Dairy Foods: Chemistry-Protein

797 Ability of Smart Nose to discriminate *tina* biofilms contributing to produce unique volatile compounds in inoculated milk.
S. Carpino*[1], I. Stampelou[2], G. Belvedere[1], C. Pediliggieri[1], and G. Licitra[3,1], [1]*CoRFiLaC, Regione Siciliana*, *Ragusa, Italy*, [2]*Wageningen University*, *the Netherlands*, [3]*DACPA, University of Catania*, *Catania, Italy*.

Wooden equipment is used in the traditional cheese making process of PDO Ragusano cheese and no starters are added in the cow raw milk. A source of aromatic components in milk might be the biofilm microflora released from the traditional wooden vat called *tina*, used during cheese making, as well as the flora naturally present in raw milk. Thus, the objectives of this work were to investigate the potential role of the *tina* biofilm to generate aroma compounds when inoculated into milk and to assess the ability of a Smart Nose to discriminate them by analyzing the aroma profiles of the inoculated milks. Pasteurized milk was used to avoid the interference of aroma compounds generated by the natural microflora present in raw milk. In this study, *tina* biofilms isolated from 3 different farms were inoculated in milk and incubated at conditions simulating the ones of the real cheese making of Ragusano before brining. The inoculated milk samples were analyzed through Smart Nose and the data were statistically treated by Principal Component Analysis (PCA). The PCA results showed first of all a good separation of the inoculated milks from the blank one (non-inoculated milk), highlighting a significant influence of *tina* biofilm on the developed milk aroma profile. In addition, all inoculated milk samples showed a clear separation among them, thus showing that each *tina* biofilm had a different behavior regarding aroma releasing when inoculated into milk under certain conditions. Certain volatile compounds were detected by GC-MS analysis in all 3 inoculated samples while these were totally absent in the blank, showing that were produced by the biofilm. Moreover, it was observed that the microbiological composition of each *tina* biofilm gave respectively a different aroma contribution. In conclusion, it was shown that Smart Nose is able to discriminate quite well aromatic profiles attributed exclusively to the biofilm bacteria during the first steps of cheese making.

Key Words: *Tina* biofilm, aroma profile, Smart Nose

798 Segmentation of scanning electron microscopy images using incremental learning.
G. Impoco[1], L. Tuminello[1], M. Caccamo*[1], and G. Licitra[1,2], [1]*CoRFiLaC, Regione Siciliana*, *Ragusa, Italy*, [2]*DACPA, University of Catania*, *Catania, Italy*.

This study tested a method for automatic quantification of digital micrographs based on statistical classification of pixels and incremental learning. Ten scanning electron microscope (SEM) images of Ragusano cheese were used as training set and the main microstructural features were gathered in 3 morphologically meaningful classes: fat globules, whey pockets, and protein matrix. A series of 10 numerical values (descriptor) was associated to each pixel. Images were partitioned into significant regions (segmentation) by clustering pixel descriptors using the k-means algorithm. According to the resulting clusters, an initial automatic classification associated each region to a specific microstructural feature. The classified images, used as reference labelings, were used to automatically learn a Bayesian statistical model which associates to each pixel its probability to belong to a certain feature class. This model was used to classify again input images. Output classifications were presented in color to a SEM specialized operator, who could select misclassified regions and associate them with a different, more appropriated label (re-labeling). New statistics obtained from the re-labeled regions were integrated into the model. The updated model was used to re-classify input images. This process of supervised re-labeling and automatic pixel classification was iterated until satisfactory results were obtained. We compared incremental learning to off-line image labeling, where the input images are manually labeled only for the initial training of the model, from a single reference data set. Experimental data showed that incremental learning gives better results than off-line learning. The training phase is less burdensome and time consuming for the user, and it can be adapted to new image samples without executing from scratch the long and tedious initial training.

