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MICROBIOLOGICAL AND NEAR INFRARED SPECTROSCOPY ANALYSIS OF BULGARIAN YELLOW CHEESE, CONTAMINATED WITH *LISTERIA MONOCYTOGENES*

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Summary

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A simultaneous microbiological and near infrared spectroscopy (NIRS) analysis of Bulgarian yellow cheese, contaminated with *Listeria monocytogenes* has been performed. The results showed that the storage of samples at room temperature resulted in elimination of a part of microorganisms and their maintenance at a low concentration in one gramme product (10^1–10^3 CFU/g) during the entire experimental period. NIRS was found to be successful in distinguishing between contaminated and non-contaminated samples. A high coefficient of correlation was found out between the near-infrared spectra of samples and the presence of *L. monocytogenes* in Bulgarian yellow cheese. The results of the study allowed classifying properly the samples contaminated and non-contaminated with *L. monocytogenes*.

Key words: *Listeria monocytogenes*, near infrared spectroscopy, yellow cheese

INTRODUCTION

The food safety requirements in the USA in the 1980-ties have implied the so-called zero tolerance with regard to the presence of *L. monocytogenes*. According to this standpoint, all *L. monocytogenes* strains are considered pathogenic. The presence of the microorganism in ready-to-eat foods is accepted as a hazard for consumers. The monitoring has shown that about 5% of ready-to-eat foods were contaminated with *L. monocytogenes* (Hitchins, 1996; Levine *et al*., 2001). Commision regulation EC 2073/2005 requires ready-to-eat products to be free of *L. monocytogenes* by the end of process of production or to contain ≤100 CFU/g at the end of shelf-life (Anonymous, 2005).

The timely detection and species identification of microorganisms in the different foodstuffs allows determination of unwanted alterations. Routine microbiological tests are time-consuming (they require at least 24–48 hours), whereas express immunological and genetic assays are rather expensive.

Naumann *et al*. (1991) have made the first attempts to introduce Fourier transform infrared (FT-IR) spectroscopy in microbiology with the purpose to identify and classify various bacterial species. The

reports about the use of FT-IR spectroscopy in microbiology have used pure bacterial cultures, and the IR spectra of bacterial suspensions, dried on zinc selenide slides or microbiological filtres, were measured.

The classic IR spectroscopy requires a preliminary preparation of samples and their drying, as samples with high water content cannot be analyzed in this spectral range. FT-IR absorption spectroscopy is not able to analyze directly biofluids, bacterial suspensions and meat and milk products with high water content.

This problem could be resolved by using the near infrared region (700–2500 nm) where the absorption of water is considerably lower and thus allows to analyze strongly absorbing materials and samples with high water content without previous chemical preparation, component separation and drying. Also, the samples are not altered during the analysis and could be then assayed by other techniques. The information from spectral data is interpreted by powerful mathematical tools for quantitative and qualitative analysis (Williams & Norris, 2001).

Within the NIR spectrum (700 to 2500 nm) Lin *et al*. (2004; 2006) have investigated the microbial spoilage of poultry meat and rainbow trout fillet and concluded that NIRS could be utilized for rapid detection of microbial contamination. Horvath *et al*. (2008) have detected the changes in pork meat stored at 4, 8 and 12 ^{o}C and established a high correlation coefficient between bacterial counts (CFU/g) and spectral data. According to the results from this study, the bacterial spoilage of meat could be determined prior to the appearance of organoleptic changes.

The purpose of this investigation was to determine the potential of NIRS for detection of *L. monocytogenes* in Bulgarian yellow cheese at a low initial level of contamination and the time course of occurring changes.

MATERIALS AND METHODS

The subject of analysis was Bulgarian yellow cheese. Purchased samples were obligatory vacuum-packed in order to avoid surface contamination in retail stores. The packs were aseptically open.

