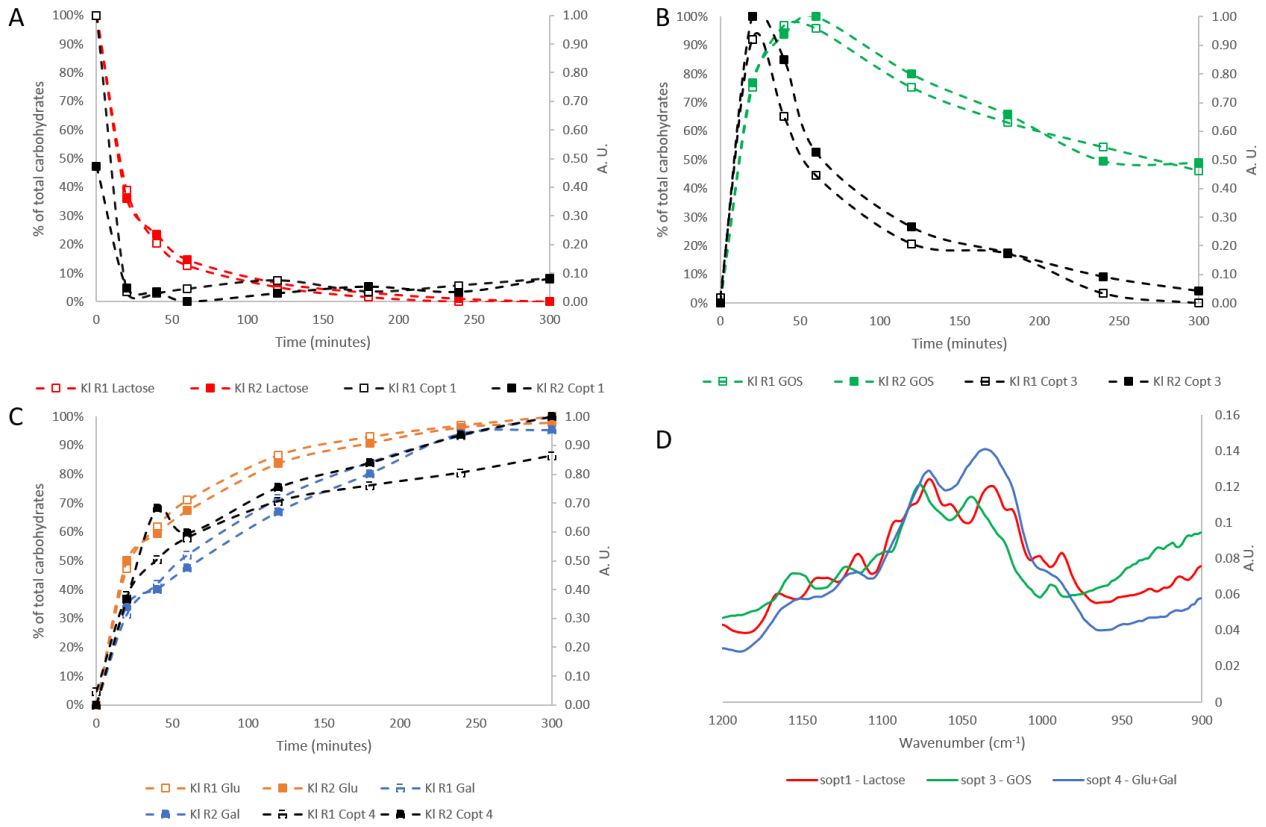

Fig. 3 - MCR-ALS results for the Kl enzymatic reaction: (A) concentration profiles of lactose and lactose concentration as determined by HPLC; (B) concentration profiles of GOS and GOS concentration as determined by HPLC; (C) concentration profiles of glucose-galactose and glucose and galactose concentrations as determined by HPLC; (D) spectral profiles of lactose, GOS and glucose-galactose.

Similar results were obtained by MCR-ALS models developed for 1A/3K (Fig. 1S) and 3A/1K (Fig. 2S). Indeed, lactose decrease, as well as the glucose and galactose production, has been detected with a faster drop by the MCR-ALS models, especially for 1A/3K duplicates. However, the GOS production was not systematically modelled by MCR algorithm, especially for 1A/3K trials. Regardless GOS typical behaviour after reaching their maximum value is to follow a reduction in their concentration due to hydrolytic enzyme property, this depletion was depicted faster than the observed HPLC data.

