SUMMARY

NATIONAL UNIVERSITY OF SCIENCE AND TECHNOLOGY POLITEHNICA BUCHAREST

FACULTY OF CHEMICAL ENGINEERING AND BIOTECHNOLOGIES

DOCTORAL SCHOOL: CHEMICAL ENGINEERING AND BIOTECHNOLOGIES

SUMMARY OF THE DOCTORAL THESIS

Analytical evaluation of chemical/biochemical markers of the nutritional quality of milk

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SUMMARY

CONTENTS OF DOCTORAL THESIS

SUMMARY

SUMMARY

SUMMARY

Milk and dairy products are one of the most important food products for the world population, considering its composition rich in nutritional compounds, which can contribute to maintaining a healthy lifestyle. Dairy products are a major source of energy and nutrients for the human body, that can be a reason of their globally consumption in very large quantities. According to Eurostat 2015, approximately 165 million tons of milk are produced in Europe, thus the EU contributes to global milk production with approximately 25%, therefore its quality becomes even more a topic of interest in an ongoing debate.

In this context, the doctoral thesis addresses an important issue in the field of milk chemistry, providing relevant technical-scientific information for the study of the fine chemical composition of milk, but also practical applications in terms of innovative nutritional strategies to improve the antioxidant status and its composition in bioactive compounds. Also, the presented thesis uses a multi-disciplinary approach combining the chemical/biochemical evaluation of the milk composition and experimental feeds, but also zootechnical approaches using precision nutrition and the elaboration of the complex experimental design for each of the three nutrition experiments.

In our country there is a milk composition analysis portfolio mainly focused on the analysis of the primary chemical composition (total protein, total fat, caseins, lactose, etc.). This can limit the studies focused on the fine chemical composition of the milk, as it presents in its composition bioactive compounds with special effects on the consumers health, which cannot be exploited at their true potential. Even at the international level, there are very few studies aimed to determine the antioxidant status of milk, a particularly important fact given that its chemical composition is rich in unsaturated fatty acids that are very susceptible to the lip-oxidation process. In this context, the main objective of the thesis was to study the antioxidant status of milk, as well as its bioactive compounds, using optimized and validated analytical methods and practical applications, such as nutrition studies based on innovative nutritional strategies (for example, the use of inactivated yeast or oilseeds). The results obtained in this PhD thesis can be important both for the dairy food industry, farmers and consumers.

The doctoral thesis is structured in nine chapters, systematized in two parts:

Part I (Chapters 1, 2 and 3) of the thesis includes three chapters that systematize the current state of knowledge in the field of milk chemistry.

SUMMARY

Chapter 1 entitled "**CHEMICAL COMPOSITION OF MILK AND THE MAIN BIOACTIVE COMPOUNDS**" presented a detailed analysis of the main interested compounds in milk. The major milk compounds (total proteins, total caseins, whey proteins, carbohydrates, total fat, minerals, enzymes, and somatic cells) that are important from the point of view of milk chemistry are classified and described. Furthermore, it provided an in-depth characterisation of the main composition of milk and its bioactive elements, encompassing water- and lipo- soluble vitamins, along with the interesting fatty acids composition (for example conjugated linoleic acid). This chapter highlighted the need of a comprehensive investigation into the composition of fine constituents in milk, as these substances may be beneficial for customers' health in addition to improving of the milk and dairy products oxidative status. Additionally, it discussed the necessity of evaluating oxidation-susceptible compounds (unsaturated lipids) as well as milk antioxidant composition, concluding with the fact that the study of the lipid oxidation is crucial considering the effects of altering the organoleptic attributes of milk and dairy products and influencing their storage time.

Chapter 2 entitled "**ANALYTICAL TECHNIQUES USED FOR THE DETERMINATION OF MILK COMPOSITION**" systematizes the analytical methodologies used for the determination of the main milk components (presented in Chapter 1). Additionally, it presented the analytical techniques utilized for determination of the milk bio-active compounds (vitamins, fatty acids, polyphenols), that are important in terms of both consumer health and milk oxidative status. Moreover, this chapter provides a comprehensive and structured overview of techniques used in the specialized research to assess the antioxidant potential of milk and dairy products.