Key Words: image analysis, SEM, cheese microstructure

799 Improvements and validation of mid-infrared predictions of milk fatty acid.
H. Soyeurt*[1,2], S. McParland[3], D. Berry[3], E. Wall[4], N. Gengler[1,2], F. Dehareng[5], and P. Dardenne[5], [1]*University of Liege, Gembloux Agro-Bio Tech, Animal Science Unit*, *Gembloux, Namur, Belgium*, [2]*National Fund for Scientific Research*, *Brussels, Brussels, Belgium*, [3]*Teagasc Moorepark Dairy Production Research Centre*, *Fermoy, Cork, Ireland*, [4]*Sustainable Livestock Systems Group, Scottish Agricultural College*, *Penicuik, Midlothian, United Kingdom*, [5]*Agricultural Walloon Research Centre, Quality Department*, *Gembloux, Namur, Belgium*.

The development of mid-infrared equations to predict the milk fatty acid (FA) content of milk allows prompt analysis of large numbers of samples. The first aim was to improve these predictions by comparing 6 statistical approaches. The second one was to validate the new equations using an independent sample set. The calibration set contained 239 spectrally different Belgian milk samples collected for over 2 years from several cows and breeds. FA were quantified by gas chromatography (GC). Statistical approaches tested were 1) partial least squares regression (PLS), 2) PLS and first derivative, 3) PLS and repeatability file (RF), 4) PLS, first derivative and RF, 5) PLS, second derivative, and 6) PLS, second derivative and RF. This last file contained spectra obtained from the same samples using 5 spectrometers. Cross-validation (CV) used 20 groups from the calibration set. Methods were compared using the ratio of the standard deviation of GC values to the standard error of CV (RPD). An external validation permitted a second comparison and was done using 362 samples collected for one year from multiple breeds and cows in Belgium, Ireland, and Scotland. Different RPD values were obtained by the 6 methods. Generally the equations developed using method 4 gave better results suggesting the adaptation of the methodology to the studied FA. It confirms by the obtained validation coefficients of determination. Highest values were observed for the equations with the highest RPD values except for C18:0. The ability to predict FA using method 4 gave superior results to those shown in previous publications.

Key Words: mid-infrared, milk, fatty acid

800 Evaluation of a faster extraction and purification procedure for the analysis of vitamin D in fortified milk.
T. C. Schoenfuss*[1] and O. Shimelis[2], [1]*University of Minnesota*, *St. Paul,* [2]*Sigma-Aldrich*, *Bellefonte, PA*.

Current methods to analyze vitamin D in milk require lengthy sample preparation steps to extract vitamin D before analysis by chromatographic methods like HPLC. The approved method from Standard Methods for the Examination of Dairy Products requires multiple days to accomplish and includes over-night saponification, liquid-liquid

extraction, dry down of over 135 mL of hexane under nitrogen, and solid phase extraction before HPLC. Various direct extraction techniques have been attempted but have not been adopted by the industry due to low extraction rates. The goal of this project was to develop a quicker, simpler method to quantify vitamin D in milk by combining new technologies. The method developed involved precipitating protein in 10 mL of milk with 10 mL of ethanol, sonicating for 1 min at 70% amplitude with a sonic horn immersed in the sample to break up complexes, and 2 liquid extractions with 15 mL hexane. The hexane extracted was dried down under nitrogen with a TurboVap LV Evaporator (Caliper LifeSciences, Hopkinton, MA) and reconstituted in 1 mL hexane. Solid phase extraction with an adsorbent designed for fatty acid methyl esters (Ag-Ion SPE tube, Sigma-Aldrich, Bellefonte, PA), and reverse phase HPLC of the eluent was done with several different columns and mobile phases that all allowed separation of D2 and D3. Vitamin D2 and D3 were observed to have different affinities for the solid phase extraction column, especially in the presence of fat, and the extraction rate from milk for D3 was up to 30% lower than D2. This extraction procedure can be completed in 1 d. This work highlights promising improvements for vitamin D analysis in milk that could reduce the time and solvents necessary.

Key Words: vitamin D, milk, extraction

801 Structural comparison of bovine and camel chymosin in relation to cheesemaking properties. K. B. Qvist*[1], J. L. Jensen[2], J.-C. N. Poulsen[2], M. Harboe[1], H. van den Brink[1], A. Mølgaard[2], and S. Larsen[2], [1]*Chr. Hansen*, *Hørsholm, Denmark*, [2]*Department of Chemistry, University of Copenhagen*, *Copenhagen, Denmark*.