4. Conclusions

Different chemometric approaches have been tested to assess a Process Analytical Technology to follow GOS production from lactose naturally present in dairy industry waste by FTIR spectroscopy. PLS regression models demonstrated to be reliable to assess lactose, glucose and galactose content in a non-destructive, fast and at line approach, which could be used for real time applications in the future. Indeed, their prediction by Kl or 3A/1K enzymatic reaction led to good fit ($R^2_P > 0.97$) and acceptable errors (RMSEP of 21.9 mg mL^{-1} for lactose, 11.1 mg mL^{-1} for glucose and 12.4 mg mL^{-1} for galactose). Unfortunately, GOS prediction by regression models did not reach the same performance. This could be linked to the development of a model considering GOS as a unique class, whereas it is quite heterogeneous accounting for di-, tri-, tetra-, penta- and hexa- saccharides.

Furthermore, an MCR-ALS model for each enzymatic combination has been developed focusing more on the process kinetics rather than the prediction of each component. Three MCR-ALS profiles were assigned to lactose, GOS and a combination of glucose and galactose. The corresponding MCR-ALS concentration profiles gave trends similar to the sugar concentrations measured by the chromatographic method, however they demonstrated not to be enough accurate to be implemented. Thus, this work should be considered as a preliminary analysis to demonstrate the potential of FTIR spectroscopy, combined with Chemometrics, to follow the kinetics of GOS production from lactose naturally present in dairy industry waste.

The future implementation of this approach to real time systems will answer the need of Circular Economy by providing a reliable monitoring of dairy waste reallocation, avoiding sample pre-processing, large volumes of organic solvents and long-time of analysis. In any case, further investigation is suggested to better assess specific GOS prediction by a single FTIR analysis.

5. ACKNOWLEDGEMENTS

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Figure captions

Fig. 1 - Lactose transgalactosylation in presence of β-galactosidase mixture: (A) Lactose consumption; (B) GOS production; (C) Glucose production; (D) Galactose production; (E) Lactose conversion to GOS.

Fig. 2 - FTIR spectra in the region 1200–900 cm^{-1} collected from different enzymatic reactions (A, Kl; B, 3A/1K; C, 1A/3K) and pure components spectra (D). In A, B and C spectra are coloured according to reaction time (min). In D spectra correspond to lactose (red, -), glucose (orange, ---), galactose (blue, -.-.) and GOS (green, ···).

Fig. 3 - MCR-ALS results for the Kl enzymatic reaction: (A) concentration profiles of lactose and lactose concentration as determined by HPLC; (B) concentration profiles of GOS and GOS concentration as determined by HPLC; (C) concentration profiles of glucose-galactose and glucose and galactose concentrations as determined by HPLC; (D) spectral profiles of lactose, GOS and glucose-galactose.

Fig. 1S - MCR-ALS results for the 3A/1K enzymatic reaction: (A) concentration profiles of lactose and lactose concentration as determined by HPLC; (B) concentration profiles of GOS and GOS concentration as determined by HPLC; (C) concentration profiles of glucose-galactose and glucose and galactose concentrations as determined by HPLC; (D) spectral profiles of lactose, GOS and glucose-galactose.

Fig 2S - MCR-ALS results for the 1A/3K enzymatic reaction: (A) concentration profiles of lactose and lactose concentration as determined by HPLC; (B) concentration profiles of GOS and GOS concentration as determined by HPLC; (C) concentration profiles of glucose-galactose and glucose and galactose concentrations as determined by HPLC; (D) spectral profiles of lactose, GOS and glucose-galactose.

