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# RESEARCHES REGARDING ANTIBIOTICS RESIDUES PRESENCE IN MILK AND MILK PRODUCTS IN CONFORMITY WITH EUROPEAN LEGISLATION

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## Abstract

*Milk and milk products represent the subject for numerous tests regarding public safety assurance and in the same time following standard composition. More, together with our country official entrance in European Community, the major objective is food security improvement and animal health developing in ecologic breeding systems. This paper present the introduction of a new modern analysis method, for screening the possible presence of antibiotics residues in milk and milk products, and also the evaluation of method selectivity and sensitivity, in accordance with ISO EN 17025/2005 stipulations.*
***Keywords:*** *screening method, Charm principle, antibiotic residue*

## Introduction

Milk and milk products are they subject for a lot various tests to accomplish public safety and, in the same time, to respect standard compositions. More, according with our country joint official character to European Community and, for a lot of activities departments, such as agricultural raw materials processing and also, food industry, since 2004, the major objective is improving food security and animal security, also in ecological processes systems for member countries and for aspirants ones. This objective can be attempt through an active opinions exchange and communication, researches results and conclusions, by those who have developed the domain politics, farmers and community interested by ecological developing, and not in the last time, through the consumers (Costin, 1999).

By the point of view ecological breeding systems it have to establish the difference between “inner” quality or “product “ quality and “external” quality or “processing” quality. The certainty for

accomplishing these objectives is sustained by a structured dialogue between farmers, processing people and the Government and, also, by responsibility and respect of each other on production chain.

The direct injection of these agents (antibiotics) in the blood current or, even, orally, to an animal for milk, leads to various content in bactericide and bacteriostatic components secreted milk. These antibiotics effect or even others chemical agents in the milk for cheese production is that of destroying the organisms responsible of cheese acidity, taste and flavour (Dan, 2001).

The problems may occur even the producers are informed for the presence of penicillin and makes use penicillinase. For example, micrococcus and clostridia are sensitive to penicillin, but the enterobacteria aren't yet, so may appear fermentation of soft cheese, as a result of coli forms majority in the milk that was treated with penicillin.

More recently, others natural or synthetic antibiotics and, even they are veterinary controlled substituted the penicillin, the antibiotic concentration may varies from a period to another. Besides the fact that the microorganisms haven't the same susceptibility to penicillin is relevant that different species of microorganisms aren't destroyed by the same antibiotic concentration. In the table 1 are presented the inhibitor concentration of penicillin for few starters used in cheese fabrication (Packard, 1982) and table 2 contains the maxim admitted limits for antibiotic substances group in milk (Walker, 1991; Sorensen, 1992)

The lactic acid production in milk – by bacteria sensitive to penicillin - may be used as test for inhibiting substances; such developing conditions for precautions must be taken to avoid acid production inhibition by bacteriophage infection. A more satisfactory test for these inhibiting substances is that using reducing colouring substances: methylen blue, resazurine or tetrasolium salts that changes colour in the presence of an active microorganisms developing (Dan, 2001).

Taking account of correlation that exists between colour changing and metabolic activity, adding a standard inocul, in standard conditions, should induce a colour change in the same period of time as for a milk batch (Costin, 1999).

**Table 1**. Penicillin limits in milk that inhibit indicated microorganisms

| No. | Microorganism | Inhibitor level of penicillin (I.U.) |
|---|---|---|
| 1. | Lactococcus lactis sub-sp.lactis | 0.1-0.25 |
| 2. | Lactococcus lactis sub-sp.cremoris | 0.05-0.1 |
| 3. | Steptococcus thermophillus | 0.01-0.05 |
| 4. | Enterococcus faecalis | 0.25-0.45 |
| 5. | Lactobacillus bulgaricus | 0.25-0.5 |
| 6. | Lactobacillus casei | 0.25-0.5 |
| 7. | Lactobacillus helveticus | 0.25-0.5 |
| 8. | Propionibacterium shermani | 0.05-0.1 |
| 9. | Starter(mixed culture) | 0.25-0.5 |

**Table 2**. Maxim admitted limits for antibiotic substances group in milk

| Residues types | Limit max. admitted |
|---|---|
| Amoxicyllin | 4 µg/kg |
| Oxacillin | 30 µg /kg |
| Enrofloxacin | 100 µg /kg |
| Erytromycin | 40 µg /kg |
| Spiramycin | 200 µg /kg |
| Tilmycosin | 50 µg /kg |
| Tylosin | 50 µg /kg |
| Thiamphenycol | 50 µg /kg |
| Chlortetracicline | 100 µg /kg |
| Oxitetracicline | 100 µg /kg |
| Tetracicline | 100 µg /kg |
| Dihydrostreptomycin | 200 µg /kg |
| Gentamycin | 100 µg /kg |
| Neomycin | 1500 µg /kg |
| Kanamycin | 150 µg /kg |
| Spectinomycin | 200 µg /kg |
| Streptomycin | 200 µg /kg |
| Novobyocin | 50 µg /kg |
| Bacitracin | 100 µg /kg |

For antimicrobial substances residues analyses were introduced a new and modern method, internationally recognized, RIA CHARM II method This method is a screening one and for this study we used it for antimicrobial substances residues detection in milk and milk

products (Banu, 2003). These new methods subscribed with actual directions concerning legislation alignment and good practice and laboratory normative, in conformity with Community Aquis.

