Ciências Agrárias
SEMINA

DOI: 10.5433/1679-0359.2022v43n4p1637

Artificial intelligence applied to enzymatic hydrolysis of lactose: improving the control of industrial processes

Inteligência artificial aplicada a hidrólise enzimática da lactose: melhorando o controle dos processos industriais

Pauline Thais dos Santos[1]; Stael Málaga Carrilho[1]; Samanta Stinghen de Abreu[1]; Fernanda Montanholi de Lira[2]; Fernanda Yuri Rodrigues Tanaka[2]; Ronaldo Tamanini[3]; Edson Antonio Rios[3]; Lycio Shinji Watanabe[4]; Rafael Fagnani[5]; Natalia Gonzaga[5]*

Highlights

Measuring lactose content is important because of globally population intolerance.
Products labeled "zero lactose" must strictly comply with this requisite.
It is crucial to evaluate the capacity of hydrolysis of enzymes used in the industry.
Artificial intelligence improves the confidence of simple tests like cryoscopy.
Artificial intelligence promotes improvements in the control of industrial processes.

Abstract

Lactose is the main carbohydrate in milk, and its absorption occurs via enzymatic hydrolysis, generating glucose and galactose. Lactose intolerance is the reduction of intestinal hydrolysis capacity due to hypolactasia, which results in the need to consume dairy foods with low levels of this carbohydrate. β-galactosidase enzymes are used in dairy industries to hydrolyze lactose, thereby allowing intolerant consumers access to dairy products without the negative health implications. Alternative and official analytical methods are used to quantify the carbohydrate content resulting from enzymatic hydrolysis. The objective of this study was to evaluate the enzymatic hydrolysis of two distinct industrial enzymes produced by the microorganisms *Bacillus licheniformis* and *Kluyveromyces lactis* using three analytical methods: enzymatic method, cryoscopy, and high performance liquid chromatography (HPLC) using artificial intelligence to improve the control of the industrial processes. After adding the enzymes to skim milk, time kinetics was performed by collecting samples at time 0, every 10 min for 1 h, and every 30 min

[1] Veterinary Resident in Dairy Products Inspection, Preventive Veterinary Medicine Department, Universidade Estadual de Londrina, UEL, Londrina, PR, Brasil. E-mail: lipoa.uel@gmail.com; stael.te@gmail.com; samanta.stinghen@uel.br
[2] M.e in Animal Science, UEL, Londrina, PR, Brasil. E-mail: fernanda.montanholi@uel.br; fernanda.yurit@uel.br
[3] Drs. in Animal Science, UEL, Londrina, PR, Brasil. E-mail: ronaldot@uel.br; edsonrios1@hotmail.com
[4] Dr. in Analytical Chemistry, UEL, Londrina, PR, Brasil. E-mail: shinjiwatanabe2003@hotmail.com
[5] Profs. Drs., Preventive Veterinary Medicine Department, UEL, Londrina, PR, Brasil. E-mail: rafaelfagnani@uel.br; nataliagonzaga@uel.br
* Author for correspondence

Received: Nov. 22, 2021 - Approved: Apr. 25, 2022

until the end of 5 h of hydrolysis. In 97% of the cases, a decrease in lactose concentration was observed by HPLC, followed by the deepening of the cryoscopic point. Glucose measurements by absorbance and HPLC quantification were correlated ($r = 0.79$; $p < 0.01$) but not concordant ($p < 0.01$). It was concluded that by means of artificial intelligence, it was possible to indirectly estimate lactose concentration using an algorithm that associates cryoscopy and glucose concentration.
Key words: Artificial intelligence. β-galactosidase. Cryoscopy. HPLC. Lactose.

Resumo

O principal carboidrato do leite é a lactose e a sua absorção ocorre devido à hidrólise enzimática, gerando glicose e galactose. A intolerância à lactose é a redução da capacidade de hidrólise intestinal devido à hipolactasia, gerando a necessidade do consumo de alimentos lácteos com baixo teor deste carboidrato. As enzimas β-galactosidase são utilizadas nas indústrias de laticínios para hidrolisar a lactose, proporcionando ao consumidor intolerante a possibilidade de ingerir os produtos lácteos sem prejuízos à saúde. Para quantificar o conteúdo de carboidratos resultante da hidrólise enzimática, são utilizados métodos analíticos alternativos e oficiais. O objetivo deste estudo foi avaliar a hidrólise enzimática de duas enzimas industriais distintas produzidas pelos microrganismos *Bacillus licheniformis* e *Kluyveromyces lactis*, por meio de três métodos analíticos: método enzimático, crioscopia e HPLC. A inteligência artificial foi utilizada para melhorar o controle dos processos industriais. Após a adição das enzimas ao leite desnatado, foi realizada a cinética de tempo coletando as amostras no tempo 0, a cada 10 minutos, até completar 1 hora de reação, e a cada 30 minutos até serem atingidas 5 horas de reação de hidrólise. Em 97% dos casos, a diminuição da concentração de lactose por HPLC acompanhou o aprofundamento do ponto crioscópico. As medições de glicose por absorbância e HPLC foram correlacionadas ($r = 0,79$; $p < 0,01$), mas não concordantes ($p < 0,01$). Concluiu-se que, por meio da inteligência artificial, é possível estimar indiretamente a concentração de lactose a partir de um algoritmo que associa a crioscopia e a concentração de glicose.
Palavras-chave: β-galactosidase. Crioscopia. HPLC. Inteligência artificial. Lactose.

Introduction

Lactose is the predominant sugar in milk and is not naturally occurring in other foods. It is a disaccharide composed of glucose and galactose, and represents approximately 4.7% of fluid milk (Beloti, 2015; Brito & Giulietti, 2007). Lactose absorption occurs in intestinal enterocytes through the action of β-galactosidase, which promotes enzymatic hydrolysis and generates the monosaccharides glucose and galactose that are assimilated into the bloodstream (Nivetha & Mohanasrinivasan, 2017). The enzyme β-galactosidase occurs in animals, vegetables, and microorganisms. However, at the industrial level, only those of microbial origin are used because of their high viability and low cost (Husain, 2010).

Lactose intolerance consists of a decrease in hydrolysis capacity due to hypolactasia, which is a reduction in the enzymatic activity of β-galactosidase present in the intestinal mucosa. Inadequate functioning of this enzyme can result in some intestinal disturbances related to the absence of lactose breakdown (Mattar et al., 2012). Studies indicate that the estimated global

prevalence of lactose malabsorption is 68%, with variations of 28% in western, southern, and northern Europe, and 70% in the Middle East (Storhaug et al., 2017). In Brazil, the prevalence of lactose intolerance is also high (>63%), especially in people of African and Japanese descent (Araújo et al., 2019), demonstrating the importance of appropriate production and quality control of lactose-free products.