By initiating milk coagulation, rennet enzymes are essential in cheesemaking. Bovine chymosin (BC) has long been considered the most suitable rennet, but recently it has been shown that *Camelus dromedarius* chymosin (CC) provides several benefits over bovine chymosin (BC), having a 7-fold higher ratio between milk clotting and general proteolytic activity. The objective of this work was to investigate the structural background for the improved selectivity of CC. Crystals of fermentation produced CC were grown with the hanging drop vapor diffusion technique. Data were collected by radiation with x-rays at 1.04 Å, and reduced with the XDS software, followed by flagging of reflections for free R-factor calculation, using the CCP4 suite. Molecular replacement was done with the Phenix software suite using the BC structure 1CMS as template. Cycles of manual fitting and refinement were done with Coot and Phenix, respectively. Validation was done with the WHAT IF package. The structure of CC, without the first 10 residues was obtained at 1.85 Å resolution, with a free R-factor of 0.21. The overall fold was similar to that of BC, but with notable differences in the details. L32V and E290D substitutions provide more space for bulky amino acids at the P1 and P1' positions, respectively. A further 7 substitutions in the extended binding region may contribute to selectivity differences. Also, in CC a surface patch, at positions 244–254, previously implicated in binding the positively charged sequence 98–102 of κ-casein (KC), was less negatively charged than in BC. This mirrors the fact that 98–102 in camel KC is more positively charged than in bovine KC. A patch near the edge of the cleft, at positions 289–300 was significantly more negative in CC. Finally, differences in the location of the N-terminal were observed. In conclusion, several structural differences likely to affect cheesemaking properties were identified.

Key Words: camel chymosin, 3-D structure, renneting

802 Detection of proteolysis in milk. A. S. Grandison*[1], L. M. Chove[2], and M. J. Lewis[1], [1]*University of Reading*, *Reading, Berkshire, UK*, [2]*Sokoine University*, *Morogoro, Tanzania*.

The quality of raw milk is affected by proteolysis during storage. It occurs through either bacterial or native proteases. Plasmin, the major protease occurring in milk, forms part of a complex system which hydrolyses the caseins and thus affects the quality of dairy products. These effects may be positive, as in cheese ripening; or negative as in the production of bitter off-flavors in dairy products or age-gelation in UHT milk. The enzyme system is very heat resistant and cannot be eliminated by pasteurization or even UHT processing. The aim of this study was to develop a simple method to detect protease activity in milk. Analysis of pH 4.6 or 6% trichloracetic acid (TCA) soluble extracts of milk by trinitrobenzene sulfonic acid (TNBS), reverse phase high performance liquid chromatography (RP-HPLC), gel electrophoresis and fluorescamine methods was carried out to determine their relative suitability for the detection of proteolysis in milk during storage for up to 7 days. Trypsin or plasmin were added to UHT sterilized milk at different levels to promote/accelerate proteolysis. This was due to the high cost of plasmin, hence trypsin was used for initial trials as it has a similar mode of action to plasmin. The TNBS, fluorescamine and RP-HPLC methods gave highly correlated results ($R^2 > 0.93$), clearly demonstrating increased proteolysis during storage. Gel electrophoresis revealed that the breakdown products from trypsin were similar to plasmin. The most obvious phenomenon was that γ-caseins, formed as a result of β-casein degradation, subsequently disappeared due to extensive proteolysis in the trypsin samples. Similar trends were found for both the pH 4.6 and 6% TCA soluble extracts. The amido methyl coumarin (AMC) method, which is specific for plasmin activity, was found to be very sensitive in the determination of proteolysis by plasmin, but is also very expensive. On balance, the TNBS method was recommended on the basis of accuracy, reliability, simplicity and cost.

Key Words: proteolysis, plasmin, trypsin

803 Genotyping of κ-casein and β-lactoglobulin genes in Chinese Holstein dairy cows, Jersey and water buffalo. D. X. Ren*[1], S. Y. Miao[1], Y. L. Chen[1], C. X. Zou[2], X. W. Liang[2], and J. X. Liu[1], [1]*Institute of Dairy Science, Zhejiang University*, *Hangzhou 310029, P. R. China*, [2]*Water Buffalo Institute, Chinese Academy of Agricultural Science*, *Nanning 530001, P. R. China*.