## Experimental

The principle of method consists of imunoenzymatic reaction between a binding agent -that is selected bacteria and possible antibiotic residues from samples (Charm technical specifications). After the reaction was done is added standard antibiotic marked with isotope, $^{3}H$ or $^{14}C$, that may react with others part of binding agent. The result of reactions is replied then by a supplementary reaction with scintillation liquid-Optiflor- that is transformed in different values for isotopes concentration and is compared with results obtained for control point that is specific determination for each antibiotic type, for each lot of reagents.

The method can be applied to residue determination from milk, milk products (pasteurised milk, skimmed milk, cream, cheese, milk with different aroma, whey, condensed milk, powder milk). Each set of samples must be accompanied by a positive and a negative sample.

The equipment needed for this determination consists of:

- Charm II apparatus that makes the final screening;
- an incubator, that allows the initial reaction, between binding agent and possible residues;
- a centrifuge as well as for plastic tubes, 50 ml as for borosilicate tubes (or Charm tubes);
- an agitator, type Vortex;
- a mixer for preparing the samples.

The reagents needed for determinations are supplied as kits and must be used as said in instructions and in the time stipulated on label.

In the final trimester of 2006 year were made a number of determinations on 20 samples of whole milk and pasteurised milk, for screening some of antibiotic substances group, as following: beta-lactamics, macrolides, amphenicols and sulphamides. The milk comes from Bacau, Constanta, Giurgiu and Covasna counties. The samples were verified at reception from the point of view their acidity, because it’s well to know that aren’t proper for determination if the protein

denaturation has began and also, they are centrifuges for eliminate a substantial quantity of fat.

## Results and Discussions

The limits of detection for beta-lactamics in milk are following: amoxicilllin - 5 µg/kg; ampicilline - 4 µg/kg; cephazoline -15 µg/kg; cephtiofur - 40 µg/kg; cephquinome - 20 µg/kg; cephapirin - 3 µg/kg; cloxacillin - 30 µg/kg; dicloxacillin -20 µg/kg; nafcillin -30 µg/kg; oxacillin -30 µg/kg; penethamate - 2 µg/kg; penicillin G - 2 µg/kg; cephadroxil - 15 µg/kg; cephotaxime - 4 µg/kg; cepharadine - 15 µg/kg; cephalexine - 15 µg/kg .

These values help us to select the kits for determination the antibiotic residues, from the point of view of reliability, sensitivity and repeatability.

In the table 3 are the results for beta-lactamics residues determination and only one of the 10 samples is found positive that means it must be verified through a quantitative method. For the determination of residues of beta-lactamics in milk, green tablet was used as substrate, and yellow tablet as beta-lactamics standard.

**Table 3**. Beta-lactamics residues determination in milk samples

| Sample number | Sample adding [ml] | Incubation [temp/time] | Centrifugation [turation/time] | Scintillation liquid [ml] | Result |
|---|---|---|---|---|---|
| 1. | 5.0 | 65°C/2 min | 3300rot/3 min | 3.0 | neg |
| 2. | 5.0 | 65°C/2 min | 3300rot/3 min | 3.0 | **poz** |
| 3. | 5.0 | 65°C/2 min | 3300rot/3 min | 3.0 | neg |
| 4. | 5.0 | 65°C/2 min | 3300rot/3 min | 3.0 | neg |
| 5. | 5.0 | 65°C/2 min | 3300rot/3 min | 3.0 | neg |
| 6. | 5.0 | 65°C/2 min | 3300rot/3 min | 3.0 | neg |
| 7. | 5.0 | 65°C/2 min | 3300rot/3 min | 3.0 | neg |
| 8. | 5.0 | 65°C/2 min | 3300rot/3 min | 3.0 | neg |
| 9. | 5.0 | 65°C/2 min | 3300rot/3 min | 3.0 | neg |
| 10. | 5.0 | 65°C/2 min | 3300rot/3min | 3.0 | neg |

The limits of detection for macrolides in milk are (results presented in table 4): eritromicina - 40 µg/kg; tilozina - 50 µg/kg; spriramicina

- 50 µg/kg; pirlimicina - 80 µg/kg; tilmicozina - 20 µg/kg; lincomicina - 100 µg/kg. To determinate of residues of macrolides in milk, white tablet was used as substrate, and green tablet as macrolide standard. From the table 4 it can be observed two samples found positive that means they have overpasses the maximum residue limit admitted.

**Table 4**. Macrolides residues determination in milk samples

| Sample number | Sample adding [ml] | Incubation [temp/time] | Centrifugation [turation/time] | Scintillation liquid [ml] | Result |
|---|---|---|---|---|---|
| 11. | 5.0 | 65°C/2 min | 3400rot/3 min | 3.0 | neg |
| 12. | 5.0 | 65°C/2 min | 3400rot/3 min | 3.0 | neg |
| 13. | 5.0 | 65°C/2 min | 3400rot/3 min | 3.0 | neg |
| 14. | 5.0 | 65°C/2 min | 3400rot/3 min | 3.0 | **poz** |
| 15. | 5.0 | 65°C/2 min | 3400rot/3 min | 3.0 | neg |
| 16. | 5.0 | 65°C/2 min | 3400rot/3 min | 3.0 | neg |
| 17. | 5.0 | 65°C/2 min | 3400rot/3 min | 3.0 | neg |
| 18. | 5.0 | 65°C/2 min | 3400rot/3 min | 3.0 | **poz** |
| 19. | 5.0 | 65°C/2 min | 3400rot/3 min | 3.0 | neg |
| 20. | 5.0 | 65°C/2 min | 3400rot/3min | 3.0 | neg |

Limits of detection for amphenicols in milk are: florphenicol - 40 µg/kg, thyamphenicol - 50 µg/kg; cloramphenicol glucuronide - 1µg/kg . The detection of amphenicols in milk is tabular presented in table 5. For putting in evidence of amphenicols in milk, white tablet was used as substrate, and black tablet as active charcoal.

Limits of detection for sulphamides in milk (the detection of them in table 6) are following: sulphadiasine - 49 µg/kg; sulphadimetoxine - 40 µg/kg; sulphametasine - 94 µg/kg; sulphatiazol - 73 µg/kg; dapsona - 20 µg/kg; PABA - 30 µg/kg; sulphacetamide - 500 µg/kg; sulphaclorpiridasine - 50 µg/kg; sulphadoxine - 70 µg/kg; sulphamerasine - 50 µg/kg; sulphametizol - 50 µg/kg; sulphametoxazol - 30 µg/kg; sulphametoxipiridazine - 50 µg/kg; sulphapiridine - 100 µg/kg; sulphaquinoxaline - 30 µg/kg; sulphisoxazol - 60 µg/kg. To determinate of residues of sulphamides in milk, white tablet was used as substrate, and pink tablet as sulphamide standard.

As we seen in table 5 and 6, the samples that have been analyzed shows no positive result for amphenicols and a single positive result for sulphamides residues. That could means that it wasn't respected the prescribed time since the latest drug administration until milking or even over passing the prescribed dose.

**Table 5**. Amphenicols residues determination in milk samples

| Sample number | Sample adding [ml] | Incubation [temp/time] | Centrifugation [turation/time] | Scintillation liquid [ml] | Result |
|---|---|---|---|---|---|
| 11. | 1.0 | 50°C/2 min | 3400rot/5min | 3.0 | neg |
| 12. | 1.0 | 50°C/2 min | 3400rot/5 min | 3.0 | neg |
| 13. | 1.0 | 50°C/2 min | 3400rot/5 min | 3.0 | neg |
| 14. | 1.0 | 50°C/2 min | 3400rot/5 min | 3.0 | neg |
| 15. | 1.0 | 50°C/2 min | 3400rot/5 min | 3.0 | neg |
| 16. | 1.0 | 50°C/2 min | 3400rot/5 min | 3.0 | neg |
| 17. | 1.0 | 50°C/2 min | 3400rot/5 min | 3.0 | neg |
| 18. | 1.0 | 50°C/2 min | 3400rot/5 min | 3.0 | neg |
| 19. | 1.0 | 50°C/2 min | 3400rot/5 min | 3.0 | neg |
| 20. | 1.0 | 50°C/2 min | 3400rot/5 min | 3.0 | neg |

**Table 6**. Sulphamides residues determination in milk samples

| Sample number | Sample adding [ml] | Incubation [temp/time] | Centrifugation [turation/time] | Scintillation liquid [ml] | Result |
|---|---|---|---|---|---|
| 1. | 5.0 | 85°C/3 min | 3300rot/3 min | 3.0 | neg |
| 2. | 5.0 | 85°C/3 min | 3300rot/3 min | 3.0 | neg |
| 3. | 5.0 | 85°C/3 min | 3300rot/3 min | 3.0 | neg |
| 4. | 5.0 | 85°C/3 min | 3300rot/3 min | 3.0 | **poz** |
| 5. | 5.0 | 85°C/3 min | 3300rot/3 min | 3.0 | neg |
| 6. | 5.0 | 85°C/3 min | 3300rot/3 min | 3.0 | neg |
| 7. | 5.0 | 85°C/3 min | 3300rot/3 min | 3.0 | neg |
| 8. | 5.0 | 85°C/3 min | 3300rot/3 min | 3.0 | neg |
| 9. | 5.0 | 85°C/3 min | 3300rot/3 min | 3.0 | neg |
| 10. | 5.0 | 85°C/3 min | 3300rot/3min | 3.0 | neg |

## Conclusions

From the results obtained from the milk samples analyzed in the course of the last trimester of 2006, we have found one positive for

beta-lactamics type, two positive for macrolide type and one for sulphamide type. For a quantitative determination the samples found positive must be analysed by Elisa method. Our country integration in European Union normative means also, modern, reliable and safety methods of analyses for rapid and objective decisions in the course of production chain.

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*** Charm.com; *Charm technical specifications*