According to the current legislation, it is mandatory to have information regarding the presence of lactose on food labels. Products with lactose content below 100 mg per 100 g should be labeled "zero lactose" (Resolução da Diretoria Colegiada nº 135, 2017; Resolução da Diretoria Colegiada nº 136, 2017). The official methods manual for food of animal origin analysis recommends high-performance liquid chromatography and the enzymatic method using pH difference to determine the lactose content in milk (Ministério da Agricultura, Pecuária e Abastecimento, 2019).

The official methods to control hydrolysis processes in industry are precise; however, they are underused because they require specific equipment and specialized professionals. Quality control of food demands rapid, economic, and robust analytical methods in relation to different sample matrices of a variety of "lactose-free" products (Morlock et al., 2014).

It is common for industries to control the lactose hydrolysis process by determining the cryoscopic index or using enzymatic methods; however, these methods are not always satisfactory. For example, Trani et al. (2017) concluded that enzymatic methods are not adequate for the quantitative determination of residual lactose in lactose-free milk, because the free galactose concentration is higher than the total galactose concentration measured after the β-galactosidase activity. In this case, the high free galactose concentration interferes with the analysis, causing abnormal behavior of β-galactosidase with the production of oligosaccharides. This results in products that are marketed as delactosed containing lactose contents greater than the limit defined by legislation for "zero lactose" products. Therefore, there is a clear need for more adequate analytical methods for monitoring the residual lactose content in these products in routine quality control analyses (Garballo-Rubio et al., 2018), which should involve changes to the current legislation by introducing stronger controls in the official methods.

Artificial intelligence using predictive models to optimize industrial processes has been implemented in many studies in the milk sector. In a study in 2006, researchers proposed the use of Near Infrared Spectroscopy (NIR) associated to the use of modified support vector machine to quantify adulterants in powder milk, such as whey, starch, and sucrose (Borin et al., 2006). Statistical methods with an artificial neural network have also been applied in a study by Jaganathan and Kuppuraj (2016), whose results showed that this technique can be used as an economic tool to predict and evaluate the performance of the ultrafiltration process of milk. Tools such as artificial intelligence have been used not only in industry but also in the field, with the main objective of producing a product with better quality, greater predictive control of future data, and consequently, increased profitability (Yan et al., 2015).

Improvements in industrial control of lactose hydrolysis processes using low-cost methods can be beneficial to both the industry and the consumer. The present study aimed to evaluate the efficiency of lactose enzymatic hydrolysis by β-galactosidase originating from two microorganisms, *Bacillus licheniformis* and *Kluyveromyces lactis*, using different analytical methods to quantify the carbohydrates resulting from the breakdown of lactose, and artificial intelligence to improve the control of the industrial processes.

Methodology

Raw material and enzymes

Skimmed ultra-high-temperature (UHT) milk samples and β-galactosidase enzymes from two different industrial brands, A and B, which originated from bacteria (*B. licheniformis* 2,600 LAU-B g^{-1}) and fungus (yeast 50,000 LCPU g^{-1}), respectively, were used to evaluate enzymatic hydrolysis. The enzymatic dosage used for hydrolysis was indicated by the manufacturers as 0.09% and 0.05%, for A and B, respectively. The experiments were performed in triplicate and on different dates.

Sample preparation

The methodology used for sample preparation was described by Moretti et al. (2016) with appropriate changes. Skimmed UHT milk (1,000 mL) was added to a volumetric flask, which was then placed in a water bath at 37 °C. The specific enzyme to be tested was added after reaching a temperature of 37 °C. At each time point, aliquots of 15 mL from the samples in duplicate were withdrawn into screw tubes and transferred to a boiling water bath for 10 min to inactivate the enzyme. The time kinetics to evaluate the efficiency of enzymatic hydrolysis was as follows: samples were collected before enzyme addition, every ten minutes in the first hour, and every 30 min from the second hour until the reaction was complete after 5 h.

Cryoscopy analysis

The cryoscope used, properly calibrated, was the PZL 7000, which operates in Hortvet degrees (ºH), and the official methodology was applied (Ministério da Agricultura, Pecuária e Abastecimento, 2019). The analyses were performed in triplicate for each time kinetic, adding 2.5 mL of the sample to the tubes at approximately 20 °C.

Enzymatic method

The enzymatic method applied was the glucose-oxidase kit (Bioliquid®) that contained a working reagent and glucose standard, which was applied to the prepared samples following the manufacturer's recommendations with modifications. Samples were diluted 1:9 (v/v) in 2 mL microtubes and homogenized. Following this, 10 µL of the sample was added to a test tube containing 1 mL of the working reagent and it was then incubated in a water bath at 37 °C for 10 min. The samples were filtered using syringe filters with a 0.22 µm nylon membrane (Filtrilo®), and 200 µL was transferred to an ELISA plate. The analysis was performed using the Bio-Rad (iMark™) microplate reader, based on absorbance at 540 nm. To

determine the unknown concentration of the samples, a standard curve was generated using a standard solution of glucose at known concentrations (0, 1, 2.5, 5, 10, 25, 50, 100, 200, and 400 mg dL^{-1}). These known points were prepared by diluting the standard solution to the necessary proportions to obtain the desired concentrations.

High performance liquid chromatography (HPLC) analysis

Carbohydrate quantification through the High Performance Liquid Chromatography - Refractive Index Detector (HPLC-RID) technique was performed with the methodology and equipment as described by Gonzaga et al. (2019), which consisted of an LC-20AT bomb, with a DGU-20A gradient management system and degasser, and an SIL-20AC automatic injector. It was used Pb (II) - Aminex HPX-87P (300 mm × 7.8 mm, 9.0 µm, Bio-Rad, USA) cation exchange column, maintained at 80 °C in a CTO-20A oven, and detection was performed using the RID-10A refractive index detector, managed by the CBM -20A controller and commanded by the LC-Solution software (Version 1.21). Ultrapure water (Milli-Q®) was used as the mobile phase. The injection volume was 10.0 µL at a flow rate of 1.0 mL min-1 and a run time of 15 min per sample was used.

Statistical analysis

At each time point, the lactose hydrolysis speed was calculated using Equation 1:

$$V = \frac{(Cf - Ci)}{(t1 - t0)}$$

V = lactose hydrolysis speed in mg dL^{-1} min^{-1};

C_f = lactose final concentration in t_1;

C_i = lactose initial concentration in t_0;

t_0 = initial time; and

t_1 = final time.

Data were analyzed for normality and homoscedasticity using the Kolmogorov-Smirnov test at a 5% significance level. The variables that did not show a normal distribution were squared and subjected to logarithmic transformation before parametric analyses. For each enzyme, the average hydrolysis speed over time was compared using analysis of variance followed by Fisher's test. The association between reduction in lactose concentration and cryoscopy deepening was analyzed using Pearson's correlation.

The association and agreement between glucose concentration measured by spectrophotometry and that by HPLC was performed using Pearson's correlation and the Bland-Altman diagram. The null hypothesis of bias was tested using the t-test for paired samples. The hypothesis of whether the variables depended on glucose concentration was assessed using Pearson's correlation.

Finally, the Support Vector Machines (SVM) tool was used to determine a predictive regression equation for the lactose concentration. For this, the cryoscopy values and glucose concentration measured by spectrophotometry were used as independent variables. To construct the algorithm, 75% of the cases were used for training. The results are presented for all cases. The association and agreement between lactose concentration

measured by HPLC and that predicted by the SVM was performed using Pearson's correlation and the Bland-Altman diagram. The null bias hypothesis was tested using the t-test for paired samples. The hypothesis of whether the variables depended on lactose concentration was assessed using Pearson's correlation.

All analyses were performed using the Statistica 13.0 program with a significance level of 5%. For a better interpretation of the results, the values of the variables are presented in the original units.

Results and Discussion

Using the lactose concentration obtained from the HPLC method, it was possible to calculate the maximum lactose hydrolysis speed of each enzyme (Figure 1). For enzyme A, the maximum lactose hydrolysis speed was achieved within 10 min, reaching 0.11 g dL min^{-1}. Subsequently, the average speed decreased ($p < 0.05$) between 30 and 90 min and stabilized ($p > 0.05$) after 120 min. For enzyme B, the maximum lactose hydrolysis speed was achieved after 20 min, achieving 0.09 g dL min^{-1}. Between 30 and 90 min, the hydrolysis speed decreased and stabilized after 90 min.

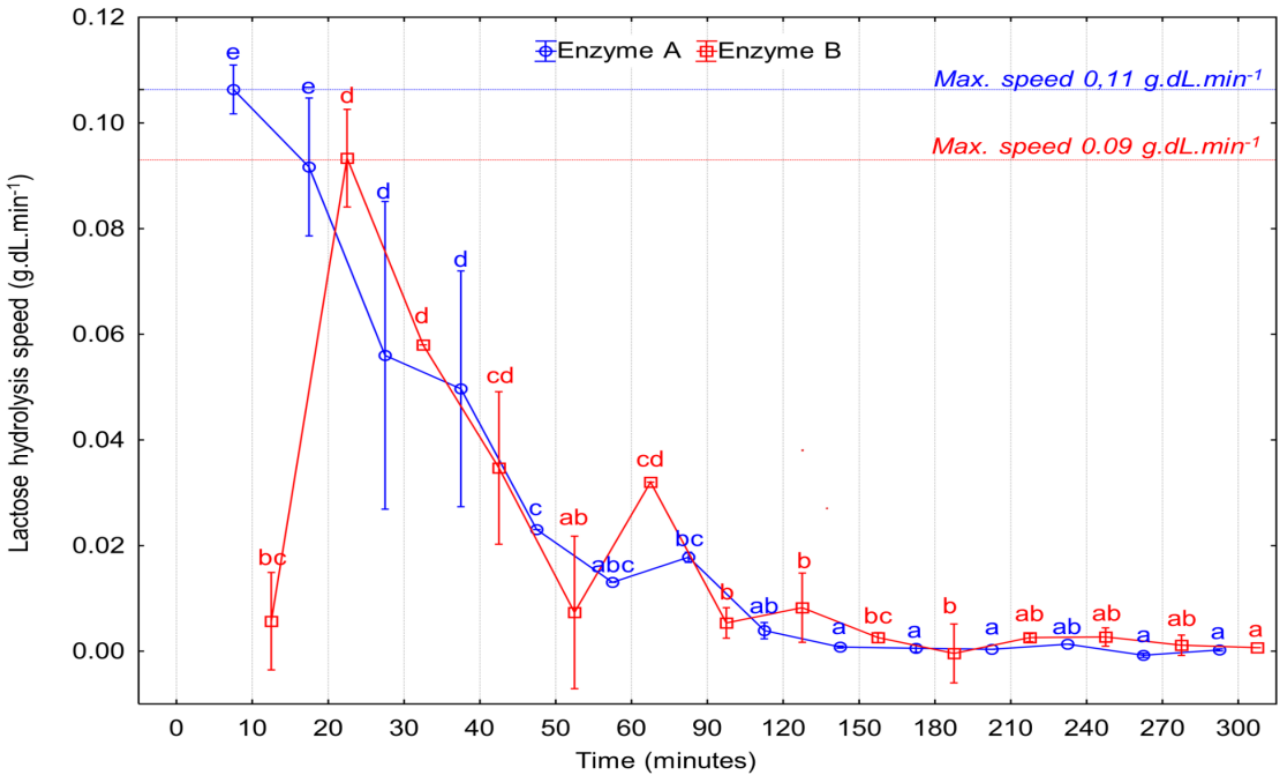

Figure 1. Means and standard deviations of lactose hydrolysis speeds over time for two brands of industrial enzymes (A and B) (n= 3; means followed by equal letters for the same enzyme do not differ by Fisher's test at 5% significance level).

For the lactose concentrations of the different enzymes (Figure 2), 50 min of hydrolysis was required to bring the lactose concentration below 1 g dL^{-1} using enzyme A under the tested conditions (initial lactose concentration of 4.23 g dL^{-1} at 37 °C), which allowed the raw material to be classified as low-lactose content milk classification (Figure 3). After 240 min of hydrolysis, the lactose concentration was below 0.1 g dL^{-1}, which is the maximum limit for classification as the "zero lactose".

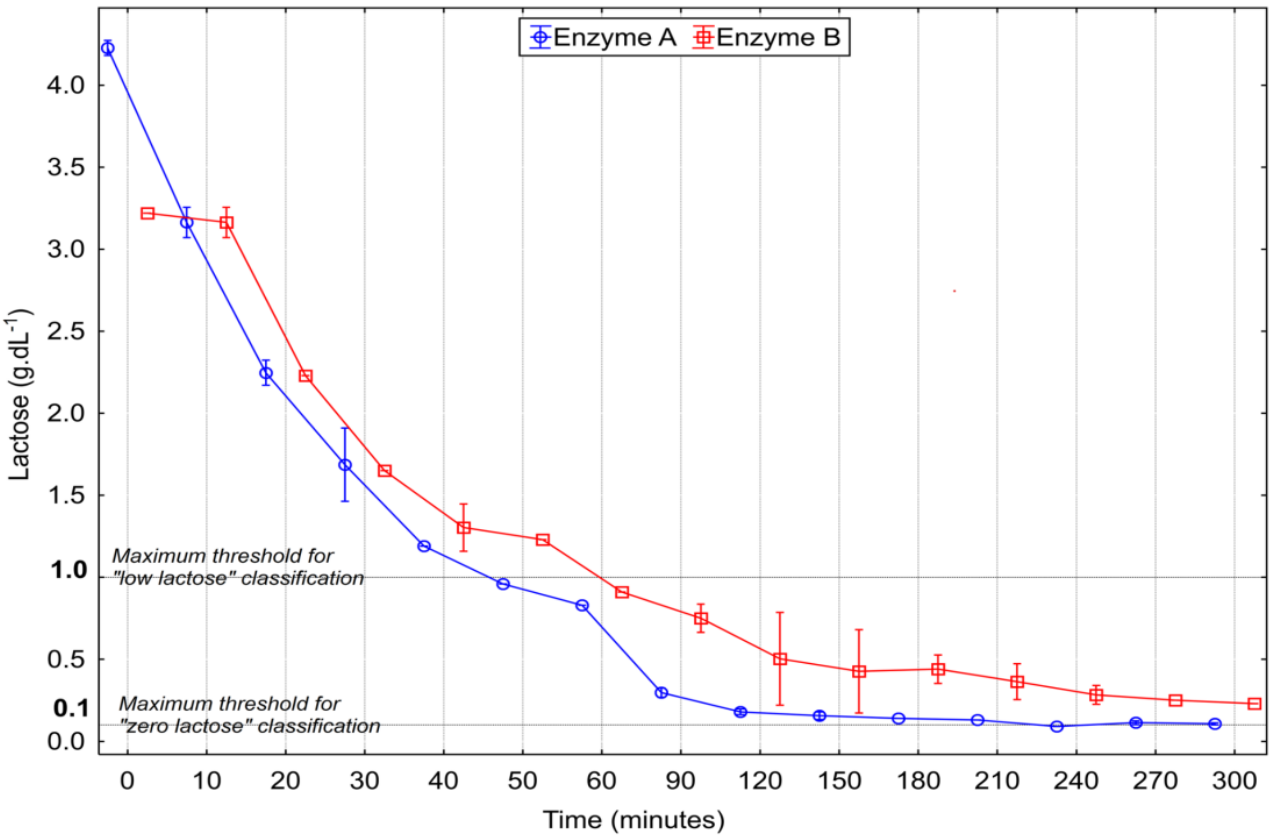

Figure 2. Means and standard deviations of lactose concentrations over hydrolysis time for two brands of industrial enzymes (A and B) (n=3).

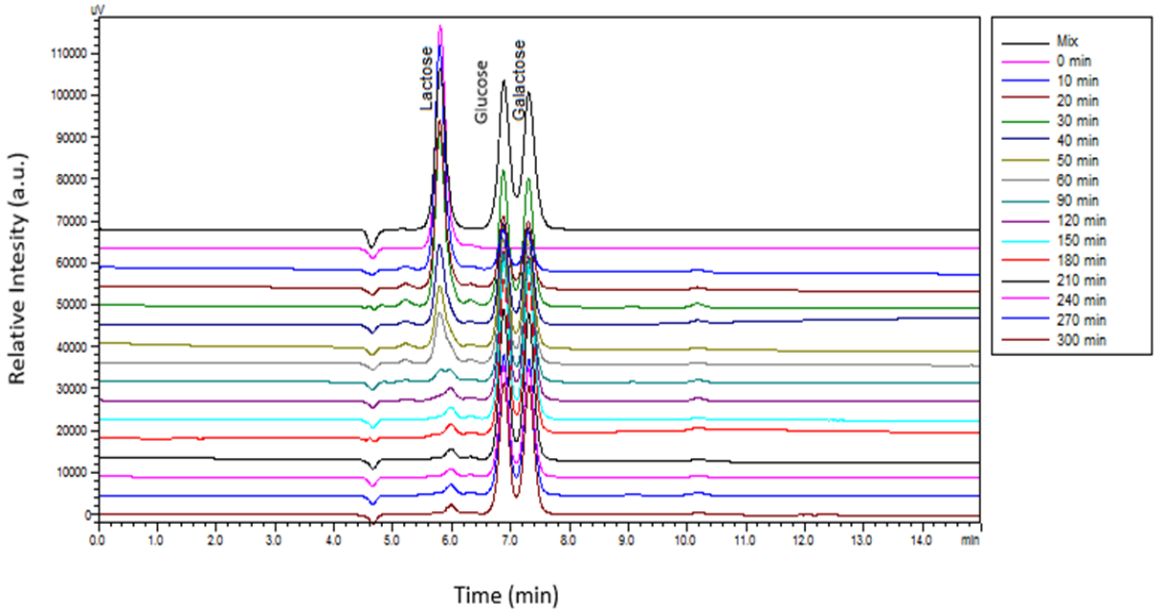

Figure 3. Overlapping HPLC chromatograms of a milk sample containing industrial enzyme A during the hydrolysis process with a standard mix and residual carbohydrates resulting from the lactose breaking process.

In contrast, using enzyme B under the tested conditions (initial lactose concentration of 3.22 g dL^{-1} at 37 °C), 60 min was required for the initial lactose concentration to decrease below 1 g dL^{-1}. After 300 minutes of hydrolysis, the mean lactose concentration was 0.23 g dL^{-1}, which was insufficient for inclusion in the "zero lactose" classification (Figure 4).

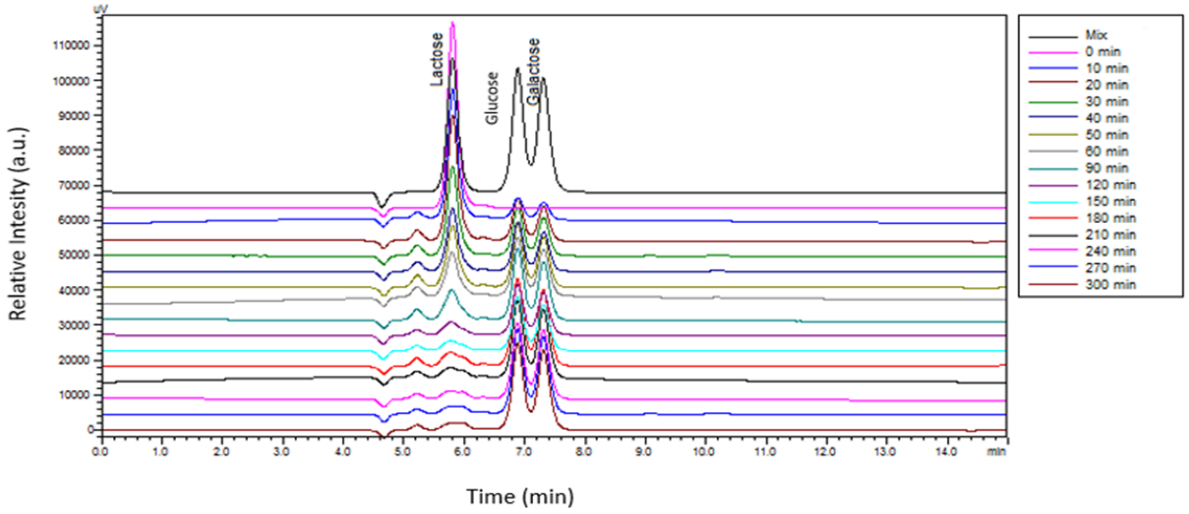

Figure 4. Superimposed HPLC chromatograms of a milk sample containing industrial enzyme B during the hydrolysis process with a standard mix and residual carbohydrates resulting from the lactose breaking process.

The results indicate that the present study was inappropriate for comparing the hydrolysis efficiencies of the two industrial enzymes. This is because, even by standardizing the time and temperature variables, lactose concentration and the pH of the raw material were uncontrolled factors. It is known that the substrate concentration and pH directly influence hydrolysis speed. In a study published by Dutra-Rosolen et al. (2015), the enzymatic hydrolysis efficiency was evaluated using commercial and industrial enzymes at different temperatures, concentrations, and pH values. Their results showed that for the same enzyme concentration after 2 h of reaction, the *K. lactis* β-galactosidase presented higher hydrolysis efficiency than the *Aspergillus oryzae* β-galactosidase. This was probably due to the raw material pH. The pH of the reconstituted milk used by those authors was approximately 6.8. The *A. oryzae* β-galactosidase used presents optimum pH in the 4.5-5.0 range, while the β-galactosidase of *K. lactis* has a 7.3-7.7 range. Maximum efficiencies in lactose hydrolysis in 2 h reaction time were obtained using the *K. lactis* β-galactosidase at 37 °C. The highest conversion rate was 73.84% with 9 U mL^{-1} of enzyme. For the *K. lactis* β-galactosidase, hydrolysis did not achieve 100% in any of the evaluated conditions, which is an important fact for intolerant consumers (Dutra-Rosolen et al., 2015).

Industrial enzyme B did not achieve a sufficiently low lactose concentration to fit with the "zero lactose" product classification. Therefore, it is possible that the decrease in lactose concentration will not reach the level of the "zero lactose" classification even when meeting the manufacturer's conditions. This suggests that it is fundamental to validate and control the hydrolysis process in each industrial setting.

A decrease in lactose concentration was positively linearly correlated to a decrease in cryoscopy results ($r=0.97$; $p<0.01$). This relationship can be described using the linear regression equation shown in Figure 5. Therefore, it is possible to predict the decrease in lactose concentration based on the difference between the initial and final cryoscopy since milk freezes at a lower temperature as lactose is broken down. This is because lactose hydrolysis produces both glucose and galactose. For example, for a 4 mg dL^{-1} decrease in lactose in the raw material, cryoscopy would decrease 0.293 °H. Considering a prediction interval with 95% confidence, a decrease in cryoscopy can change between 0.250 °H and 0.337 °H. This range can be a limiting factor for the required precision in industrial processes. To overcome this shortcoming, it is suggested that the smallest value in the precision range be taken as the reference.

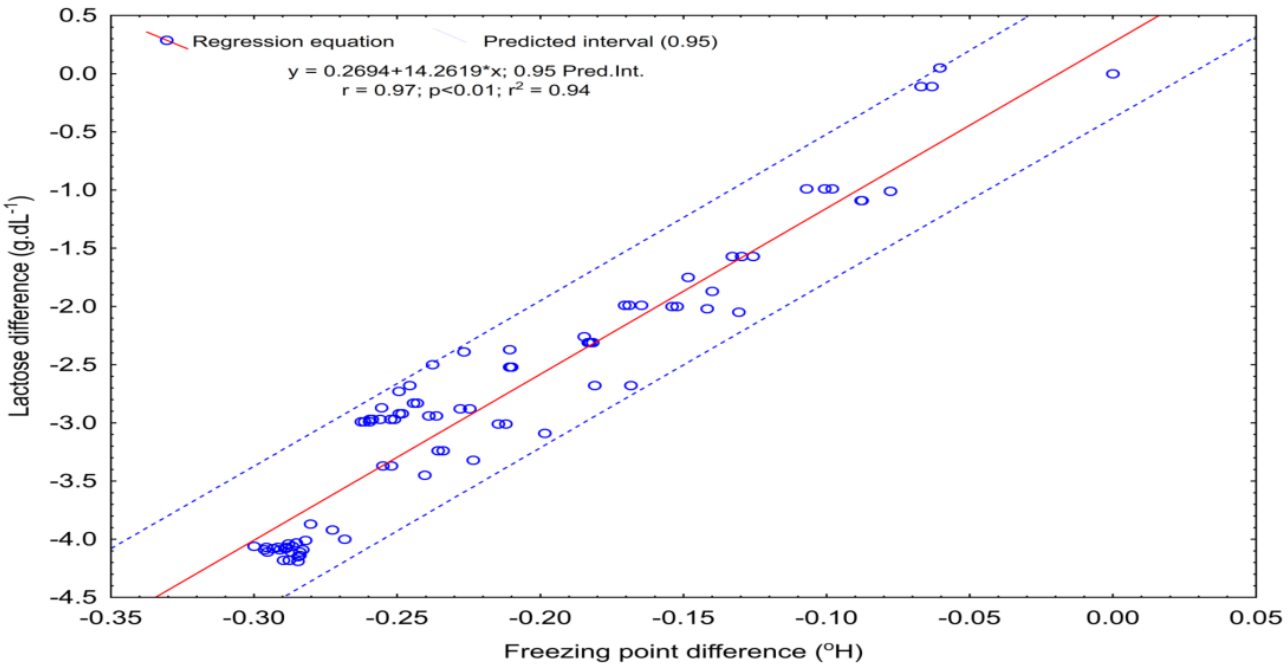

Figure 5. Linear regression equation and prediction intervals between the decrease in lactose concentration and the decrease in cryoscopy from two lactose hydrolysis trials using two types of industrial enzymes (A and B).

In conjunction with cryoscopy, the measurement of glucose concentration by the enzymatic method can provide more reliable information about lactose concentration improving control of industrial processes involving enzymatic hydrolysis. Therefore, it is necessary to establish a relationship between lactose hydrolysis and glucose production. A decrease in lactose concentration was negatively linearly correlated to an increase in glucose concentration ($r = -0.94$, $p < 0.01$). This relationship is shown in the linear regression equation of Figure 6. Thus, for every 1 g dL^{-1} of hydrolyzed lactose there was 0.57 g dL^{-1} of glucose.

Since this relationship is constant and has been confirmed by many authors, it is possible to predict lactose concentration from glucose concentration. Theoretically, blood glucose measurement kits could be used to estimate the lactose concentration of milk, which would allow the industry to take advantage of the cost, speed, and simplicity of this method. However, since the kits measure glucose by absorbance, an inter-method agreement analysis is needed to compare the glucose measurements obtained by absorbance with those of the reference method (HPLC).

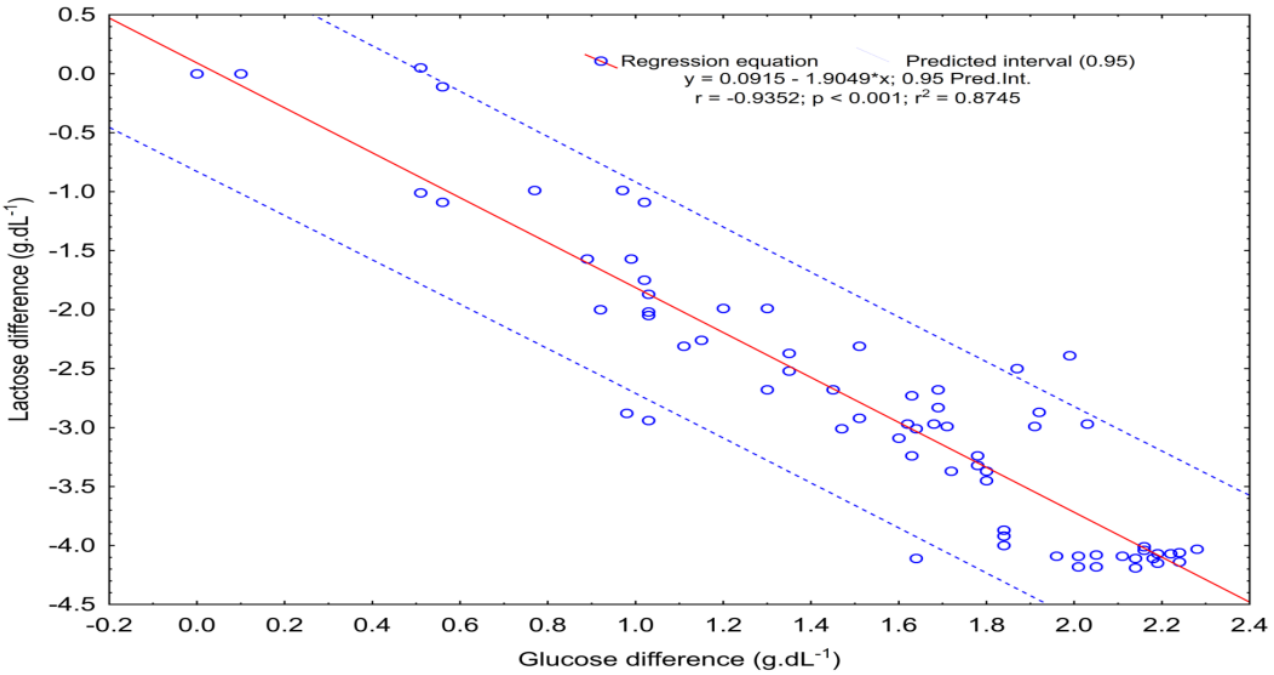

Figure 6. Linear regression equation and prediction intervals between the decrease in lactose concentration and the increase in glucose concentration from two lactose hydrolysis trials using two types of industrial enzymes (A and B).

The glucose measurements obtained by absorbance and HPLC were correlated ($r = 0.79$; $p < 0.01$), but not concordant ($p < 0.01$) (Figure 7). This means that although the values between the methods are correlated, the absorbance method overestimates the glucose concentration by an average of 0.17 g dL^{-1}. Churakova et al. (2019) quantified the residual lactose values in low-lactose milk using different analytical methods and also observed this phenomenon. Some of the methods used were enzymatic, and they measured the amount of lactose retained after hydrolysis. It is speculated that galacto-oligosaccharides present in the samples are also hydrolyzed by β-galactosidases, resulting in an overestimation of the amount of glucose or galactose that relates to an overestimation of the lactose concentration. This clearly illustrates the disadvantage of using an indirect method, such as a commercially available enzyme kit, to measure low lactose concentrations in milk (Churakova et al., 2019).

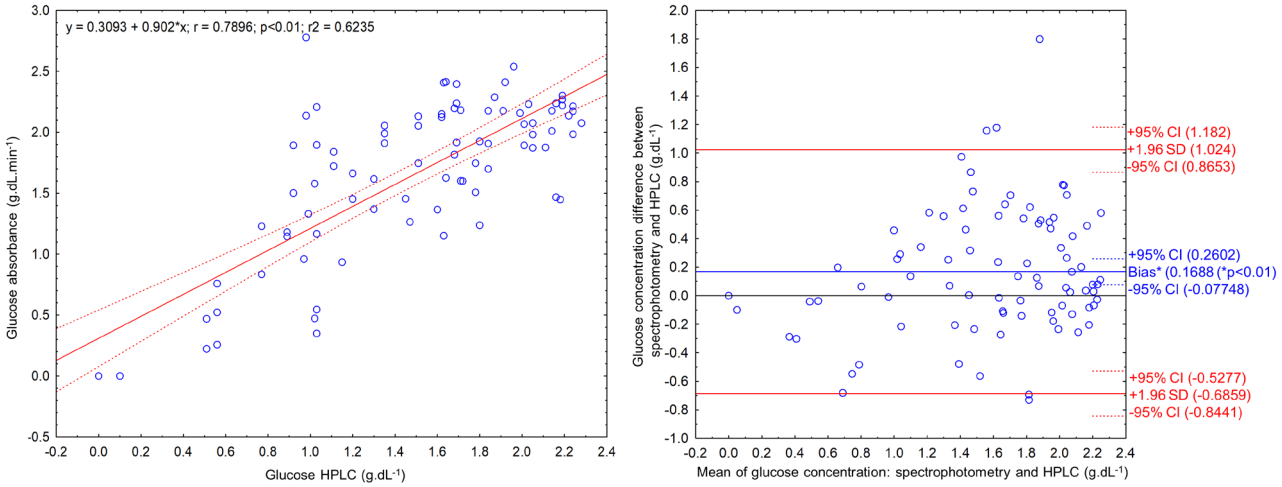

Figure 7. Relationship and agreement between glucose concentration measured by spectrophotometry and by HPLC from 90 observations of two lactose hydrolysis assays using two types of industrial enzymes.

Figure 7 shows that this bias is constant (it does not relate to glucose concentration), which allows the use of the 0.17 correction factor. Therefore, to equate the glucose value obtained by spectrophotometry to the HPLC value, 0.17 g dL^{-1} must be subtracted. Considering the prediction interval with a 95% confidence interval, the margin of error for this correction is ± 0.86 g dL^{-1}.

Cryoscopy and glucose concentration measured by absorbance alone cannot predict lactose concentration with the precision required by industry. To improve the reliability of internal quality control analyses, an alternative is to combine these variables in a predictive regression model for indirect lactose estimation using Support Vector Machines (SVMs), for example.

When a SVM was applied, the generated algorithm allowed the prediction of lactose concentration with greater precision. The mean difference between the observed and predicted values was only 0.001, which was concordant with the actual lactose values measured by HPLC (p=0.03). In addition to concordance, the values also showed a strong correlation ($r = 0.97$; $p < 0.01$) (Figure 8). Considering the prediction interval with 95% confidence, the algorithm-predicted lactose concentration has ±0.51 g dL^{-1} as a margin of error.

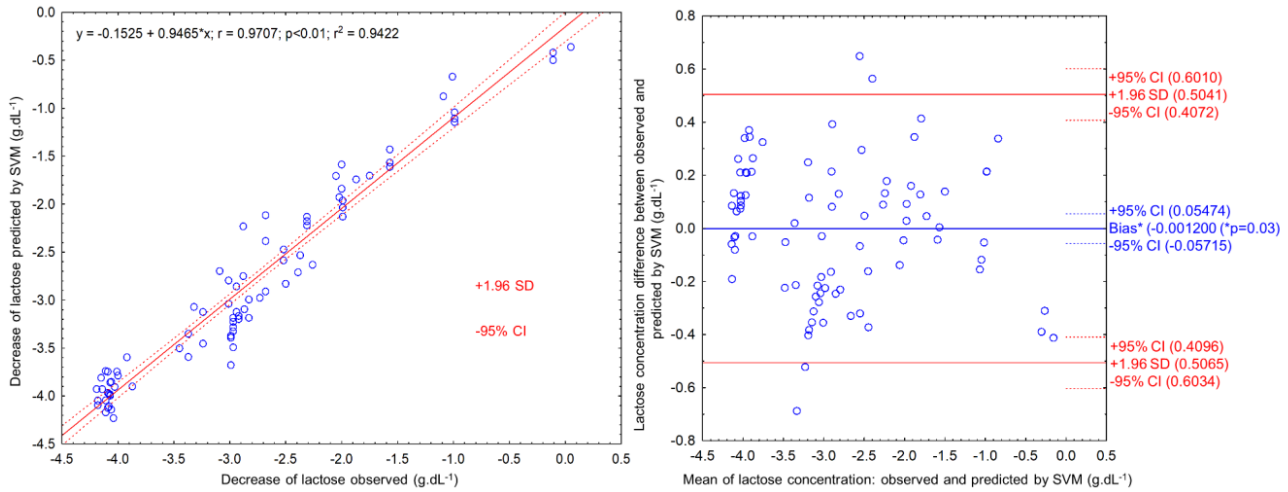

Figure 8. Relationship and agreement between the concentration of lactose measured by HPLC and that predicted by Support Vector Machines (SVMs) from 90 observations of two lactose hydrolysis assays using two types of industrial enzymes.

Conclusion

It was concluded that based on the reaction time, temperature, and enzymatic concentration conditions evaluated, the *B. licheniformis* enzyme could achieve the maximum limit to comply with the "zero lactose" classification after 240 min of reaction. In contrast, the *K. lactis* enzyme did not achieve a sufficient residual lactose concentration to fit into this classification. Lactose enzymatic hydrolysis is an error-prone industrial process, even with strict fulfillment of the recommendations provided by enzyme manufacturers. Consequently, some products do not meet the current regulations for delactosed milk and dairy products.

Furthermore, alternative analytical methods, such as cryoscopy and enzymatic kits, can be used to measure enzymatic hydrolysis in milk; however, it is necessary to perform a concordance analysis between the tests in order to compare glucose measurements obtained by absorbance and cryoscopic values determined using the HPLC method, which has more reliable precision and accuracy. Through artificial intelligence, it is possible to indirectly estimate lactose concentration by using an algorithm that associates cryoscopy with glucose concentration. Although the margin of error is a limiting factor, data processing with an SVM can improve the control of industrial processes using enzymatic lactose hydrolysis.

Acknowledgments

The authors thank the Instituto Nacional de Ciência e Tecnologia para a Cadeia Produtiva do Leite, the Inspeção de Produtos de Origem Animal, and the Desenvolvimento de Instrumentação e Automação Analítica laboratories of the Universidade Estadual de Londrina.

References

Araújo, E. M. Q., Almeida, D. O., Coutinho-Lima, C. R. O., Santos, L. A., Brandão, N., Sacramento, J. M., & Vázquez, M. R. (2019). Why is there a high prevalence of lactose intolerance in Brazil? - A mini review. *Current Research in Diabetes & Obesity Journal, 11*(5), 1-4. doi: 10.19080/CRDOJ.2019.11.555822

Beloti, V. (2015). Fatores que interferem na quantidade e composição do leite produzido. In V. Beloti, R. Tamanini, L. A. Nero, M. A. S., Moreira, L. C. C., Silva, R. da, Fagnani, & K. T. M. G. Reis (Eds.), *Leite: obtenção, inspeção e qualidade* (Cap. 2, pp. 35-50). Londrina.

Borin, A., Ferrão, M. F., Mello, C., Maretto, D. A., & Poppi, R. J. (2006). Least-squares support vector machines and near infrared spectroscopy for quantification of common adulterants in powdered milk. *Analytica Chimica Acta, 579*(1), 25-32. doi: 10.1016/j.aca.2006.07.008

Brito, A. B. N., & Giulietti, M. (2007). Study of lactose crystallization in water-acetone solutions. *Crystal Research and Technology, 42*(6), 583-588. doi: 10.1002/crat.200610867

Churakova, E., Peri, K., Vis, J. S., Smith, D. W., Beam, J. M., Vijverberga, M. P., Stora, M. C., & Winter, R. T. (2019). Accurate analysis of residual lactose in low-lactose milk: Comparing a variety of analytical techniques. *International Dairy Journal, 96*, 126-131. doi: 10.1016/j.idairyj.2019.02.020

Dutra-Rosolen, M., Gennari, A., Volpato, G., & Volken de Souza, C. F. (2015). Lactose hydrolysis in milk and dairy whey using microbial β-Galactosidases. *Enzyme Research, 2015*, 1-7. doi: 10.1155/2015/806 240

Garballo-Rubio, A., Soto-Chinchilla, J., Moreno, A., & Zafra-Gómez, A. (2018). Determination of residual lactose in lactose-free cow milk by hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry. *Journal of Food Composition and Analysis, 66*(1), 39-45. doi: 10.1016/j.jfca.2017.11.006

Gonzaga, N., Watanabe, L. S., Mareze, J., Madeira, T. B., Tamanini, R., Rios, E. A., Nixdorf, S. L., & Beloti, V. (2019). Green method using water for lactose and lactulose extraction and determination in milk by high-performance liquid chromatography with refractive index detection. LWT - *Food Science and Technology, 113*, 1-6. doi: 10.1016/j.lwt.2019.108288

Husain, Q. (2010). β Galactosidases and their potential applications: a review. *Critical Reviews in Biotechnology, 30*(1), 41-62. doi: 10.3109/07388550903330497

Jaganathan, A., & Kuppuraj, S. (2016). Artificial neural networks methods for predicting the performance and process in the milk industry. International Research *Journal of Engineering and Technology, 3*(11), 1127-1132.

Mattar, R., Mazo, D. F. de C., & Carrilho, F. J. (2012). Lactose intolerance: diagnosis, genetic and clinical factors. *Clinical and Experimental Gastroenterology, 2012*(5), 113-121. doi: 10.2147/CEG.S32368

Ministério da Agricultura, Pecuária e Abastecimento (2019). *Manual de métodos oficiais para análise de alimentos de origem animal.* Brasília: Secretaria de Defesa Agropecuária, 2019. https://www.gov.br/agricultura/pt-br/assuntos/laboratorios/credenciamento-e-laboratorios-credenciados/legislacao-metodos-credenciados/arquivos-metodos-da-area-poa-iqa/ManualdeMtodosOficiaisparaAnlisedeAlimentosdeOrigemAnimal2ed.pdf

Moretti, M. M. S., Perrone, O. M., Nunes, C. D. C. C., Taboga, S., Boscolo, M., Silva, R., & Gomes, E. (2016). Effect of pretreatment and enzymatic hydrolysis on the physical-chemical composition and morphologic structure of sugarcane bagasse and sugarcane straw. *Bioresource Technology, 219*, 773-777. doi: 10.1016/j.biortech.2016.08.075

Morlock, G. E., Morlock, L. P., & Lemo, C. (2014). Streamlined analysis of lactose-free dairy products. *Journal of Chromatography A, 1324*, 215-223. doi: 10.1016/j.chroma.2013.11.038

Nivetha, A., & Mohanasrinivasan, V. (2017). Mini review on role of β-galactosidase in lactose intolerance. IOP Conference Series: *Materials Science and Engineering, 263*(2), 1-5. doi: 10.1088/1757-899X/263/2/022046

Resolução da Diretoria Colegiada n. 135, de 8 de fevereiro de 2017. *Altera a Portaria SVS/MS nº 29, de 13 de janeiro de 1998, que aprova o regulamento técnico referente a alimentos para fins especiais, para dispor sobre os alimentos para dietas com restrição de lactose.* https://www.in.gov.br/materia/-/asset_publisher/Kujrw0TZC2Mb/content/id/20794561/do1-2017-02-09-resolucao-rdc-n-135-de-8-de-fevereiro-de-2017-20794490

Resolução da Diretoria Colegiada n. 136, de 8 de fevereiro de 2017. *Estabelece os requisitos para declaração obrigatória da presença de lactose nos rótulos dos alimentos.* https://www.in.gov.br/materia/-/asset_publisher/Kujrw0TZC2Mb/content/id/20794620/do1-2017-02-09-resolucao-rdc-n-136-de-8-de-fevereiro-de-2017-20794494

Storhaug, C. L., Fosse, S. K., & Fadnes, L. T. (2017). Country, regional, and global estimates for lactose malabsorption in adults: a systematic review and meta-analysis. *The Lancet Gastroenterology and Hepatology, 2*(10), 738-746. doi: 10.1016/S2468-1253(17)30154-1

Trani, A., Gambacorta, G., Loizzo, P., Cassone, A., Fasciano, C., Zambrini, A. V., & Faccia, M. (2017). Comparison of HPLC-RI, LC / MS-MS and enzymatic assays for the analysis of residual lactose in lactose-free milk. *Food Chemistry, 233*, 385-390. doi: 10.1016/j.foodchem.2017.04.134

Yan, W. J., Chen, X., Akcan, O., Lim, J., & Yang, D. (2015). Big data analytics for empowering milk yield prediction in dairy supply chains. *Proceeding of the 2015 IEEE International Conference on Big Data (Big Data)*, Santa Clara, CA, USA.