Chapter 3, entitled "**FACTORS INFLUENCING THE CHEMICAL COMPOSITION OF MILK**" presents a comprehensive examination of the main factors that can influence the milk constituents. These factors included breed, environmental conditions, and the stage of lactation. Among these, the diet of the animal is one of the most crucial elements that can be capable of significantly impacting the composition of milk. Milk can be enhanced with a variety of nutritional substances through precise animal nutrition, which may improve consumer health or can influence the milk processing technologies. In addition, feeding techniques aimed to enriching the milk composition in nutrients such as innovative feeding strategies based on the reusing of by-products from various industries (which are thereby given economic value) that can improve milk in bioactive compounds (essential fatty acids, antioxidant compounds) are also presented.

SUMMARY

Part II of the paper included original experimental studies carried out during the doctoral internship. In each of the six chapters, the objectives, the materials and methods, the results, and discussions on the relevance of the data obtained, as well as the conclusions of the study are presented.

Chapter 4 entitled "**DEVELOPMENT / VALIDATION OF THE METHOD FOR THE PREPARATION AND SIMULTANEOUS DETERMINATION OF THE VITAMIN E ISOMERS USING RP-HPLC**" was aimed to develop and validate a simple and rapid method for the simultaneous determination of α-, δ-, γ-tocopherols in milk samples. This presented the validation of a reversed-phase high-performance liquid chromatography (RP-HPLC) method that can be used to simultaneously determination of the vitamin E isomers (δ, γ, and α) from biological samples (milk). This chapter also described the validation process for the milk samples preparation. The calibration curves obtained for each compound demonstrated a coefficient of determination greater than 0.99, indicating that the method exhibited adequate linearity. Furthermore, low quantification and detection limits were found, which might make it possible to identify and quantify vitamin E isomers in milk samples at low concentrations (LODδ - 0.29 mg/L, LODγ - 0.28 mg/L, LODα - 0.07 mg/L; LOQδ - 0.33 mg/L, LOQγ - 0.32 mg/L, LOQα - 0.10 mg/L). Additionally, all three analytes showed a high degree of recovery with values above 95%. A representative chromatogram obtained for the assessment of the vitamin E isomer calibration curve is shown in figure 1.

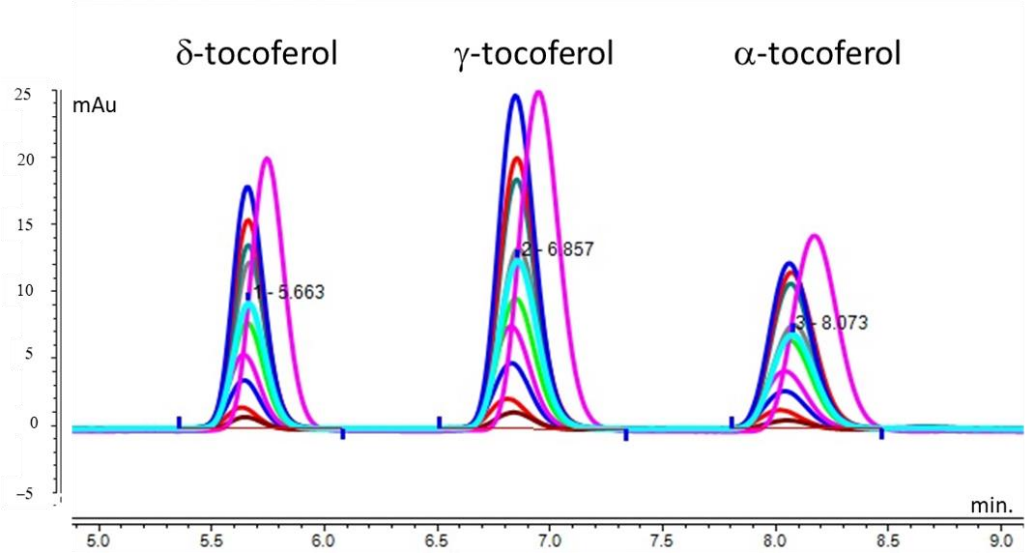

Figure 1. A representative chromatogram obtained for the assessment of the vitamin E isomer calibration curve (0.5 mg/L – 10 mg/L).

SUMMARY

The method of preparing milk samples for the analysis of vitamin E isomers was validated for determination of the α-tocopherol isomer, by using a reference material Infant/Adult Nutritional Formula 1849a, which presents a known and exact concentration of α-tocopherol, obtaining a recovery rate of 101.57%.

