For antioxidant peptides, the optimal conditions proposed by the model were a digestion time of 3 minutes, a temperature of 42°C, and an E/P ratio of 1/40. Only the last factor significantly affects the peptides intensity (P = 0.04), the other factors and interactions did not show any statistically significant effect. The optimal pepsin digestion conditions to obtain peptides with antimicrobial activity determined through B. *subtilis* inhibition were a digestion time of 3 min, a temperature of 30°C, and E/P ratio of 1/40.
Regarding α-glucosidase inhibitor's peptides, the optimal conditions recommended by the model were a digestion time of 5 minutes, a temperature of 35°C, and an E/P ratio of 1/40. Only the E/P ratio (P = 0.004), showed a statistically significant effect over bioactive peptide formation. and the most influential factor in pepsin digestion was the ratio of enzyme to protein.
Previous studies have demonstrated that MAD can yield peptides with AChE inhibitory, α-glucosidase inhibitory, and antimicrobial activities. Hall and Liceaga [50] conducted a study on Cryllodes sigillatus and observed antihypertensive and antidiabetic effects using a 10-minute microwave-assisted digestion (MAD) method. In contrast, Srinivas and Prakash [5] examined the inhibitory activity of AChE, antioxidant capacity, and antimicrobial activity of peptides derived from α-casein. However, their digests were obtained through conventional digestion, which involved a minimum of 2 hours for the digestion process.

4. CONCLUSIONS

This study presents a novel approach utilizing MAED and HPTLC-bioassay to obtain and detect bioactive peptides from α-casein digestion with pepsin and trypsin enzymes. The optimization of α-casein digestion was achieved through chemometric tools employing a central composite design, allowing to establishment a high throughput, precise, and sustainable MAED method to generate bioactive peptides from α-casein digestion. HPTLC-bioassay proved to be a unique technology capable of detecting different in situ bioactivities. Through this analytical technique, it was possible to detect peptides generated from pepsin- and trypsin digestion with very promising bioactivities, i.e., AChE inhibitory peptides, α-glucosidase inhibitors, antioxidant, and antimicrobial peptides. These peptides will be identified soon to be studied as functional ingredients or nutraceuticals.

5. ACKNOWLEDGEMENTS

This work was supported by National Fund for Scientific & Technological Development (FONDECYT), projects Nº1201658 and 1211803, the International Cooperation Program from the National Agency of Research and Development (ANID) projects REDES Nº180178, FOVI210081, and Academy Installation Grant Nº85220091.