The present work is carried out to analyze the polymorphism of κ-casein and β-lactoglobulin genes in Chinese Holstein dairy cows, Jersey and water buffalo. The DNA was extracted from the blood samples of 82 Holstein, 56 Jersey and 48 buffalo. Identification and genotyping of κ-casein and β-lactoglobulin gene was conducted by PCR-RFLP assay. Different restriction endonucleases, *HinfI* and *Hae*III, were used. The PCR product of the primer specific for κ-casein and β-lactoglobulin gave the specific band at size 379, and 252 bp, respectively. After digestion of PCR product with restriction enzymes, different genotypes of κ-casein and β-lactoglobulin were obtained, the fragment size of which is shown in Table 1. Among the examined Holstein cows, κ-casein genotype of AA, BB, and AB was 45.83, 8.34, and 45.83%, and corresponding genotype for β-lactoglobulin was 19.1, 20.6, and 60.3%, respectively. For Jersey, the κ-casein genotype of AA, BB, and AB was 0, 80, and 20%, and the corresponding value was 36.8, 22.8, and 40.4%for β-lactoglobulin, respectively. However, all buffalo samples were homozygous for the κ-casein and β-lactoglobulin, with all genotype as BB. In summary, allele of κ-casein gene was mainly A in Holstein dairy cows and B in Jersey, while β-lactoglobulin was mainly A in both Holstein cows and Jersey. However, water buffalo were monomorphic

for the κ-casein and β-lactoglobulin genes, possessing only allele B in homozygosis form.

Table 1. Fragment size corresponding to different genotypes of κ-casein and β-lactoglobulin after digestion of PCR product with restriction enzymes

Milk protein	Genotypes	Size of fragments from digestion of PCR product with restriction enzymes (bp)
κ-casein	AA	156, 132, 91
	BB	288, 91
	AB	288, 156, 132, 91
β-lactoglobulin	AA	144, 108
	BB	108, 74, 70
	AB	144, 108, 74, 70

Key Words: genetic polymorphism, κ-casein, β-lactoglobulin

804 Impact of plasmin hydrolysis of caseins on the minimum coagulation temperature observed for milk during renneting. B. Coude*, Y. Lu, and J. Lucey, *University of Madison*, *Madison, Wisconsin*.

The rennet coagulation of milk involves 2 different stages. The primary stage is the enzymatic phase in which rennet hydrolyzes κ-casein. The second stage is an aggregation of renneted micelles but this aggregation reaction is highly temperature dependent and does not occur at temperatures < 18°C. It is not clear why the aggregation reaction is so temperature dependent. We believe that at low temperature β-casein may protrude from the surface of casein micelles thereby, creating an additional barrier to aggregation. We hypothesized that removal of β-casein, that is close to the micelle surface, should allow rennet coagulation to occur at lower temperatures. We used plasmin enzyme to hydrolyze β-casein since it is more susceptible to hydrolysis than α-caseins while κ-casein is resistant to breakdown. We studied the impact of plasmin hydrolysis of caseins on the minimum temperature at which the rennet coagulation reaction could occur in milk. Human plasmin (0.01mg/ml) was added to reconstituted skim milk. Milk samples were incubated with plasmin at 37°C for 0.5 to 6 h. Hydrolysis was terminated by adding soybean trypsin inhibitor. The extent of degradation of caseins was determined with SDS-PAGE. Degradation of α- and β-caseins after incubation with plasmin for 0.5 to 6 h ranged for about 10 to 40%, and about 30 to 60%, respectively. Rennet was added to milk at different temperatures: 32, 25, 20, 15, 10 and 5°C. Coagulation was visually determined (Berridge method) as well as monitored by dynamic low amplitude oscillatory rheology. The minimum rennet coagulation temperature for control milk (without plasmin hydrolysis) was ~20°C. Milk that was hydrolyzed with plasmin at 37°C for 0.5 or 1 h could undergo rennet coagulation at ~15°C. Milk that was hydrolyzed with plasmin at 37°C for 2 h and up to 6 h could undergo rennet coagulation at temperatures as low as 5°C. In conclusion, these results support the hypothesis that at low temperature, β-casein on the micelle surface inhibits the aggregation of renneted micelles.

Key Words: plasmin, hydrolysis, rennet coagulation