Figure 1

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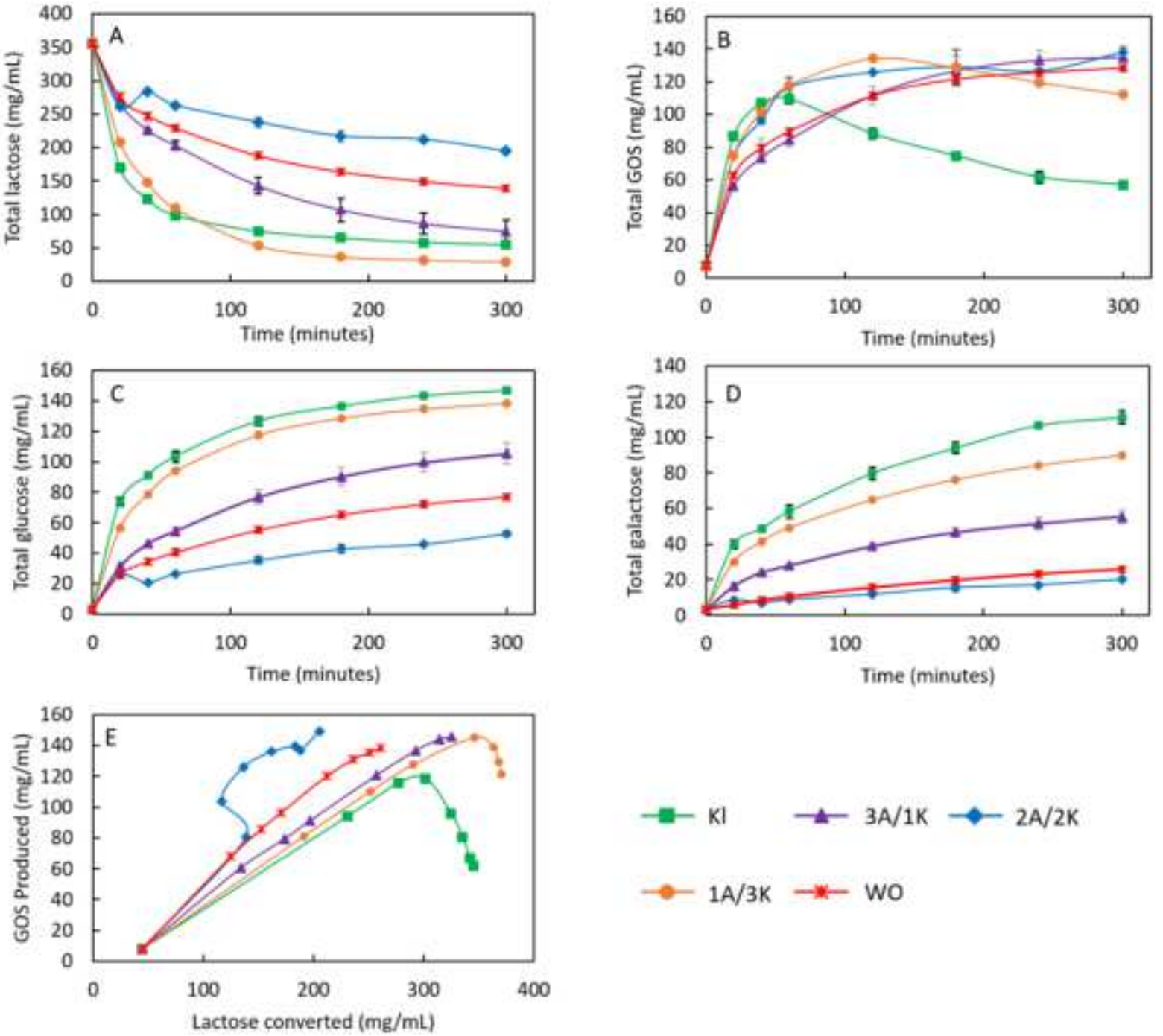