Chapter 5, entitled "**OPTIMIZATION OF TBARS PARAMETERS FOR ASSESSING THE OXIDATIVE STABILITY OF MILK**" was aimed to optimize a TBARS method for the determination of lipid oxidation products specific to the milk fatty acid profile. Optimizing the TBARS method was essential for measurement of the secondary compounds deviating from milk lipid oxidation, considering the milk's unique fatty acid composition, differing from the other food types. Such variations in fatty acid profiles resulted in distinct secondary compound formation during lipid oxidation compared to similar processes in other animal-derived food items. The optimized TBARS method allows the quantification of the absorbances of these classes of compounds and involves treating 5 mL of milk with 10 mL of 20% trichloroacetic acid, without addition of the synthetic antioxidant, centrifugation at 3000 rpm, for 5 minutes and filtered. After that, 2.5 mL of the filtrate were incubated with 1.5 mL of 0.8% thiobarbituric acid, for 90 minutes at 80 °C. After cooling the samples were performed absorption spectra readings at specific wavelengths corresponding to degradation products of milk (450 nm for saturated aldehydes, 495 nm for dienals, and 532 nm for malondialdehyde). The oxidation status of six samples from two distinct ruminant species (sheep and cow) was assessed using the TBARS technique optimized in this study. Sheep milk samples had higher absorbance values for saturated aldehydes and dienals, compared with cow milk, which is consistent with the specific fatty acid profile of it (sheep milk having a higher concentration of unsaturated fatty acids). The results obtained in this study included an optimized TBARS method for the determination of the oxidative status of milk, article published in an ISI rated international journal (Applied Sciences - IF 2.7).

Chapter 6, entitled " **NUTRITIONAL CHARACTERIZATION OF DIFFERENT VEGETABLE SOURCES WITH POTENTIAL USE IN RUMINANT NUTRITION**" presents the nutritional evaluation of various alternative feed resources that may have a high potential for use in ruminant rations. The aim of this study was to investigate the nutritional composition of five feed resources (hemp seed, linseed, mustard, hemp meal and inactivated brewer's yeast) that may have the inclusion potential in ruminant diets. The feed resources studied (oilseeds, by-products from various industries) were chosen to be representative for the various categories of feedstuffs used in ruminant nutrition. These were examined with a focus

on bioactive compounds (polyphenolic compounds, vitamins, essential fatty acids, antioxidant capacity) capable of being transferred through animal nutrition into milk, thereby enhancing its quality. Additionally, consideration was given to the intake of nutrients essential for the optimal functioning of the animal body. In this study, linseeds were noted to have high concentrations of essential fatty acids, such as α-linolenic, and conjugated linoleic acids, particularly important for human health, but also for the potential to positively influence the quality of milk fat. Also, mustard seeds were highlighted to be important feedstuffs for ruminants' nutrition. In addition to having a unique fatty acid profile, mustard seeds also demonstrated a strong antioxidant potential. These had significant levels of total polyphenols (28.47 mg/g gallic acid equivalents), vitamin E (244.49 mg/kg), and ferulic acid (8.638 mg/g), as well as a strong antioxidant capacity (33.78 μM Trolox equivalents). Brewer's spent yeast was selected for practical investigations among various industrial by-products due to its classification as waste within the brewing sector and due to its composition rich in nutrients. The result of this study revealed its substantial content of bioactive compounds, such as vitamin E (125.69 mg/kg), and notable antioxidant activity (13.26 μM equivalents to Trolox). Consequently, the selected feedstuffs for practical applications consist of linseeds, mustard seeds, and inactivated brewer's yeast.