Samples were contaminated with a reference *Listeria monocytogenes* H3 4B strain, obtained from the Centre of Hygiene, Sofia, Bulgaria. The microorganism was maintained on Triptone Soya Broth Yeast Extract (TSAYE) agar and periodically re-inoculated and stored at 0–4 ^{o}C. Then, a broth culture (Triptone Soya Broth Yeast Extract, TSBYE) was prepared for 18–20 hours at 30 ^{o}C with a concentration of 10^{9} CFU.mL^{-1}. Serial dilutions were made in tubes with 9 mL Maximum Recovery Diluent (MRD) in each. In the first, second and third experiment, dilutions of 10^{5} CFU.mL^{-1}, 10^{4} CFU.mL^{-1} and 10^{3} CFU.mL^{-1} were used. Yellow cheese samples were contaminated with 0.1 mL of the respective dilution, spread on sample surface by a Drigalski spatula. Control samples were processed same way with sterile MRD. After the contamination, the samples were placed in Petri dishes, were vacuumized and incubated in a thermostat at room temperature (18 ^{o}C). Experimental samples were measured at various time intervals. In the first and the third experiment, measurements were performed on post contamination hours 0, 6, 24, 30, 48, 54, 72 and 78, and in the second experiment – at 24-hour intervals from post contamination hours 0 to 192.

The pH of yellow cheese samples was determined in previously prepared extracts of the product with Sartorius pH-metre. In the three experiments, pH values were 5.95; 5.96 and 5.95, respectively.

The microbial counts in 1 mL were determined after inoculations of 0.1 mL of working dilutions in Petri dishes with TSAYE and PALCAM agar. At the same time, after the spectrum determinations of each of control and contaminated samples, microbial counts in 1 g product were determined on PALCAM agar.

The spectral analysis was performed with spectrophotometer InfraAlyzer 450, working in the NIR region and allowing obtaining spectral data on 19 wavelengths within the range from 1445 to 2348 nm. Diffuse reflectance spectra of studied samples were obtained. Prepared samples in Petri dishes were placed into a specially designed device into the spectral instrument. For each sample, diffuse reflectance spectra were obtained for several positions of the Petri dish, and the average was recorded as log (1/R), where R was the sample reflectance. For each measurement, 2 control and 3 contaminated samples were analyzed. A total of 120 samples (48 control and 72 contaminated) were analyzed.

Spectral data were saved in a text file that was then open in Microsoft Excel. Then spectral data were processed with Pirouette 2.0 (Infometrics, Inc., Woodinville, WA, USA), used further for quantitative analysis and classification. The Partial Least Square Regression (PLS) method was used for quantitative analysis, whereas samples were classified on the basis of their spectra by the Soft Independent Modeling of Class Analogy (SIMCA) method. In this method, samples are divided into classes on the basis of some parameter. In this study, samples were divided in two classes: contaminated and non-contaminated as per microbiological testing results. For each class, a model was designed on the basis of spectral data of samples from the respective class and their transformation via principal components analysis.

The potential of used classification method was verified by calculating its specificity and sensitivity. Specificity (Sp) indicates the percentage of correctly found non-contaminated samples and sensitivity (Sn) – the percentage of correctly detected contaminated samples.

$$\mathrm{Sp} = \frac{\text{true negative (TN)}}{\text{true negative (TN)} + \text{false positive (FP)}}$$

$$\mathrm{Sn} = \frac{\text{true positive (TP)}}{\text{true positive (TP)} + \text{false negative (FN)}}$$

RESULTS

Microbiological analysis

In the broth culture used for contamination, the average cell counts in 0.1 mL on PALCAM agar were 3.2×10^4 CFU.mL^{-1} in experiment 1; 3.4×10^3 CFU.mL^{-1} in experiment 2 and 2.1×10^2 CFU.mL^{-1} in experiment 3. The results from the microbiological assay of control and contaminated samples of yellow cheese are shown in Table 1. The average number of living cells in contaminated samples varied between 10 and 14 000 per 1 g product. In 15 contaminated samples from experiment 3, living *L. monocytogenes* cells were not detected.

NIR spectral analysis

The multiple correlation coefficient for *L. monocytogenes* quantitation obtained in PLS regression was 0.96, with standard

Table 1. Results from the microbiological testing of yellow cheese samples

Hour	Non-contaminated, CFU/g	Contaminated, CFU/g
Experiment 1		
0	0	3.1×10^2
6	0	3.6×10^2
24	0	3.3×10^2
30	0	3.4×10^2
48	0	2.5×10^3
54	0	6.2×10^3
72	0	1.2×10^4
78	0	1.4×10^4
Experiment 2		
0	0	1.9×10^2
24	0	2.0×10^2
48	0	$1.8.\times0^2$
72	0	6.0×10^1
120	0	1.4×10^2
144	0	1.1×10^2
168	0	2.0×10^2
192	0	2.3×10^2
Experiment 3		
0	0	0
6	0	1.10^1
24	0	5.10^1
30	0	0
48	0	0
54	0	0
72	0	1.10^1
78	0	0

error of calibration SEC=0.29 log CFU/g and standard error of cross validation SECV=0.36 log CFU/g. Fig. 1 presents the relationship between log CFU/g obtained in the microbiological assay and from spectra of samples.