REFERENCES

1. P. Koirala, M. Dahal, S. Rai, M. Dhakal, N. P. Nirmal, S. Maqsood, F. Al-Asmari, A. Buranasompob, Current Nutrition Reports (2023)
2. D. D. Kitts, Trends in Food Science & Technology 16, 549, (2005)
3. C. M. Urista, R. Fernandez, F. R. Rodriguez, A. Cuenca, A. T. Jurado, Food Science and Technology International 17, 293, (2011)
4. D. Mohanty, R. Jena, P. K. Choudhury, R. Pattnaik, S. Mohapatra, M. R. Saini, Int. J. Food Prop. 19, 837, (2016)
5. S. Srinivas, V. Prakash, International Journal of Peptide Research and Therapeutics 16, 7, (2010)
6. Korhonen, Pihlanto, International Dairy Journal 16, 945, (2006)
7. P. Patil, S. Mandal, S. K. Tomar, S. Anand, European Journal of Nutrition 54, 863, (2015)
8. N. P. Moller, K. E. Scholz-Ahrens, N. Roos, J. Schrezenmeir, European Journal of Nutrition 47, 171, (2008)
9. M. Gobbetti, F. Minervini, C. G. Rizzello, Int. J. Dairy Technol. 57,173, (2004)
10. S. G. Rival, C. G. Boeriu, H. J. Wichers, Journal of Agricultural and Food Chemistry 49, 295, (2001)
11. M. Moreno-Montoro, M. Olalla-Herrera, J. A. Rufian-Henares, R. G. Martinez, B. Miralles, T. Bergillos, M. Navarro-Alarcon, P. Jauregi, Food & Function 8, 2783, (2017)
12. F. Jan, S. Kumar, R. Jha, Veterinary World 9, 1152, (2016)
13. Korhonen, Journal of Functional Foods 1, 177, (2009)
14. R. Hartmann, H. Meisel, Current Opinion in Biotechnology 18, 163, (2007)
15. J. Pavón-Pérez, K. Henriquez-Aedo, R. Salazar, M. Herrero, M. Aranda, J. Food Sci. Technol. 58, 2914, (2021)
16. J. Pavón-Pérez, K. Henriquez-Aedo, M. Herrero, M. Aranda, Food Addit. Contam. B 13, 268, (2020)
17. Ulug Sule Keskin, Jahandideh Forough, W. Jianping, Trends in Food Science & Technology 108, 27, (2021)
18. B. N. Pramanik, U. A. Mirza, Y. H. Ing, Y. H. Liu, P. L. Bartner, P. C. Weber, M. K. Bose, Protein Science 11, 2676, (2002)
19. S. Tadesse, S. Emire, Heliyon 6, e04765, (2020)
20. C. Wen, J. Zhang, H. Zhang, Y. Duan, H. Ma, Trends. Food Sci. Technol. 105, 308, (2020)
21. S. P. Mane, S. K. Johnson, M. Duranti, V. K. Pareek, R. P. Utikar, Trends in Food Science & Technology 73, 1, (2018)
22. J. Biller, L. Morschheuser, M. Riedner, S. Rohn, J. Chromatogr. A 1415, 146, (2015)
23. R. Ł. Gwarda, T. H. Dzido, Journal of Chromatography A 1534, 179, (2018)
24. M. Treblin, T. von Oesen, L.-C. Class, G. Kuhnen, I. Clawin-Rädecker, D. Martin, J. Fritsche, S. Rohn, Journal of Chromatography A 1653, 462442, (2021)
25. S. M. Derayea, E. Samir, Microchemical Journal 156, 104835, (2020)
26. 26 J. Jaxel, M. Guggenberger, T. Rosenau, S. Böhmdorfer, Talanta 217, 121072, (2020)
27. M. G. Weller, Sensors 12, 9181, (2012)
28. G. Morlock, W. Schwack, Journal of Chromatography A 1217, 6600, (2010)
29. I. O. Oresanya, M. A. Sonibare, B. Gueye, F. O. Balogun, S. Adebayo, A. O. T. Ashafa, G. Morlock, Journal of Food Biochemistry 44, (2020)
30. I. Klingelhofer, G. E. Morlock, Analytical Chemistry 87, 11098, (2015)
31. O. Galarce-Bustos, J. Pavon, K. Henriquez-Aedo, M. Aranda, Phytochemical Analysis 30, 679, (2019)
32. M. Aranda, M. H. Vega, R. F. Villegas, JPC J. Planar Chromatogr. - Mod. TLC 18, 285, (2005)
33. L.-J. Min, Y. Kobayashi, M. Mogi, K. Tsukuda, A. Yamada, K. Yamauchi, F. Abe, J. Iwanami, J.-Z. Xiao, M. Horiuchi, PloS one 12, e0171515, (2017)
34. M. Abdel-Hamid, J. Otte, C. De Gobba, A. Osman, E. Hamad, International Dairy Journal 66, 91, (2017)
35. Wu, W. Xu, K. Liu, Y. Xia, Shuangquan, Journal of Dairy Science 102, 5913, (2019)
36. Bamdad, Shin, Suh, Nimalaratne, Sunwoo, Molecules 22, (2017)
37. B. Hernandez-Ledesma, B. Miralles, L. Amigo, M. Ramos, I. Recio, Journal of the Science of Food and Agriculture 85, 1041, (2005)
38. M. Q. Li, S. W. Xia, Y. J. Zhang, X. L. Li, Lwt-Food Science and Technology 98, 358, (2018)
39. P. Bove, P. Russo, V. Capozzi, A. Gallone, G. Spano, D. Fiocco, Microbiological Research 168, 351, (2013)
40. E. Miquel, J. Ángel Gómez, A. Alegría, R. Barberá, R. Farré, I. Recio, European Food Research and Technology 222, 48, (2006)
41. P. Bove, A. Gallone, P. Russo, V. Capozzi, M. Albenzio, G. Spano, D. Fiocco, Applied Microbiology and Biotechnology 96, 431, (2012)
42. O. Galarce-Bustos, J. Pavon-Perez, K. Henriquez-Aedo, M. Aranda, Journal of Chromatography A 1608, (2019)
43. M. Aranda, J. Carrasco, K. Henríquez Acetylcholinesterase (ACHE) and α-Glucosidase Inhibitory Assay by Effect-Directed Analysis on High Performance Thin-Layer Chromatography Coupled to Mass Spectrometry, In: Mass Spectrometry for Food Analysis, Koolen H (ed) Springer US, New York, NY, 2022; 213-218.
44. M. Jamshidi-Aidji, G. E. Morlock, Journal of Chromatography A 1420, 110, (2015)

*Corresponding author email: kahenriquez@ubiobio.cl ; mario.aranda@uc.cl

45. M. F. Angulo, M. Flores, M. Aranda, K. Henriquez-Aedo, Food Chem. 309, (2020)
46. O. Galarce-Bustos, L. Novoa, J. Pavon-Perez, K. Henriquez-Aedo, M. Aranda, Food Anal. Methods 12, 448, (2019)
47. P. M. Reddy, W. Y. Hsu, J. F. Hu, Y. P. Ho, J Am Soc Mass Spectrom 21, 421, (2010)
48. Pavón, Jessy, K. Henriquez, R. Salazar, M. Herrero, M. Aranda, Journal of Food Science and Technology(2020)
49. H. K. Hustoft, L. Reubsaet, T. Greibrokk, E. Lundanes, H. Malerod, J Pharm Biomed Anal 56, 1069, (2011)