Figure 2

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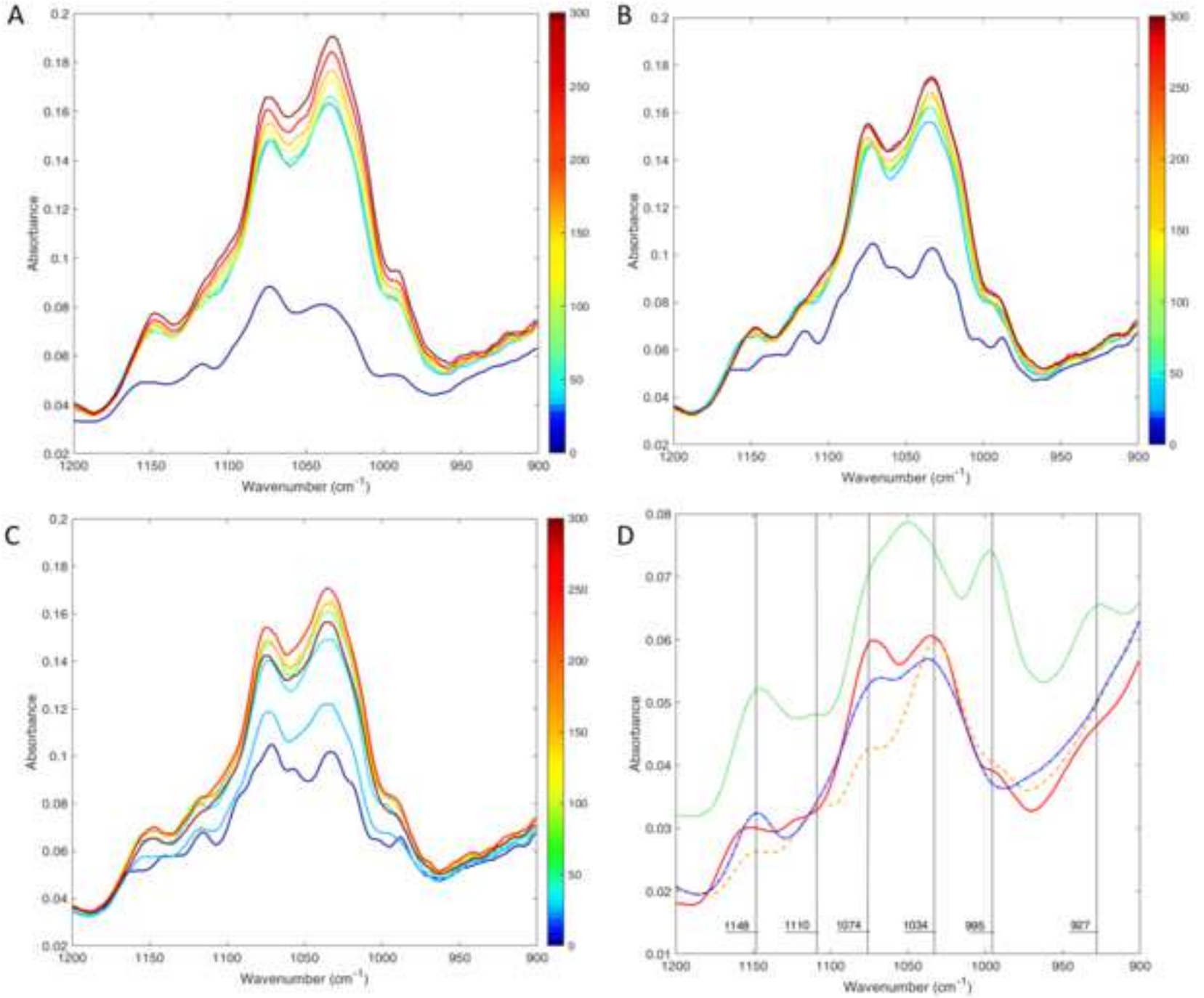