SIMCA models were developed using 57 contaminated and 63 non-contaminated samples according to results from microbiological analysis. The data from SIMCA classification showed that 49 samples from class ”contaminated” were correctly classified and 8 samples were classified as “non-contaminated”. For class “non-contaminated” samples 47 samples were classified as “non-contaminated” and 16 samples – as “contaminated”. The calculated specificity was 74.60%, and the sensitivity – 85.96%.

The plot of PLS regression vector (Fig. 2) and the parameter “modeling power” from SIMCA procedure (Fig. 3) were used to investigate the NIR spectral information, significant for quantitative or qualitative determination of *L. monocytogenes* contamination.

DISCUSSION

The microbiological analysis of samples showed that after contamination of experimental samples with the respective dilution of the *L. monocytogenes* broth culture, a sharp reduction of microbial counts had occurred. In the third experiment, where the initial bacterial density in 0.1 mL was very low, they practically disappeared. In experiments 1 and 2, microorganisms were consistently detected but their density was low and did not increase considerably despite the temperature of 15 ^{o}C, appropriate for their development. The pH of the product was 5.95 and it had an insignificant effect on microbial reproduction. The elimination and discontinuation of the growth of *Listeria* in some of the contaminated samples was attributed to the presence of lactic acid microflora and its metabolites (bacteriocins) (Zhu *et al.,* 2005). These assumptions are however subject of additional investigations.

The PLS regression equation for quantitative determination of microbial counts in studied samples yielded a high coefficient of correlation, thus evidencing a relationship between NIR spectra of samples and *L. monocytogenes* contamination. The obtained precision of estimation is

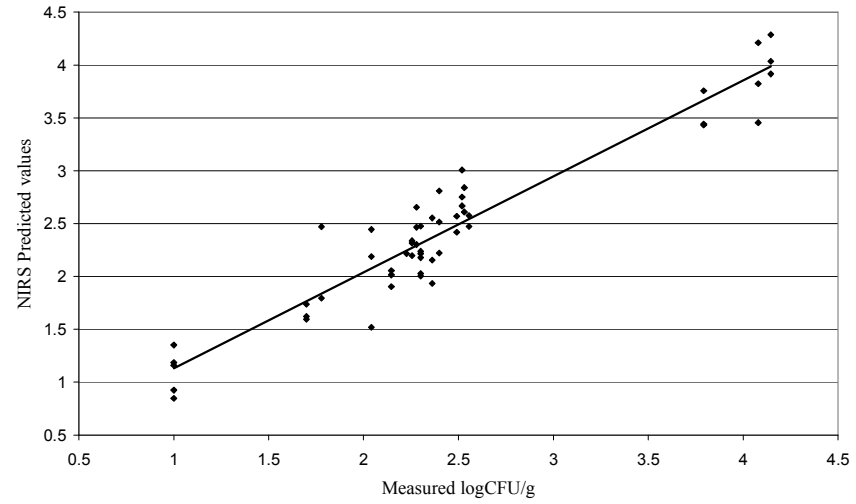

Fig. 1. Correlation of *Listeria monocytogenes* occurrence, determined microbiologically (in log CFU/g) and by means of spectral analysis.

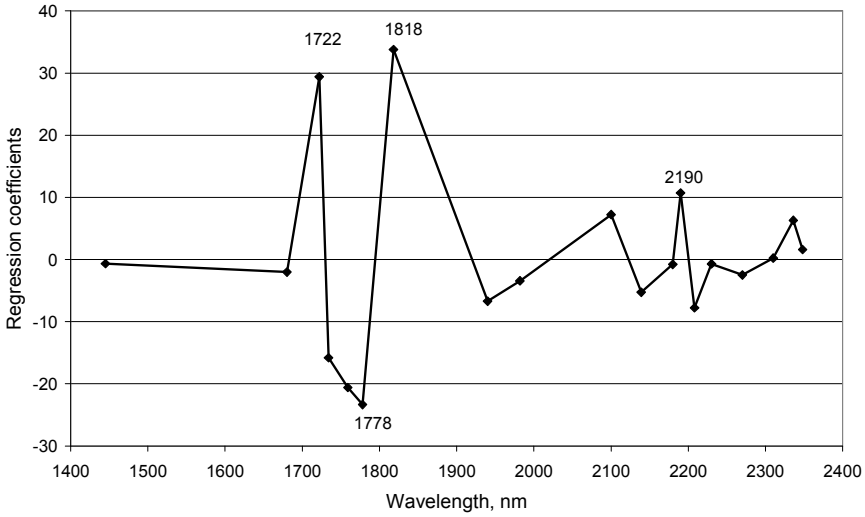

Fig. 2. Graph of the vector regression for quantitation of microbial contamination of Bulgarian yellow cheese samples.

comparable to data reported by Lin *et al.* (2004) in poultry meat, where the correlation coefficient between spectra and the number of microbial colonies was 0.91 with standard error of prediction 0.48 log CFU.g^{-1} and those of Lin *et al.* (2006) for rainbow trout fillet (standard error of prediction 0.38 log CFU.g^{-1} and correlation coefficient 0.97).

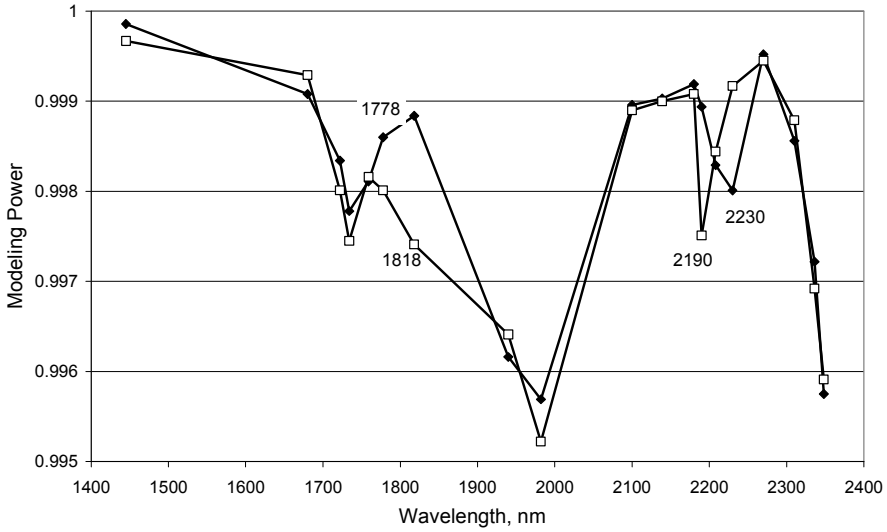

Fig. 3. Graph of SIMCA modeling power for samples belonging to class control (–◆–) and class contaminated (–□–).

The results of SIMCA classification also showed a good precision. One part of non-correctly classified samples were contaminated samples from experiment 3. In 15 samples, there were no living *L. monocytogenes* organisms. The SIMCA model has determined some of these samples as contaminated. These samples were initially contaminated and the microbial metabolism or the residual bacterial biomass was probably responsible for these results.

The analysis of spectral information, utilized in PLS equation for determination of microbial colonies has shown that the highest positive coefficients in the regression equations were observed at wavelengths of 1722, 1818 and 2190 nm, whereas the negative ones have occurred at 1734, 1759, 1778 nm (Fig. 2). The greatest differences in SIMCA models for samples from class contaminated and class control were observed at 1778, 2190 and 2230 nm (Fig. 3). The absorptions at these wavelengths were mainly due to protein and carbohydrate components. Therefore, the development of bacteria in yellow cheese samples has changed them in a specific way and this circumstance was spectrally detectable.

It could be concluded that the observed high coefficient of correlation between NIR spectra of samples and the content of *Listeria monocytogenes* in Bulgarian yellow cheese, permitted to obtain an equation for quantitation of the degree of contamination. The results evidenced also a good precision in the classification of samples as contaminated with or free from *L. monocytogenes*.

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