In **Chapter 7**, " **THE EFFECTS OF INACTIVATED BREWER'S YEAST (BY-PRODUCT OF THE BREWING INDUSTRY) INCLUSION IN THE SHEEP RATIO ON MILK QUALITY AND RUMEN FERMENTATION PARAMETERS** " the influence of the of inactivated brewer's spent yeast inclusion in sheep diets, as a by-product of the beer industry, on the nutritional quality of milk and on its oxidative status is presented. The objective of this study was to investigate the way several milk quality parameters and ruminal fermentation parameters were influenced as a result of inactivated brewer's yeast inclusion in sheep's diet. The principal hypothesis proposed that inactivated brewer's yeast could have a beneficial effect on rumen fermentation, enhance the antioxidant potential of milk, and influence other minor constituents of milk with bioactive characteristics. Through analysis of the fatty acid profile of milk, it was observed that including brewer's spent yeast in sheep diet can improve the quality of milk by lowering the content of omega 6 fatty acids and the ratio of omega 6 / omega 3, a higher quality of milk fat being characterized by a low ratio between them. An increased in the content of total polyphenols was also determined (2.383 mg/g gallic acid equivalents vs. 1.556 mg/g gallic acid equivalents), a fact that can also be correlated with a significant decrease ($p = 0.004$) of the concentration of conjugated dienes in milk, as an indicator of the primary oxidation stage of milk lipids. Moreover, the diet including brewer's spent yeast also presented

an increased in calcium content of the milk (1.182% vs. 1.063%), fact that can be important from the point of view of consumers' health. The results of the research suggest that brewer's spent yeast can be utilized as an alternative protein source, enhancing milk's antioxidant status and its bioactive composition. Article published in an ISI rated international journal (IF 3.6).

Chapter 8, entitled "**THE EFFECTS OF LINSEEDS AND MUSTARD SEEDS INCLUSION IN GOAT DIET ON THE NUTRITIONAL COMPOSITION OF MILK** " presents a complex approach regarding the potential of linseeds to positively influence the composition of polyunsaturated fatty acids, but also the ability of the mustard seeds to counteract the peroxidation effects of polyunsaturated fatty acids. The aim of this study was to nutritionally characterize the milk obtained from goats fed linseeds and mustard seeds, considering the ability of linseeds to increase the concentration of polyunsaturated fatty acids and the rich composition of mustard seeds in antioxidant compounds, which can influence the antioxidant status of milk. The inclusion of linseeds led to a significant increase in the content of polyunsaturated fatty acids (8.291% vs. 5.738%), which is particularly important for the quality of milk fat. Simultaneously, there is an increase in the concentration of omega 3 fatty acids (1.186 %) and a decrease in the omega 6/ omega 3 ratio (6.316). Also, in case of inclusion of a mixture between linseeds and mustard seeds (9% linseeds and 3% mustard seeds), there was observed a significant decrease in the omega 6/ omega 3 ratio (5.936). Also, in this group was observed an increase of the vitamin E content (32.92 mg/L), a fact that can be explained by the high intake of vitamin E that mustard seeds bring to the diet. Also, the comparative study of the two diets highlighted the potential of the seeds mixture to positively influence the concentration of p-anisidine, a secondary parameter of lipid oxidation. Both experimental rations resulted in a decrease in this parameter at fresh time (t0). After 24 h of storage at room temperature, the p-anisidine index was influenced, increasing for the linseeds diet, which may be caused by the higher content of polyunsaturated fatty acids. However, no differences were recorded between the control group and the seed mixture group, an explanation can be the fact mustard seeds has a rich composition of antioxidant, as presented in chapter 6. The study's conclusions highlight the facts that linseeds can be used in goats' diet to improve the quality of milk fat, and the mixture of mustard seeds and linseeds can be used to counteract the undesirable effects of lipid oxidation in milk, following the increase of polyunsaturated fatty acids.

Chapter 9, entitled "**THE EFFECTS OF THE RAPESEEDS INCLUSION IN DAIRY COWS DIETS ON THE MILK PROTEIN FRACTIONS**" discusses a problem that is frequently observed in zootechnical practices: feeding recipes to improve the fatty acid

profile of milk without considering the impact on protein fractions after supplementation. The aim of this study was to evaluate the quality of milk in terms of protein fractions following the replacement of soybean meal with rapeseed. The study included the evaluation of the effects regarding the inclusion of rapeseeds in ruminants' diets (a feed ingredient which is frequently utilized to influence the quality of milk lipids), by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to observe the effects on milk proteins fractions. The study revealed decreases in the content of the important protein fractions, such as k-casein (25883 U.A. vs. 32275 U.A.), β-lactoglobulin (52694 U.A. vs. 61256 U.A.), bovine serum albumin (16934 U.A. vs. 19114 U.A.) and immunoglobulin G (17997 US vs. 21554 US). These effects can be attributed to the high amount of fat provided in the ration, which can lead to an increase in the milk quantity produced per day, thus influencing the protein components of the milk. Figure 2 shows the representative electrophoretic pattern for the milk samples used in this study.

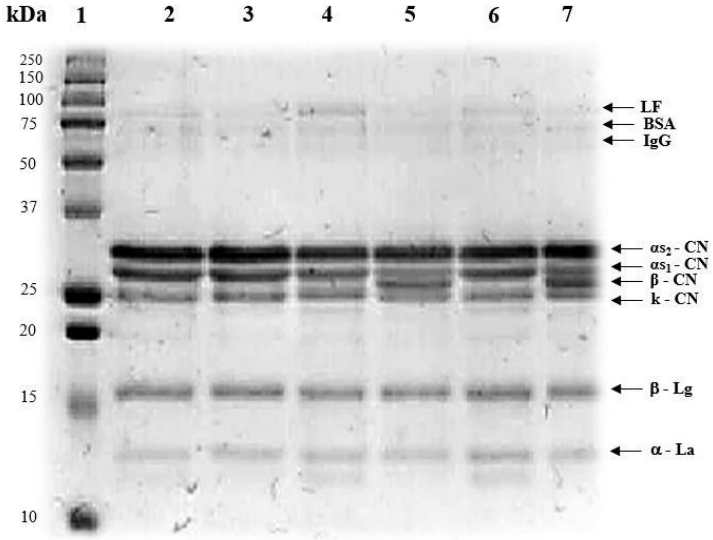

Figure 2. Representative electrophoretic pattern for the milk samples used in this study.

The effects on milk proteins fractions were attributed exclusively to feeding diets, considering that the cows breed did not significantly influence the milk protein profile. The conclusions of the study reveal the importance of evaluating the effects of rapeseeds on the composition of milk peptides, since an increase in milk fat content can negatively influence protein fractions. The study was published in an ISI-listed international journal (IF 1,169).

In the final chapter are presented the **GENERAL CONCLUSIONS. PERSONAL CONTRIBUTIONS. DEVELOPMENT PERSPECTIVES** relevant from the point of view of the obtained results. Among them, the most scientifically relevant are the following:

SUMMARY

- Two quick and easy analytical techniques that enable the precise determination of biochemical analytes important from the perspective of milk quality were developed or improved as part of the PhD thesis. Lipid oxidation is a common issue presented in milk and dairy products that is not specifically assessed in accordance with its complex fatty acid profile, in this context one simple and rapid TBARS method was optimized in this study that addressed this problem. Also, in this doctoral thesis was presented the validation of RP-HPLC method aimed to simultaneous determination of vitamin E isomers of α-, δ- and γ- tocopherol in milk. Moreover, during the doctoral stage was validated the milk samples preparation to determine the alpha-tocopherol using the RP-HPLC method.

- Innovative feeding strategies have been established to improve the quality of milk fat in a natural way (through animal feeding) by using feeds with a special composition in bioactive compounds as well as by utilizing waste from certain industries (beer industry), evaluating at the same time the impact of these strategies on the oxidative status and composition in antioxidant compounds of milk.

- In this doctoral thesis was presented the secondary effect of enriching the composition of milk in polyunsaturated fatty acids on the milk protein fractions, that can have economic and medical importance.

Also, the thesis brings to attention an important biological phenomenon from the perspective of the consumer health and the authorities. The results of this thesis can also be exploited in the direction of completing the normative acts with the parameters that describe the evolution of the fat quality (relevant especially in the case of dairy products enriched in unsaturated fatty acids susceptible to oxidation). Furthermore, the presence of certain antioxidant compounds due to feeding alternatives ingredients to ruminants opens new research opportunities to improve milk from this perspective as well.

Finally, both the methodology and the results of the thesis lead to the perspective of exploiting an extended range of feed resources, leading to the diversification of feed strategies (based on new combined feed recipes) that allow obtaining dairy products enriched in various nutrients. Thus, the opening of new opportunities for innovation in the milk production industry, generating benefits both for producers and processors, as well as for consumers. These perspectives underline the intention of the thesis to have both an in-depth character, based on laboratory research, and an applicative character, based on nutrition tests, in conditions of zootechnical practice, thus ensuring concrete results.

SUMMARY

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