Figure 3

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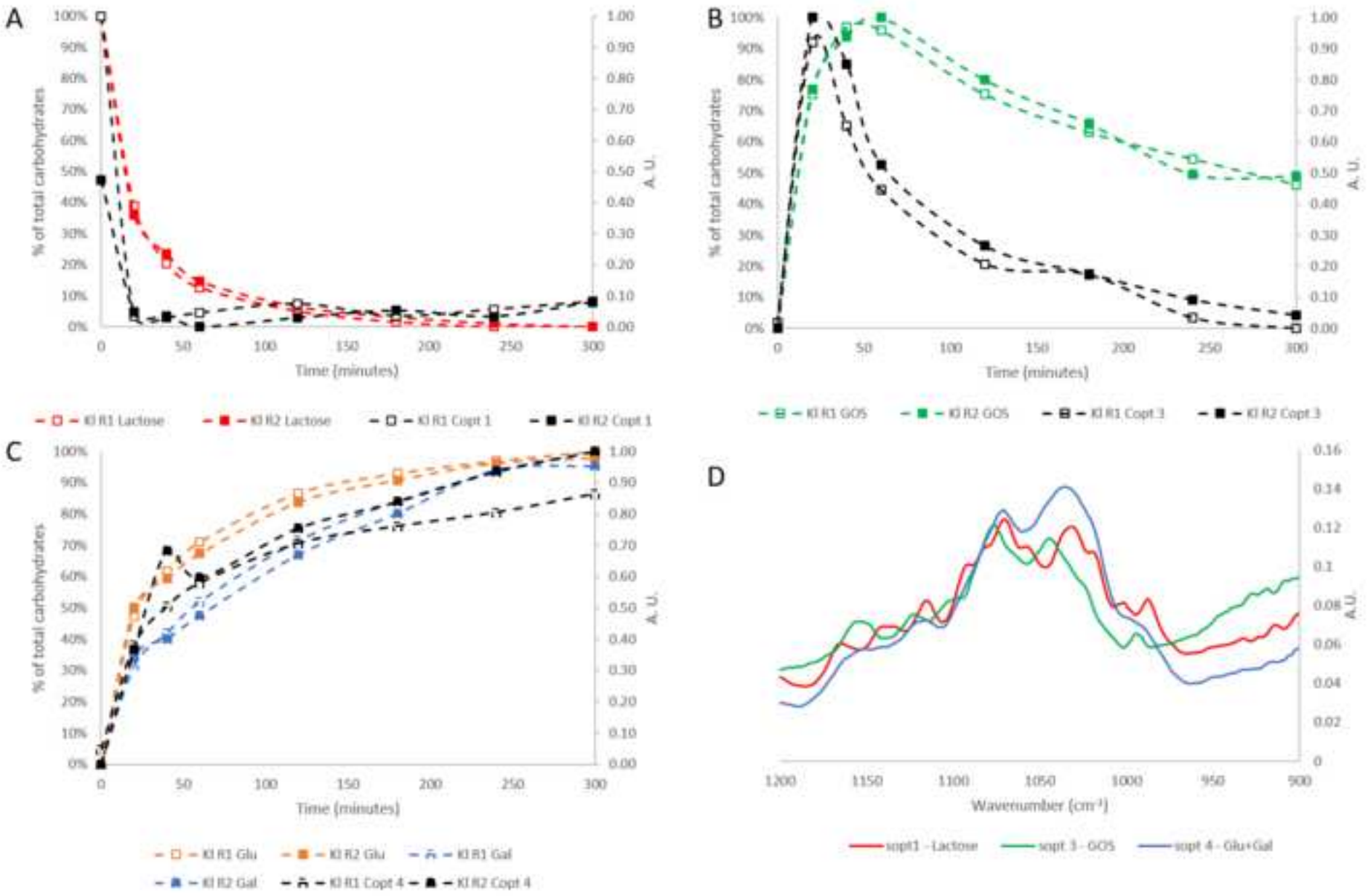

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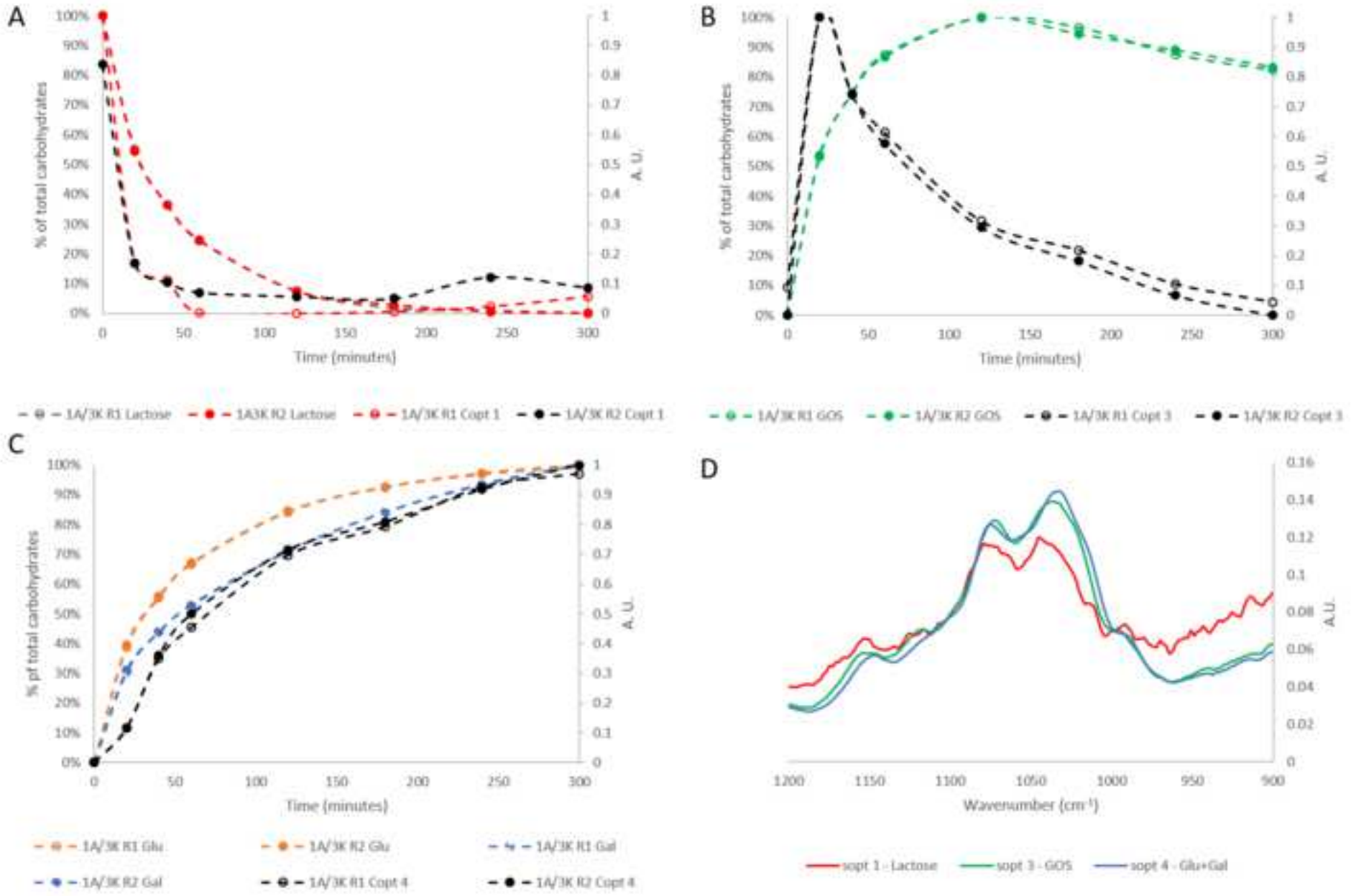

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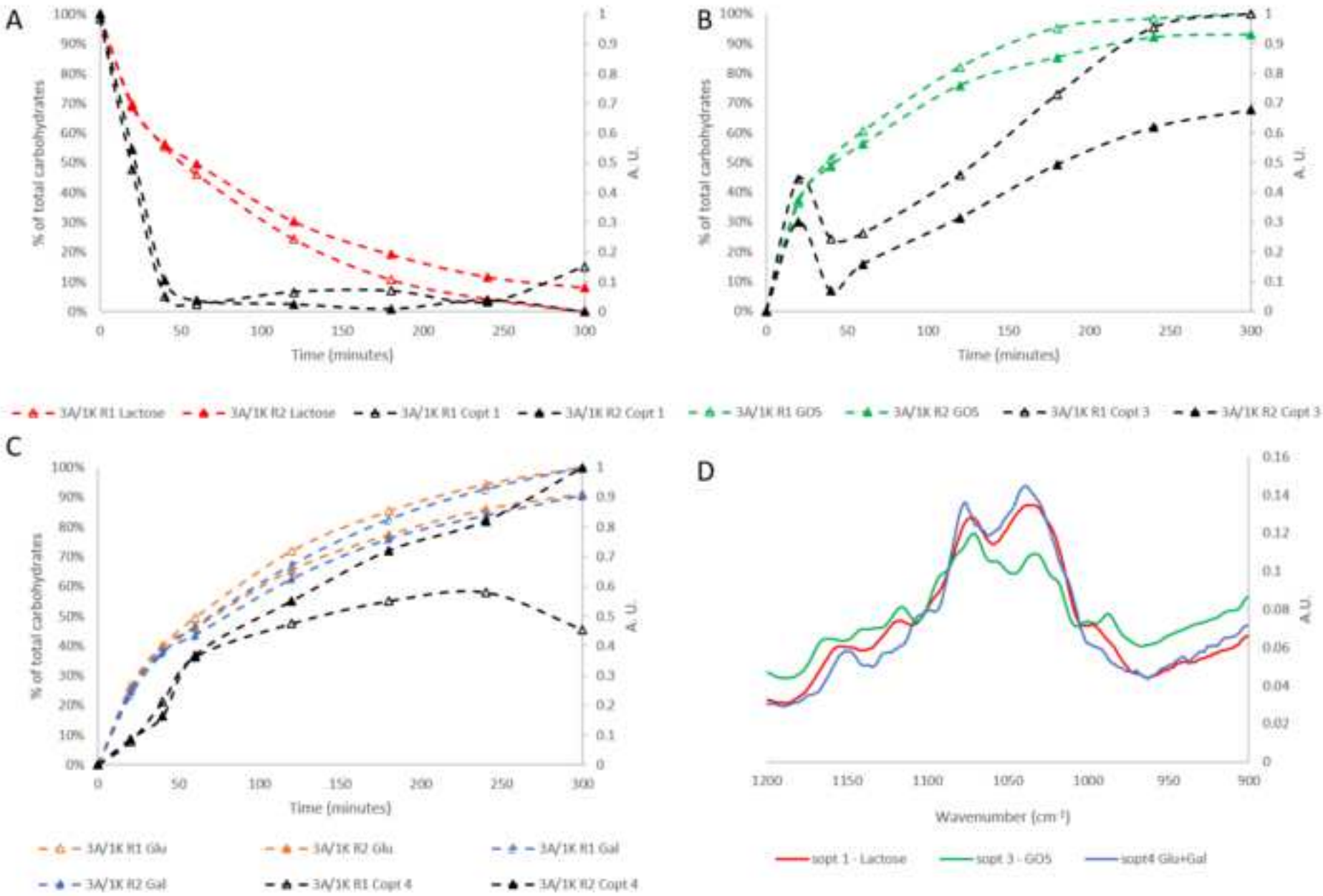

Table 1 - Reaction arrays and dose for each assay

Assay	Alias	Hal Dose (U)	Enz Dose (U)
Enz:Hal (100:0)	WO	37.8	0
Enz:Hal (75:25)	W1/4	28.4	0.9
Enz:Hal (50:50)	W1/2	18.9	1.9
Enz:Hal (25:75)	W3/4	9.9	2.8
Enz:Hal (0:100)	WK	0	3.8

Table 2 - PLS regression models for lactose, GOS, glucose and galactose content prediction from FTIR spectra.

	Test set	LV	Pre-treatment	RMSEL (mg mL^{-1})	RMSEC (mg mL^{-1})	RMSECV (mg mL^{-1})	RMSEP (mg mL^{-1})	R^2_{Cal}	R^2_{CV}	R^2_P
Lactose	**Kl**	**6**	**Baseline correction**	8.2	**14.9**	**19.2**	**21.9**	**0.98**	**0.97**	**0.98**
	3A/1K	3	1st Derivative		24.7	28.3	28.4	0.94	0.92	0.98
	1A/3K	5	Baseline correction		16.6	20.3	22.3	0.98	0.97	0.96
GOS	Kl	7	SNV	3.2	9.4	17.9	18.7	0.95	0.84	0.76
	3A/1K	**7**	**Baseline correction**		**10.2**	**14.3**	**16.0**	**0.94**	**0.89**	**0.88**
	1A/3K	7	1st Derivative		11.7	17.3	19.5	0.92	0.84	0.92
Glucose	Kl	2	1st Derivative	8.0	12.6	13.7	14.5	0.91	0.89	0.96
	3A/1K	**6**	**Raw**		**8.1**	**10.6**	**11.1**	**0.97**	**0.94**	**0.99**
	1A/3K	5	Baseline correction		8.7	10.3	12.1	0.97	0.96	0.93
Galactose	Kl	2	1st Derivative	8.1	11.9	12.9	23.6	0.8	0.76	0.95
	3A/1K	**6**	**SNV**		**9.6**	**11.5**	**12.4**	**0.91**	**0.88**	**0.97**
	1A/3K	5	Baseline correction		10.1	12.5	12.6	0.92	0.88	0.75

LV, latent variables; SNV, Standard Normal Variate; RMSEL, Root Mean Square Error of Laboratory; RMSEC, Root Mean Square Error of Calibration; RMSECV, Root Mean Square Error of Cross-Validation; RMSEP, Root Mean Square Error of Prediction; R^2, coefficient of determination in calibration (Cal), cross-validation (CV) and prediction (P).

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: