DETERMINATION OF ANTIBIOTICS IN RAW AND UHT MILK SAMPLES BY THE IMAGE FORMING METHOD OF BIOCRYSTALLIZATION

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ABSTRACT

DETERMINATION OF ANTIBIOTICS IN RAW AND UHT MILK SAMPLES BY THE IMAGE FORMING METHOD OF BIOCRYSTALLIZATION

This study aims to investigate a new technique for the antibiotic residue analysis that can able to detect a wide range of antibiotic residues directly in raw and drinking milk by producing reliable and definitive results. "Biocrystallization method" was selected as a new technique to distinguish the raw and UHT milk samples containing antibiotic residues from the antibiotic free ones. This method is based on the crystallographic phenomenon that occurs after adding ionic substances to an aqueous solution of dihydrate $CuCl_2$ and drying in a constant temperature and relative humidity.

In this study, the raw and UHT milk samples was screened for antibiotic residues using New SNAP* Beta-Lactam (IDEXX Lab. USA) test kits. Then, the chemical properties of milk samples (e.g. fat%, protein%, lactose%, minerals%, SNF%) were determined. At the same time, biocrystallization method were optimized with antibiotic free raw milk samples. Evaluation of biocrystallograms was performed via a panel. The best biocrystallogram images were obtained for 6 ml of milk samples prepared from 0.5 % milk and 5% $CuCl_2.2H_2O$ solution by mixing at a ratio of 3 (milk) to 1 ($CuCl_2.2H_2O$) dried at 30 °C ,60% relative humidity for 22 h.

After optimization, the raw and UHT milk samples were spiked with Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb) to generate biocrystallogram images at the optimized conditions. All the images were evaluated with a visual inspection. Biocrystallization method was successfully used to distinguish raw and UHT milk from the samples spiked with Penicillin G and Ampicillin. This method gave better results when discriminating naturally contaminated raw milk from antibiotic free raw milk.

ÖZET

BİOKRİSTALİZASYON GÖRÜNTÜ OLUŞTURMA METODU İLE ÇİĞ VE UHT SÜT ÖRNEKLERİNDE ANTİBİYOTİKLERİN TESPİT EDİLMESİ

Bu çalışma kalıntı analizinde geniş antibiyotik kalıntı yelpazesine sahip, çiğ ve içme sütlerinde doğrudan ölçüm yapabilen, kesin ve güvenilir sonuç veren, yeni bir tekniğin araştırılmasını hedeflemiştir. "Biyokristalizasyon metodu" çiğ ve içme sütlerinde antibiyotik kalıntılarının belirlemek için yeni bir yöntem olarak seçilmiştir. Bu metod, ionik maddelerin bakır klorür dihidratın sulu çözeltilerine eklenip sabit sıcaklık ve bağıl nemde kurutulduklarında oluşturdukları kristalografik olguya dayanır.

Bu çalışmada, çiğ ve UHT süt örneklerinin antibiyotik kalıntıları New SNAP* Beta-Lactam test kiti ile belirlenmiştir. Süt örneklerinin kimyasal özellikleri (% yağ, % protein, % laktoz, % mineral, % yağsız kuru madde) belirlendikten sonra biyokristalizasyon metodu antibiyotiksiz çiğ süt örnekleri kullanarak optimize edilmiştir. Biyokristalogramların değerlendirilmesi bir panel ile gerçekleştirilmiştir. En iyi biyokristalogram görüntüleri % 0,5'lik süt konsantrasyonu % 5'lik $CuCl_2.2H_2O$ konsantrasyonu ile 3'e 1 oranında 6 ml karışım hazırlanıp 30°C sıcaklıkta, % 60 bağıl nemde 22 saat kurutularak elde edilmiştir.

Optimizasyondan sonra, biyokristalogram görüntüleri, çiğ ve UHT süt örneklerine penisilin g (2, 4, 8 ppb) ve ampisilin (2, 4, 8 ppb) eklenerek optimize edilen koşullarda oluşturulmuş ve görsel olarak analiz edilmiştir. Biyokristalizasyon metodu, çiğ ve UHT sütleri sonradan penisilin ve ampisilin eklenen sütlerden ayırt etmede başarılı olmuştur. Bu metot, antibiyotiksiz sütlerle doğal yolla antibiyotik bulaşmış sütleri ayırmada daha iyi sonuçlar vermiştir.

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CHAPTER 1

INTRODUCTION

Milk is very important food supplying nutritious elements for feeding physiology and health. Some chemical residues cause contamination of milk which result in serious health problems and negatively affect dairy processing. Antibiotics are among those materials that are used for treatment of a cow especially when they suffer from mastitis. Low levels of antibiotics have been also allowed to be utilized in order to increase the rate of weight gain or improve feed efficiency in cattle breeding (Gustafson and Bowen, 1997).

The usage of antibiotics in excess amount may lead to have residues in milk following 12- 96 hours after the injection based on a variety of factors (Santos, et al. 2006). The antibiotic residue, even though it is found in milk at a level higher than its allowable limits (maximum residue limits (MRL)), is degraded by applying heat processes. They may cause allergic reactions in sensitive individuals and their exposure may lead to an increase in the resistant of a number of antibiotics in individuals. In addition, antibiotic interference with starter cultures used for dairy products negatively influences the coagulation process (Rinken and Riik, 2006).

To protect consumers, Maximum Residue Limits (MRLs) for veterinary medicinal products in milk have been established by the EU Council Regulation (EEC) No: 2377/90 (Zvirdauskiene and Salomsskiene, 2007). The residue levels of veterinary drugs in the raw and drinking milk must not exceed the limits stated in the 6^{th} part of Turkish Food Codex Regulation No: 2002/30 (KKGM 2008).

Residue analysis must be periodically used to screen the milk and milk products in the market. It includes both screening and confirmatory methods. Present methods for the detection of antibiotic residues are classified as microbial growth inhibitor tests, microbial receptor assays, enzymatic assays, immunologic assays or receptor-based methods and chemical-physical methods such as spectrophotometric, chromatographic, and fluorimetric methods (Le Breton, et al. 2007). Chromatographic analysis is a confirmatory method which sensitively identifies and quantitifies the presence of antibiotic residues. But this method is time consuming, necessitates complex steps, not

environment-friendly and requires expert people (Rinken and Riik, 2006). That's why, more simple and rapid test kits were developed and started to use for a quick response in dairy industry. On the other hand, in spite of the advantages, there are some drawbacks of rapid tests. They can not quantify the antibiotics present in the milk and they usually do not have wide antibiotic spectrum. They may also give false-positive or false-negative results. False-positive test kit results might lead to unjustified waste of milk and several economic losses. The data on a rate of true false-positive tests or data on how much milk was discarded because of false-positive test results was not been able to found (Kang, et al. 2005).

In our previous study, we explored that the beta-lactam group of antibiotics such as Penicillin G, ampicillin and amoxicillin are commonly used for treatment of dairy cattles (Yıldız, 2008). The origin of the idea of this study based on an interest to develop a new method to detect the existence of antibiotic residues of penicillin G and ampicillin in milk. For this purpose "biocrystallisation" method was investigated. The biocrystallization method, also called sensitive crystallization or copper chloride crystallization, includes determination of the structure of crystallization pattern of a sample i.e. milk. The basic principle of the method is based on the crystallographic phenomenon which is arised from mixing of an aqueous solution of $CuCl_2.2H_2O$ (copper chloride dihydrate) and organic or inorganic substance in a glass petri dish dried at a constant temperature and relative humidity. After drying, the crystallogram composed of reproducible dendritic structures forms in a Petri dish. Any changes in the structure of the organic substrate cause the changes in the dendtritic structure of the crystallagrom. Since this method is very sensitive, in the first step, an optimization study was carried out to generate the best biocrystallogram images. For this purpose milk and $CuCl_2.2H_2O$ concentration, their mixing amount and ratio (milk to $CuCl_2.2H_2O$ concentration), drying temperature, relative humidity and time are investigated for optimization. In the second step, biocrystallogram images using raw and UHT milk samples spiked with penicillin G (2, 4, 8 ppb) and ampicillin (2, 4, 8 ppb) were generated at the optimized conditions and evaluated with a visual inspection and computerized image processing. For the visual inspection, panel composed of trained people was set up for different milk samples.

CHAPTER 2

LITERATURE REVIEW

2.1. Milk Chemistry and Physics

Milk is a liquid which has a complex chemical composition (Table 2.1). Milk contains 88 % of water. The rest of the other part was composed of lactose, fat, protein (mostly casein), minerals and vitamins which their level may vary considerably depending on cow breed and time of lactation (Otter, 2003). Three physical phases including dilute emulsion, a colloidal dispersion, and a solution are observed in milk. The emulsion phase is composed of lipid and aqueous part. The colloidal dispersion is formed from casein micelles and some other proteins, such as lactoferrin (Neville and Jensen, 1995). Lactose, mineral salts and some of the lactalbumin constitute dispersion phase or true solution phase. (Eckles, et al. 1951).

Table 2.1. Chemical composition of bovine milk
(Source: Otter, 2003)

Component	**Concentration (gl^{-1})**
Lactose	36-55
Fat	
Triacyglcerols	36-38
Diacylglycerols	0,1-0,23
Monoacylglycerols	0,006 -0,015
Sterols	0,09 -0,16
Sterol esters	Trace
Unesterified fatty acids	0,04 -0,17
Hydrocarbons	Trace
Phosoholipids	0,08 -0,39
Proteins	30-35
Caseins	24 -28
α_{s1}-Casein	12-15
α_{s2}-Casein	3 -4
β - Casein	9 -11
κ - Casein	2 -4

(cont. on next page)

Table 2.1. (cont.)

Whey	5 -7
β – Lactoglobulin	2 -4
α – Lactalbumin	0,6 -1,7
Bovine serum albumin	0,2 – 0,4
Immunoglobulins	0,5 -1,8
Casein fragments	
γ – Casein	1 -2
Proteose – peptones	0,6 -1,8
Milk fat globule membrane	0,4
Salt	0,7 -0,8
Calcium	1,1 -1,3
Chloride	0,9 -1,1
Iron	0,3 -0,6
Magnesium	0,09 -0,14
Phosphorus	0,9-1,0
Sodium	0,35 -0,9
Potassium	1,1 -1,7

2.1.1. Chemical Properties of Milk

The main compound of milk is water, fat, proteins, lactose (milk sugar) and minerals (salts). In addition, milk includes trace amounts of other substances such as enzymes, pigments, vitamins, phospholipids (substances with fat like properties), and gases. Chemical composition of milk shown in % was tabulated in Table 2.2 (Eckles, et al. 1951).

Table 2.2. Chemical composition of milk
(Source: Eckles, et al. 1951)

Composition	**Percentages**
Water	87,25
Dry Matter	12,75
Fat	3,80
Protein	3,50
Sugar	4,80
Ash	0,65
Total	100

2.1.1.1. Milk Fat

Milk fat is the most valuable component of milk. Milk fat appears as an emulsion of globules dispersed in the milk serum. The number and the sizes of the fat globules show variability according to stage of lactation. They are generally large during the first phase of lactation period.

Milk fat consists of different types of glycerides. Triglycerides containing 1 molecule glycerol and 3 molecule fatty acids typically make up approximately 98% of the total milk fat; other components are consisted of di- and monoglycerides, fatty acids, sterols, carotenoids vitamins (fat-soluble vitamins A, D, E, K) and phospholipids. Saturated fatty acids are in solid form at room temperature, only unsaturated oleic acid is in liquid form at room temperature. More than 400 fatty acids have been detected in milk (Otter, 2003). The volatile fatty acids in milk are myristic, palmitic, stearic oleic,and small amounts of a few others. The nonvolatile fatty acids are butyric, caproic, caprilyc, capric, lauric and small amount of others. The fatty acids are synthesized in the mammary gland. Thus seasonal and dietary variations of animals affect the fatty acids of milk and thus affecting the milk compositions (Eckles, et al. 1951).

2.1.1.2. Proteins of Milk

Proteins are other complex organic substances consist of amino acids (Eckles, et al. 1951). Milk proteins are composed of casein, whey proteins and non-protein fractions (NPN). The protein content of milk is approximately 3% (Eckles, et al. 1951).

Although casein is a yellowish-white granular substance, pure casein has a snow-white colour, odorless and tasteless. It is found in milk together with calcium. and found in colloidal form (Eckles, et al. 1951). The casein constitutes about 80% of milk protein and contains high amounts of essential amino acids including phenylalanine, methionine, leucine, valine, lysine, isoleucine, threonine, tryptophan, histidine (Hui, 1993). Lysine is one of the abundant essential amino acids in milk proteins (Otter, 2003).

The whey proteins are called soluble proteins or milk serum proteins. Their fractions are β-lactoglobulins, α-lactalbumins, bovine serum albumin and

immunoglobulins (Hui, 1993). Lactalbumin is one of the important fractions of whey. Powder form of Lactalbumin is tasteless and consists of carbon, oxygen, hydrogen, nitrogen and a small quantity of sulfur (Eckles, et al. 1951). α- Lactalbumin has a vital role in milk composition. β-lactoglobulin has been known as the allergenicity of milk (Rosenthal, 1991).

2.1.1.3. Lactose

Lactose, a disaccharide consisting of galactose and glucose linked by a β1-4 glycoside bond, is the main carbonhydrate component of milk. It is also called milk sugar and its level varies with the breed of cow, individual factors, udder infection and stage of lactation. Although milk consists of about 4.8 percent of lactose, powdered milk has a 38% of lactose (Eckles, et al. 1951). Lactose is less sweet and less soluble than sucrose. Lactic acid is the metabolite of lactose. It is produced microbiologically in milk and the main source of energy for microbial metabolism (Rosenthal, 1991).

2.1.1.4. Vitamins

Milk is a good source fulfilling the daily vitamin requirement of an adult person. It contains fat-soluble vitamins i.e. A, D, E and K, and water soluble B group vitamins e.g. B_1, B_2, niacin, biotin, panthothenic acid, B_6, folate and B_{12} and vitamin C (ascorbic acid). The amount of each vitamin varies with stage of lactation and diet or health of the animal (Otter, 2003).

2.1.1.5. Minerals

Minerals are considered to be essential for human diet and milk contains 22 different minerals including three types of salt. The first type includes sodium (Na), potassium (K) and chloride (Cl). A second one includes colloidal calcium (Ca), magnesium (Mg), inorganic phosphorus (Pi) and citrate. The third one comprise of diffusible salts of Ca, Mg, citrate and phosphate (Hui, 1993).

2.1.2. Physical Properties of Milk

2.1.2.1. Appearance

The color of milk changing from white to yellow depends on the amount of carotene. Its opaque appearance is caused by suspended particles such as fat and casein micelles. The greenish color of milk serum and whey is due to the presence of riboflavin (Otter, 2003).

2.1.2.2. Density

The specific gravity of milk is 1.021 – 1.037 usually measured at 60 °F (15.5 °C). Composition of milk affects the specific gravity of milk. Constituents of milk have different specific gravity value, e.g. specific gravity of fat, lactose, proteins, casein, and salts are 0.93; 1.666; 1.346; 1.31 and 4.12, respectively. (Eckles, et al. 1951). The density of milk varies between 1.027 g/ml – 1.035 g/ml at 20 °C and may change based on the composition i.e. decreases with increasing fat content and increases with increasing protein, lactose and mineral content. Rising the temperature causes declining of the density of milk (Metin, 2001).

2.1.2.3. Acidity

Acidity, one of the most important parameters, controls the quality and processing of milk. Milk acts as a buffer. This buffer is a chemical system. It resists changes in the concentration of hydrogen ions under internal and external influences (Rosenthal, 1991). Fresh bovine milk has no lactic acid. Mostly, the titratable acidity is because of the casein and phosphates. Lactic acid can be produced by bacterial contamination (Neville and Jensen, 1995). For this purpose titratable acidity is measured and reported according to amount of alkali required to bring the pH to neutrality using phenolphthalein indicator. This property can be used to determine bacterial growth during fermentations, such as during cheese making, as well as detecting the compliance of cleanliness according to adopted standards.

Milk is usually slightly acidic, with a pH value between 6.5 and 6.7. But if the pH value of cow milk is measured to be above 6.8, it may point out the mastitis disease or a neutralized substance added in milk. If pH value is smaller than 6.5, it means colostrum may be present or bacterial growth or spoilage may be occurred in milk.

2.2. Antibiotics

Antibiotic is a molecule that stops the growth of microbes (both bacteria and fungi) or kills them (Al-Jabri, 2005). Generally, farmers and veterinarians have used antibiotics to control and treat the infectious diseases of dairy cattle and enhance the growth performance of animals. When the antibiotics are used at low levels, they increase the rate of weight gain and improve feed efficiency in cattle breeding (Yıldız, 2008).

Mastitis is a mammary gland infection caused by bacteria. Treatment of this disease can be done with implementation of antibiotics applied as orally, infusion into the udder directly and intravenous injection. Failure usage of antibiotics may result in unacceptable residues in milk (Albright, et al. 1961).

Antibiotic residues are undesirable in milk and milk products from public health point of view and because of their potential impact on manufacturing process (Ruegg and Tabone, 2000; Yamaki, et al. 2004). The presence of antibiotic residues in milk was considered primarily a manufacturing problem related to inhibition of dairy starter microorganisms and cause economic losses in cheese and fermented milk industries (Al-Jabri, 2005; Kang'ethe, et al. 2005).

The presence of antibiotics in milk has been prohibited, due to the fact that they are sometimes associated with adverse effects on host which comprise hypersensitivity, depletion of beneficial gut and mucosal microorganisms, immunosuppression and allergic reactions (Al-Jabri, 2005). Even very small concentrations of Penicillin found in milk may cause allergic reactions causing skin rashes, asthma, anaphylactic shock and even death in highly sensitive individuals (Albright, et al. 1961; Chenh Chen and Chain Chang, 1994; Yamani, et al. 1999).

Furthermore, any exposure of the intestinal micro flora of humans to antibiotics may lead to increase their resistivity to antibiotics (Yamani, et al. 1999; Adesiyun and Webb, 1997). Some antibiotics are directly toxic, e.g. chroramphenicol which destroys

blood-forming cells. Allergic reactions and toxic side effects may have fatal results (Hall, et al. 2003).

Dairy manufacturing companies those that produce fermented milk products are directly affected by the presence of antibiotics in milk. Especially cheese production depends on lactate fermentation. When the starter culture is inoculated into milk which contains traces of antibiotic residues, suitable or active cultures cannot be maintained. Such contaminated milk constitutes a great economic risk. Producer is required to follow withholding recommendations. If milk which contains antibiotic residues is used in the manufacturing of dairy products, it will contaminate these products too. For example when the milk is dried, evaporated, or made into ice cream, the antibiotics will be found in more concentrated form in these products. Despite the fact that no manufacturing problems result from the presence of antibiotics in the above-mentioned products, it is obvious that the consumption of these will create health problems in sensitive individuals (Albright, et al. 1961).

2.2.1. Regularity Control of Antibiotic Residues in Milk

Milk producers must guarantee their milk product are not contaminated by any veterinary drugs found in the list of banned antimicrobials or the level of these materials are not exceeding the Maximum Residue Limits (MRLs) (Zvirdauskiene and Salomsskiene 2006). MRLs mean that the drug may be safely used without harming the consumer (Hall, et al. 2003).

Maximum Residue Limits (MRLs) and Acceptable Daily Intake (ADI) values are two interpretations of residues in food. The MRL is the maximum concentration of a residue, expressed as mg per kg food, legally permitted in or on food commodities and animal feeds. The Acceptable Daily Intake (ADI) value is an estimate of the amount of residue, expressed as mg per kg body weight ingested daily over a lifetime without significant health risk. The ADI is based on a toxicological evaluation for a range of many criteria tested on animals and contains safety factors to account for inter-species differences (normally x 10) and differences between humans (normally x 10), such as vulnerable (sick) individuals, infants, elderly, etc (O'Keeffe and Kennedy, 1998).

The Codex Alimentarius Commission of the Food and Agriculture Organization (FAO) and World Health Organization (WHO) of the United Nations stated in 2001 that

the scientific literature about the impacts of processing on drug residues in milk is insufficient to permit clear determination of the effect. Additional studies are required in this area (Yıldız, 2008). The EU Maximum Residue Limits (MRLs) for veterinary medicinal products in milk were established by Council Regulation (EEC) No.2377/90 (Zvirdauskiene and Salomsskiene, 2006). The residue levels of veterinary drugs in the raw and drinking milk must not exceed the limits stated in the 6th part of Turkish Food Codex Regulation No:2000/6 (ABGS 2008), (Table 2.3).

Table 2.3. MRLs at EU Codex and at Turkish Food Codex
(Source: Copan Sciences, 2008)

Antimicrobial Agents	EU/ Codex MRL[1] (ppb)	Turkish Food Codex MRL (ppb)
Beta Lactams		
Penicillin G	4	4
Ampicillin	4	4
Amoxicillin	4	4
Cloxacillin	30	30
Dicloxacillin	30	30
Oxacillin	30	30
Naficillin	30	30
Ceftiofur[2]	100[3]	100
Cefquinom[7]	20	20
Cefapirin	10	10
Cefoperazon	50	50
Cefalexin	100	100
Cefazolin	50	50
Tetracyclines		
Chloetetracycline[2]	100[4]	100
Oxytetracycline[2]	100[4]	100
Tetracycline[2]	100[4]	100
Doxycycline[2]	100[4]	100
Sulphonamides		
Sulfathiazole	100[6]	100
Sulfamethazine[5]	100[6]	100

(cont. on next page)

Table 2.3. (cont.)

Sulfadimethoxin	100[6]	100
Sulfadiazin	100[6]	100
Sulfamonometossina	100[6]	100
Aminoglycosides		
DH-Streptomycin	200	200
Streptomycin	200	200
Neomycin	500	500
Gentamicin	100	100
Spectinomycin	200	200
Macrolides		
Erythromycin	40	40
Spiramycin	200	200
Tylosin	50	50
Tylmicosin	50	50
Other antibiotics		
Dapson	0[7]	0
Trimethoprim	50	50
Tiamfenicol	50	50
Chlorampheniol	0[7]	0

1. Regulation 2377/90 ff EEC, 2. Mother compound , 3. Mother compound and metabolites
4. Mother compound and 4-epimer, 5. Sulfadimidine, 6. Sum of all substance of this group, 7. Not allowed

2.2.2. Classes of Antibiotics

Veterinary drugs are classified as sulphonamides, beta-lactams (e.g. penicillin), tetracyclines, aminoglycosides (e.g. streptomycin), macrolids (e.g. erythromycin), peptide antibiotics (e.g.virginiamycin) and ionophores (e.g. monensin) (O'Keeffe and Kennedy, 1998).

A research conducted in the years between 1997 to 2003 shows that beta-lactams (combined total beta-lactams and cloxacillin) are most widely detected antibiotics in milk. Figure 2.1 depicts that Tetracyclines and gentamycin/neomycin- type aminoglycosides are the second and third group of antibiotics found in cows milk (Hall, et al. 2003).

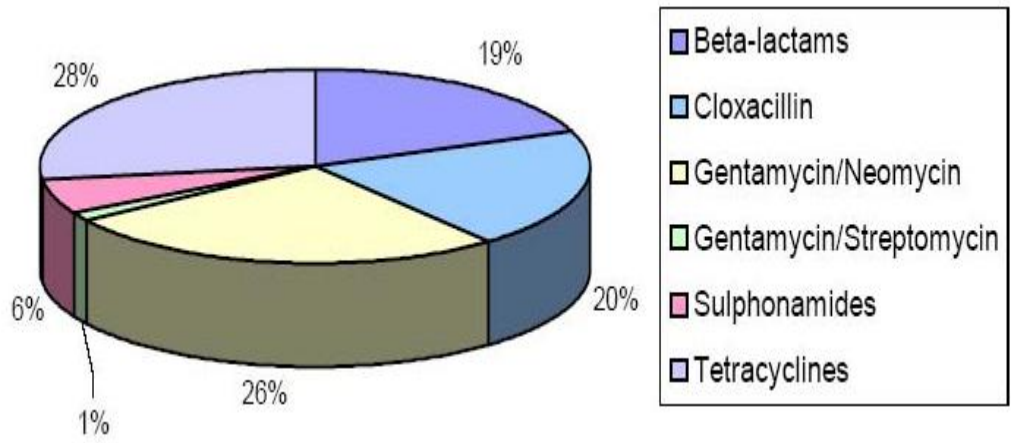


Figure 2.1. Antibiotic residues detected in milk 1997-2003 by percentage (Source: Hall, et al. 2003)

2.2.2.1. Beta-Lactam Group of Antibiotics

The beta-lactam group of antibiotics (namely Penicilin-G, Amoxicillin, Ampicillin and Cloxacillin), are extensively used for treatment of bacterial infections. They are the preferred drugs for the treatment of clinical mastitis in dairy cows. This group comprises the major source of antibiotic residues in milk (Lamar and Petz, 2007).

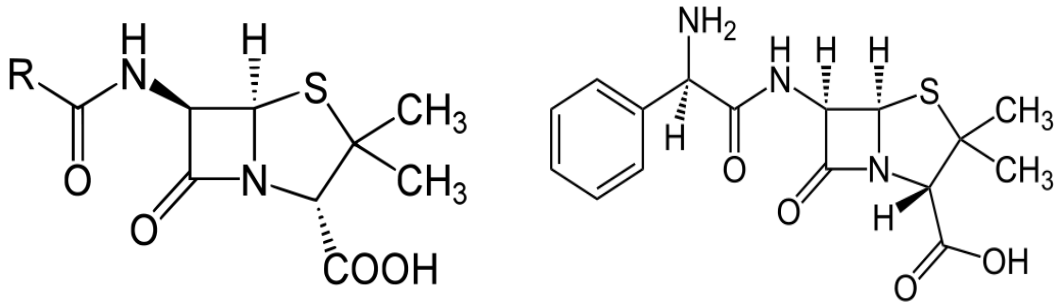
The presence of beta-lactam residues in food may causes allergic reactions in sensitive individuals. The beta- lactam ring system containing a highly strained and reactive cyclic amide is a feature of these antibiotics. The beta lactam ring makes them susceptible to degradation processes. Beta lactam ring is opened by means of reaction with hydroxide ions to produce an inactive compound. Also, beta-lactam has sensitivity for acids and degrades at low pH by means of a more complex mechanism. Therefore alcoholic solutions of these antibiotics are unstable because of the acidic character of alcohols (Santos, et al. 2006).

Penicillins are a member of beta-lactam group of antibiotics and cause inhibition of bacterial cell wall synthesis. They show high sensitivity for heat, acids and penicillinases. The degradation of penicillin is affected by a variety of factors such as temperature, pH, ionic strength, metal ions, degree of crystallization and solvent composition (Michnik, et al. 2004).

Moreover, penicillins are one of the oldest groups of antibiotics used extensively in clinical treatments of bovine mastitis disease which causes economic loss of about 1

billion € per year according to estimate of the German Veterinary Society. Penicilins are not intrinsically very toxic. But they can cause strong allergic reactions in sensitive humans and the usages at concentrations over the MRL inhibit the growth of bacteria used in the fermentation processes in dairy industry (Grunwald and Petz, 2003).

The name "penicillin" can also be used in reference to a specific member of the penicillin group. All penicillins possess the basic Penam Skeleton, which has the molecular formula R-C9H11N2O4S, where R is a variable side chain (Figure 2.2), (Ashnagar and Gharib, 2007).



a) Penicillin G b) Ampicillin

Figure 2.2. Structure of some beta-lactams, Penicillin G (a) and Ampicillin (b) (Source: Kennedy, et al. 1998)

Ampicillin is a beta-lactam antibiotic that has been used widely for treatment of bacterial infections since 1961. It can sometimes cause allergic reactions in the same way like penicillin. Ampicillin, belonging to the group of beta-lactam antibiotics, is able to penetrate gram-positive and some gram-negative bacteria (Ashnagar and Gharib, 2007).

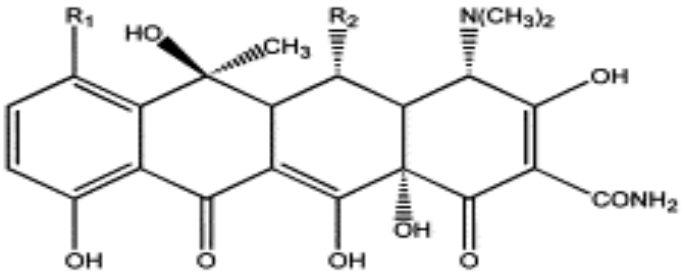
2.2.2.2. Tetracyclines

Tetracyclines, chloetetracycline, doxycycline and oxytetracycline are broad-spectrum of antibiotics used for the treatment of mastitis and added to cattle feeds to increase growth rate more than 50 years (Santos, et al. 2006).

Tetracycline residues in milk may stimulate harmful effects on humans, such as allergic symptoms, liver damage, yellowing of teeth, and gastrointestinal disturbance because of their selectivity on human gut micro flora or may lead to financial losses in the dairy industry by inhibiting starter cultures in food technology processes (Reid, et al.

2006). Moreover, trace amounts of antibiotic residues in milk favor the development of antibiotic-resistant bacteria (Fritz and Zuo, 2007).

Figure 2.3 shows the chemical structures of tetracycline. However not only the concentration of tetracycline residues but also their degradation products in animal fluids and tissues have important potential effects in human and animal health (Fritz and Zuo, 2007).



Compound	R_1	R_2
Oxytetracycline	H	OH
Tetracycline	H	H
Chlortetracycline	Cl	H

Figure 2.3. Structure of tetracycline
(Source: Kennedy, et al. 1998)

2.2.3. Identification and Quantification of Antibiotic Residues in Milk

Determination of antibiotic residues in food is vital due to assurance of the quality and safety of the foodstuff. Generally, antibiotic residue analysis contains either screening methods or confirmatory methods.

For screening, several commercial test kits are available on the market for antibiotic residue detection in milk. The screening methods are inhibitory tests, receptor assay or immunoassays and confirmation methods, such as chromatography with UV, fluorescence or mass spectrometry (Le Breton, et al. 2006; Setford, et al. 1999). Rapid screening tests are commercially used to detect the presence of antibiotic residues in milk but they can not be used to determine the quantity of antibiotics present in the milk. Additionally the outcomes of many of these tests may lead to false-positive or false-negative results. For instance, the presence of high somatic cell counts causes false positive results of these test kits. False-positive test kit results might cause waste of milk

and several economic losses. In contrast to chromatographic methods, they may detect antibiotic residues at levels far below the safe levels (Schenck and Callery, 1998). But false-positive results create negative image among consumers, producers, veterinarians and regulatory personnel (Coffman, et al. 1999).

The government regulatory agencies accept chromatographic methods as official methods in determination and confirmation of identity and quantity of antibiotic residue present in foodstuff. .The most widely used chromatographic methods are carried out by liquid chromatography (LC) and gas chromatography (GC) and they are more sensitive and specific than rapid screening methods (Schenck and Callery, 1998). On the other hand these methods are costly in time and necessitate equipments and specific chemicals for the full procedure and the methodology for confirmatory analysis. Moreover, they require trained personnel with high expertise.

2.2.3.1. Rapid Screening Methods for Antibiotic Residues

The rapid tests were designed to be completed in a short time and simple to be used. For this reason, several different screening methods are available for detection antibiotic residues in milk. These methods are inhibitory tests, receptor assay or immunoassay tests. The most widely used tests are microbial inhibitor tests where the specific microorganisms were utilized as inhibited agents. Delvotest SP (DSM, Netherlands), Copan Test (Copan, Italy), Charm Farm-960 Test (Charm Sciences, Inc., USA); with *Streptococcus thermophilus*-Valio T 101-test, Valio T 102-test (Valio, Finland); enzymatic tests- Penzyme, Penzyme S (UCB Bioproducts, Belgium); immunological tests- Delvo-X-Press β-Lactam (DSM, Netherlands), Snap (IDEXX lab, Inc, USA), ROSA test (Charm Sciences, Inc.,USA) are counted among these commercial rapid tests. The brief scheme of the inhibitor tests is presented in Table 2.4.

The rapid tests were evaluated in terms of the test procedures, the shelf life of the test, ability for use at laboratories and other features of the tests. These features of tests were established by EN ISO 13969:2004 [(Milk and milk products-Guidelines for a standardized description of microbial inhibitor tests (ISO 13969:2003)] and EN ISO 18330:2004 [(Milk and milk products-Guidelines for the description of immunoassays or receptor assays for the detection of antimicrobial residues (ISO 18330:2003)]. The short and brief evaluations of some of these tests are displayed on Table 2.5.

Table 2.4. Milk tests for determining for antibiotic residues
(Source: Zvirdauskiene and Salomskiene, 2007)

Type of test	**Test**	**Producer**	**Principle of method**	**Inhibitor detected and sensitivity**
Microbial inhibitor test	Delvotest SP	DSM, Netherlands	Microbiological method with *Bacillus stearothermophilus* var. *colidolactics* C953 spores	Penicillin G 0,003-0,004IU/ml, Ampicillin 0,003-0,004, sulfamethazine 0,1-0,2µg/ml and others
	LPT	State Laboratory For Milk Control Lithuania	Microbiological method with *B. stearothermophilus* var. *colidolactics* C953 spores	Penicillin G 0,004±0,001IU/ml, sulfamethazine not <1µg, dapsone not < 0,003µl/ml
	Mal-1	KTU Food Ins. Lithuania	Microbiological method with *B. stearothermophilus* var. *colidolactics* C953 spores	Penicillin G 0,004±0,001IU/ml, sulfamethazine not <1µg, dapsone not < 0,003µl/ml
	Copan Single Test P&S 100	Copan, Italy	Microbiological method with *B. stearothermophilus* var. *colidolactics* C953 spores	Penicillin G 0,002±0,001IU/ml, Sulfamethazine 0,15±0,05µg/ml, dapsone 0,003±0,001µl/ml and others
	Valio T 101 test	Valio, Finland	Microbiological method with *Streptococcus thermophilus*	Penicillin G 0,004±0,001IU/ml, tetracycline-more than 0,2±0,1µg/ml, sulfamethazine 1-0,5µg/ml and others
Rapid tests	Delvo-X-Press β-II	DSM, Netherlands	Receptor- enzyme assay	Penicillin G 0,002 µg/ml, ampicillin 0,004 µg/ml, amoxicillin 0,004 µg/ml and others
	SNAP test	IDEXX Lab. Ins.USA	Enzyme immunoassay	Penicillin G 0,004 µg/ml, ampicillin 0,004 µg/ml, amoxicillin 0,004 µg/ml and others
	Rosa test	Charm Sci. Inc. USA	Receptor assay	Penicillin G 0,004 µg/ml, and others
	Penzyme S	UCB Bioproducts Belgium	Enzymatic method	Penicillin G 0,005-0,006 IU/ml

Table 2.5. Comparison of different tests according to usage
(Source: Zvirdauskiene and Salomskiene, 2007)

Type of test	Test	Incubation temperature	Incubation time	Notes
Microbial inhibitor test	LPT	63.5°C±0.5°C	4h 15 min-4h 30 min	One multiple for 96 samples. Duration of test is long but test is sensitive for many groups of inhibitory substance. Short shelf life 5 days from the date of manufacture. Suitable for screening of milk in a big laboratory
	Mal-1	63.5°C±0.5°C	4h 15 min-4h 30 min	One test tube for 1 sample. Duration of test is long but test is sensitive for many groups of antibacterial substances. Shelf life 3 months from the date of manufacture. Suitable for single samples.
	Copan	64.5°C±0.5°C	3 h	Test is sensitive for some groups of antibacterial substance but for the smaller number of them than LPT and MaI-1. It is simple to use and read the result. Suitable for single samples. Shelf life 12 months
	Valio T 101	42°C±1°C	4h 30 min	It is necessary to heat milk for 5 min at 92°C ± 2 °C before testing. The heating takes an additional time
Rapid tests	SNAP	45°C±5°C	10 min	It is important to press the activator at the proper moment. The test is appropriate for a small number (2-4) of samples. It can be difficult to read the results because of the similarity of the control and test sample spots.
	Penzyme S	47°C±0,5°C	25 min.	Reagent No.1 is colourless so after adding 10 µl of it into eppendorf type vial it is difficult to catch sight of it in the vial. It takes time to divide the tablets of Reagent 2 into the vials. The reading of results should be performed quickly. This method is not suitable for testing a large number of samples (>10) at once.
	ROSA	56°C±1°C	8 min	The use of ROSA reader is recommended because, without it, it can be difficult to determine which strip (test or control) is more intense.

2.2.3.2. Chromatographic Methods

The high performance liquid chromatography (HPLC) has been widely used for screening and confirming of antibiotic residues in milk. It detects residues by means of a detector. Choice of the detection system is very vital for selectivity and sensitivity of screening. The screening of residues follows a solid-phase extraction clean-up step then filtration and finally injection step into reverse-phase HPLC combined with UV diode array detection. The main advantages and drawbacks of HPLC are compared in Table 2.6. (Toldra and Reig, 2006).

Table 2.6. Main advantages and drawbacks of HPLC
(Source: Toldra and Reig, 2006)

Advantages	**Drawbacks**
Short time to analyze	Expertise required
Sensitive	Need for sample preparation (extraction, filtration)
Automatisation of leading higher productivity (injection, elution, washing of column, detection)	High initial equipment
Possibility to find more information from spectra when using diode array detector	Cost of column

Liquid chromatography and gas chromatography are other confirmatory methods. They coupled to mass spectrometry (LC/MS and GC/MS). They are highly specific and need complex equipment and well-qualified laboratory personnel (Okerman, et al. 2003). A clean-up is required before the chromatographic analysis of antibiotics in milk. Antibiotics are typically polar constitutes and extracted in polar organic solvents. Precipitation of the milk proteins is necessary step for determination of antibiotics in milk (Schenk and Callery, 1998). The requirement of chemo-metrics analysis is the main disadvantage of these two methods (Reid, et al. 2006).

2.3. Somatic Cells in Milk

Monitoring microbial and somatic cells (white blood cells) is essential for assurance of the safety of milk and dairy products. The number of somatic cells is an important indicator for detection of mastitis (Kehrli, et al. 1994; Dosogne, et al. 2003). In addition to this, it is a useful method when it is run together with rapid screening methods to reduce false-positive outcomes of antibiotic residue tests (Kang, et al. 2005).

All milk contains white blood cells known as leukocytes which constitute the majority of somatic cells. These cells consist of neutrophils, lymphocytes and macrophages. Macrophages comprise the major cell type in milk obtained from uninfected cow (Hamed, et al. 2008). The cell count for "normal" milk is nearly always less than 200,000 cells/ml (lower for first lactation cows). Higher counts are considered abnormal and indicate probable infection e.g mastitis caused by a pathogenic bacteria like *Staphylococcus aureus*, tissue damage or other inflammation processes affecting the mammary tissue (Lindmark, et al. 2006 and Rysanek, et al. 2001). The increase in somatic cell count causes to transfer white blood cells from blood to mammary gland. (Hamed, et al. 2008). As a result, the level of neutrophils present in milk increases significantly to fight with disease and to repair the damaged tissue. Their main function is to protect the udder from bacterial infections. During the mastitis, neutrophils are transported from the peripheral blood system into milk via the mammary epithelium in response to chemotactic stimuli produced locally as a reaction against microorganisms.

Currently, the enumeration of somatic cells relies on two methods including direct microscopic counting and automatic counting such as flow cytometry in raw milk (Gunsakera, et al. 2003). Direct microscopy is a time consuming method. It does not provide complete quality and safety assurance and the instrumentation is limited in its range of application (Vasvada, 1993).Variations in the results related with the skill level of an operator can be another drawback of this method. Discrepancy in the results is also caused by lack of specificity between cells and cytoplasmic particles (Gonzalo, et al. 2003).

There are some automated somatic cells counting (SCC) systems developed based on a staining of the sample and direct microscopic counting. Flow cytometry offers an automatic and objective counting of somatic cells in milk samples. It minimizes the discrepancy of the results via its highly-sensitive property (Feng and

Zheng, 2004). It can also give information about viability of cells. Automation of this process means that large number of samples can be analyzed per hour in milk-testing laboratories (Gonzalo, et al. 2004).

2.3.1. Somatic Cell Count (SCC) Methods

2.3.1.1. Flow Cytometry

Flow cyometer can be used for enumeration of somatic cells in raw milk. Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from cells. It works with a combination of fluorescent stains or fluorogenic substrates and the sample pumped through a flow cell of very small diameter which allows only one cell to pass at a time (Feng and Zheng, 2004; Gonzola, et al. 2004**)**.

Somatic cells are counted according to the number of their nucleus DNA in flow cytometer. DNA is stained with fluorescent dyes such as acridine orange (AO), ethidium bromide (EtBr) or propidium iodide (PI) or syto 13 (Gunesekera, et al. 2003; Wallen, et al., 1982). According to dye property, different pre-processing procedures can be applied. For instance, EtBr and PI can penetrate only DNA of dead cells. In order to evaluate the live and dead somatic cells together, it is necessary to create pore on the cell wall by using a detergent with a salt solution. On the other hand, AO can penetrate into both live and dead cells; only salt solution can be used for AO to penetrate into DNA (Sierra, et al. 2006; Wallen, et al. 1982).

Stained cells pass individually in flow cytometer which is composed of many parts including fluidics, optics, detectors, and electronics. In fluidic part, when a cell passes through the laser beam, forward-scattered light (FSC) measure the surface area or size of cell, side- scattered light (SSC) measure the granularity or internal complexity of a cell. Light emitted from the interaction between the cell particle and the laser beam is collected by a lens in optic part. Specified wavelengths are then routed to optical detectors. These detectors separate the emission wavelength of fluorochrome from other confounding light. In electronic part, light analog signals are converted digital values via a graphic drawn on the computer. The data can be exhibited on 1, 2 or 3 dimensional

plot format via Fax Diva Version; 5.0.3 software (FACSCANTO BD) (Figure 2.3) (Shapiro, 2003).

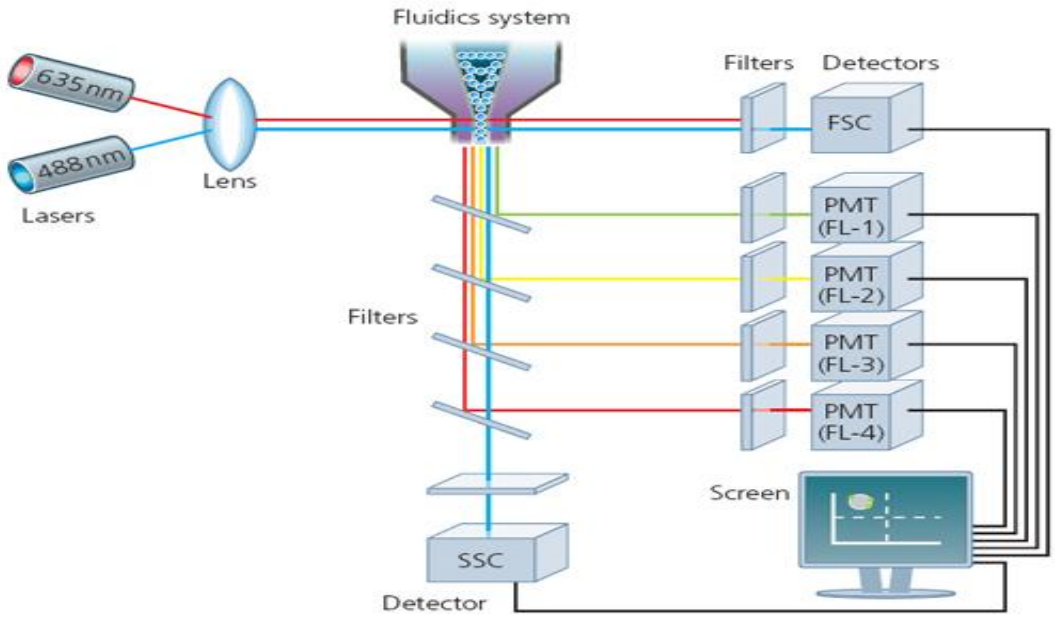


Figure 2.4. General view of flow cytometry instrumentation
(Source: Shapiro, 2003)

Flow cytometry allows the enumeration of large number of somatic cells in a certain time and eliminates variations in the results arising from operator skills. Being expensive, requiring an expertise and lacking of a standard method for staining the cells are the drawbacks of this method.

2.3.1.2. Direct Microscopy

Direct microscopic counting is known as a conventional and culture method to detected and enumerate the somatic cells or microbial contamination. Currently direct microscopic counting is recommended as a confirmation method to control the results obtained through an automated somatic cell counter like flow cytometry. This well-known method is based on staining milk with trypan blue, spreading uniformly as a thin film onto a foursquare area marked on the surface of a piece of microscope slide and examining under a light microscope (Gonzola, et al. 2004).

However this method is time consuming does not provide complete quality and safety assurance (Vasvada, 1993), or the instrumentation is limited in its range of application. The variations in the results of SCC caused by the skills level of operator can be one of the drawbacks of this method. The discrepancy in the results is also induced not to specify between cells and cytoplasmic particles (Gonzalo, et al. 2003).

2.4. Biocrystallization

2.4.1. Biocrystallization Method

The biocrystallization method, also named “sensitive crystallization” or “copper chloride crystallization”, was originally introduced by E. Pfeiffer in the 1930’ies. It is based on the crystallographic phenomenon that occurs after adding specific inorganic ionic or organic substances to an aqueous solution of dihydrate copper chloride. Hereby crystallization pictures with reproducible textures are formed during crystallization. The method has been applied for examining the effects of different farming systems, fertilization practices and processing on the pictomorphological properties of agricultural and horticultural samples (Engqvist, 1989; Balzer-Graf, 1996; Weibel, et al. 2000).

Despite the fact that crystallograms produced using pure $CuCl_2$ exhibit a merely peripherical distribution of crystals on the circular glass surface (Figure 2.5.a), biocrystallograms produced from biological substances, such as plant extracts, fruits, vegetables and milk, display crystal structures covering the whole of the glass underlay exhibiting a variety of macro and microscopical morphological features reflecting the specific admixed substances (Figure 2.5. b,c).

a) copper chloride solution b) wheat sample c) carrot sample

Figure 2.5. The crystallogram image of copper chloride (a), biocrystallogram images of wheat sample (b), carrot sample (c) (Source : Meelursarn, 2007).

The phenomenon of biocrystallograms is based on ramification structure. The ramification structure extending from a center and developing in all directions until reaching to the periphery of the image can be divided into three major stages (Figure 2.6). In the initial or 1-zonal biocrystallogram, the transparent needles in star-like formations are extending in all directions to the periphery by increasing concentrations of biological substances with fixed concentration of $CuCl_2$. The second, 2-zonal structure, the needles are pointed predominantly on the vertical and horizontal axis going through the crystallization center, transparent and relatively equal length in the middle zone. These morphological features can be described via plant morphological terms, such as stems, branches and needles. The last stage of the biocrystallogram is divided into a 3-zonal structure: the central zone around the crystallization centre, the median zone containing the major ramification structure, and marginal zone. In the third stage the biocrystallogram exhibits various macro and microscopic morphological features which reflect the quality of sample in question. (Andersen, 2001; Engqvist, 1970). The location of the crystallisation centre generally does not equal to the geometrical centre. All the biological or agricultural substances have unique biocrsytallogram patterns with changing centre co-ordination, distribution of branches and variety of needles. Neuhaus (1957) indicated that among numerous single organic compounds examined, proteins and N-containing compounds exhibited unique abilities to co-ordinate the crystal structures (Neuhaus, 1957).

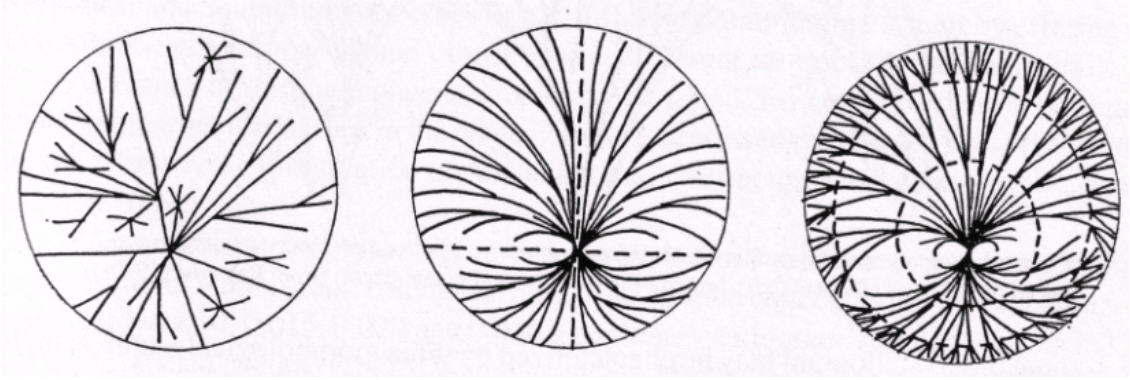


Figure 2.6. The phenomena of biocrystallogram image, divide into 3 stages of image on the basis of increasing concentration of additives at the giving amount of $CuCl_2$. On the left side are 1-zonal structure; 2- zonal structure (middle) and 3 - zonal structure on the right side (Source: Andersen, 2001).

2.4.2. Evaluation of Biocrystallization

The quality of the products can be assessed by evaluating biocrystallogram pictures employing both visual and computerized analysis. The visual assessment method is evaluating and interpreting the pictures visually by trained people according to ISO – Norms (Huber, et al. 2010). Meanwhile, the computerized image analysis is applied to the texture of images for discrimination purposes. Image processing is widely used in remote sensing, medical diagnostic applications and food quality evaluations etc.

2.4.2.1. Visual Evaluation of Biocrystallization

The visual evaluation is based on ranking or scoring of individual scales of morphological properties in the crystal structure. The trained specialist can discriminate the pictures at 5 levels. These levels are:

1. Quantifiable evaluations about single morphological and local properties (e.g. length of the sideneedles).

2. Qualitative descriptive evaluations, connected to single morphological properties (e.g. regulation of ramification).

3. Qualitative descriptive evaluations of a higher order characterizing gestures in the whole picture (e.g. coordination, integration of the features).

4. Qualitative interpretative evaluations about plant-physiological aspects (e.g. ripeness, root typical) based on references of defined samples.

5. Qualitative interpretative judgments of the highest order related to clearly defined concepts of food quality (Huber, et al. 2010).

2.4.2.2. Computerized Evaluation

The computerized image analysis has become an optional method for evaluation of biocrystallogram images after some developments in the computer science. This method is based on reflection of all the characteristics of a biocrystallogam in a three dimensional form. Generally the previous studies used texture analysis applying 32 grey level and a single type of a circular region-of-interest (ROI) approaches (Meelursarn, 2007). This method does not provide only the information representing the visual characteristics, but also discriminates images which cannot visually be differentiated (Basset, et al. 2000).

2.4.3. The Main Factors Affecting Biocrystallization

2.4.3.1. Concentration Matrix

Concentration matrix is comprised of some factors affecting biocrystallisation such as concentration of sample extract and $CuCl_2$ solutions, mixing ratio of sample extract and $CuCl_2$ solution, and total volume of a blend. The optimum levels of concentration matrix need to be investigated to generate biocrystallograms with desired morphological features to be used in differentiation of various samples (Meelursarn, 2007).

2.4.3.2. The Physical Conditions in the Crystallization Apparatus

In addition to concentration matrix, the physical conditions such as air temperature, air relative humidity (%RH), air movements also affect the texture of biocrystallization images. Air temperature and %RH affect the structure of the

ramification and length of the branches. Air movement effects distribution of the needles on the surface of petri dish.

Furthermore, the mechanical vibrations have a significant impact causing development of more than one central zone in the biocrystallograms. The cleaning procedure and the surface properties of glass petri dishes also have an effect on biocrystallisation. Busscher, et al. (2010) used a series of cleaning process for a special Float-Glas petri plates before performing biocrystallization (Busscher, et al. 2010).

2.4.4. Applications of Biocrystallization in Science

Biocrystallization method was firstly introduced by E. Pfeiffer in 1930ies. He called this method "sensitive crystallization" or "copper II chloride crystallization". It was developed a point of view that live organisms are not only a material but also have an ability of control its own function and situation. As mentioned before, it is based on crystallographic phenomena performed by mixing plant extract and $CuCl_2$ solutions and evaporating the blend under suitable conditions (i.e.temperature and %RH) in order to generate re-producible crystal structure which is called biocrystallogram. On the light of this, biocrystallization method has been used for differentiation of organic and conventional samples and medical investigations. Moreover, the effect of degradation in carrots during storage was investigated by using biocrystallization method (Meelursam, 2007).

Morris and friends (1941) modified the cupric chloride crystallization using with purified egg albumin, the water soluble globulin of oats, white blood and tobacco mosaic virus. The biocrystallogram images showed that their protein properties affect the biocrystallization patterns.

In another studies introduced by Koepf in 1963, biocrystallograms formed by using whole and germ extracts of bean and oat seeds indicated different maturation time. The number of ramification and branches increased and became closer with increasing maturation time.

Biocrystallization method was also applied to distinguish the effects of different treatment methods on milk samples (Figure 2.6). There were significant differences between raw, UHT milk and milk samples homogenized at two different pressures. The

structure of the needles became invisible with increasing homogenization pressure. (Huber, et al. 2007).

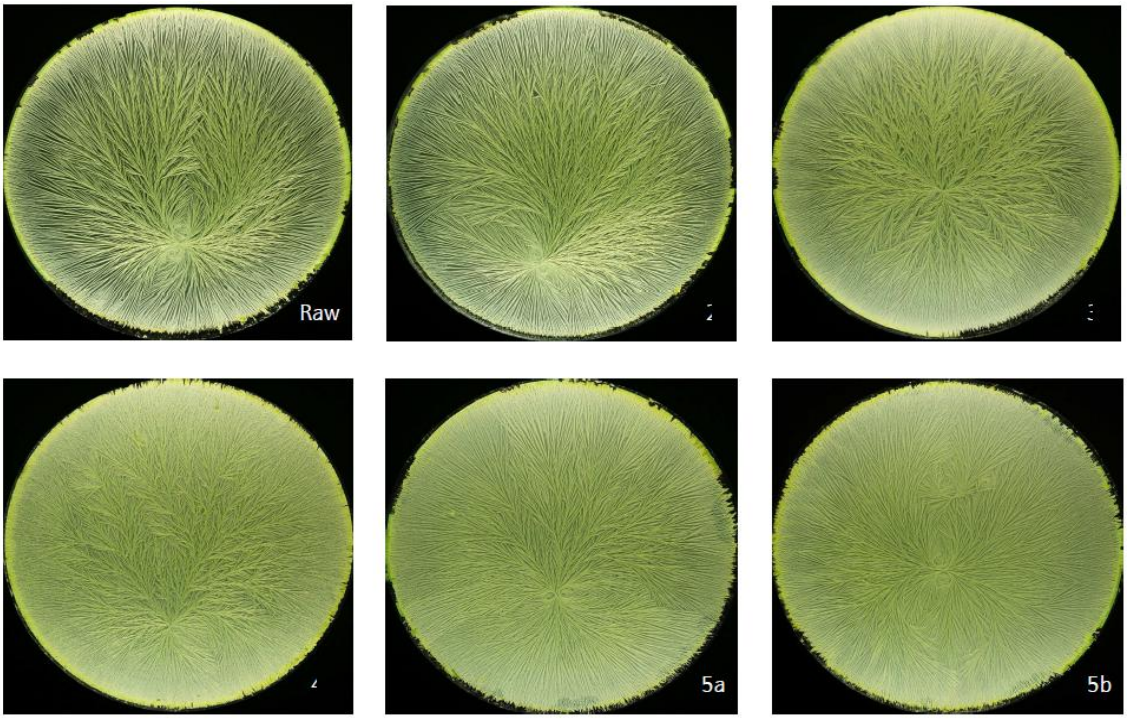


Figure 2.7. Crystallizations originating from the raw milk samples and the 5 differentiated treatments. 1-Raw, 2=UHT past.; 3=50Bar hom.; 4=200Bar hom.; 5ab=200Bar hom. and subs. 76°C (a) or 90°C (b).(Source : Huber, et al. 2007)

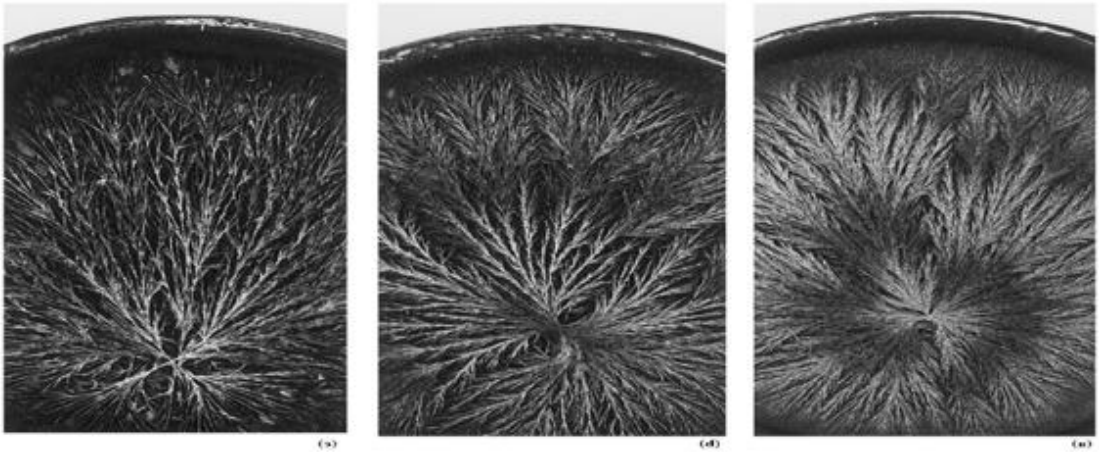


Figure 2.8. Enlarged photographic sections from biocrystallograms from the carrot extract degradation series representing: (a) Day 1; (b) Day 4; and (c) Day 7. (Source: Andersen, 1999).

Anderson, et al. (1999) investigated the effect of storage time on carrots by means of biocrystallisation method combined with a computerized image analysis (Figure 2.7). They classified the samples based on first- and second order grey colour statistics focusing on an area around the crystalization center.

Ünlütürk, et al. (2011) applied a feed-forward neural network model along with computer vision techniques to discriminate sweet red pepper products prepared by different methods such as freezing and pureeing.. The differences among the fresh, frozen and pureed samples are investigated by studying their bio-crystallogram images (Figure 2.8). A methodology called process neural network (ProcNN) reached 100% recognition proved to be a useful method in discrimination of red pepper products.

(a) (b) (c)

Figure 2.9. Biocrystallogram images of (a) fresh, (b) pureed, and (c) frozen sweet red peppers (Source : Ünlütürk, 2011)

In recent years, investigators focused on the standardized and validated biocrystallization method. Busscher, et al. (2010) investigated the evaporation effect on biocrystallization images and found the wetting conditions to be important in the growth of the crystals. It was indicated that surface tension of the solutions also affect the crystallization process during the evaporation. The surface properties and application of cleaning procedure of the glass plate also influenced the crystallization during evaporation in connection with the adsorption and precipitation of the additives. Huber, et al. (2010) tried to adapt the main norm, ISO-Norm 11035, to evaluate biocrystallograms visually. A panel was performed, mainly morphological criteria were selected and defined, a scale with references was established then the panel trained and tested to discriminate biocrystallogram images obtained for carrots having different qualities. The panel has been validated for the evaluation of carrots with a defined set of criteria (Huber, et al. 2010).

In another study, biocrystallization method was applied in the medical science. It is reported that the specific dentric crystal growth patterns in the presence of hemolysate from diabetic and healthy blood samples differ significantly based on evaluation of biocrystallogram images (Shibata, et al. 2000)

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

UHT whole cow milk of the same brand was purchased from a local market in Izmir, Turkey through the interval period from 2010 and 2011. The production date, expiry date, serial number, energy and nutrition values of UHT milk samples were recorded. The raw milk samples were collected during the period from March 2010 to October 2011 from a farm located in Gulbahçe, Izmir, Turkey. Prior to measurements, raw milk samples were preserved with a final concentration of 0.02% Bronopol (PESTANAL® Code:32053, Sigma-Aldrich) and stored at +4 ^{0}C.

Firstly, the flow cytometer was used to detect somatic cells in raw milk. Two different dyes i.e. acridine orange (Code: 158550, Sigma-Aldrich) and ethidium bromide (Code: 32221, Sigma-Aldrich) were applied for counting somatic cells by means of the flow cytometer.

Secondly, all the milk samples were screened by using New SNAP Beta-Lactam Test Kits (Idexx Laboratories, USA) for antibiotic residues employing a procedure recommended by the manufacturer. Thereafter, physico-chemical properties of the samples were analyzed by using Lactostar (Funke Gerber, Berlin, Germany).

Finally, the biocrystallization method was optimized. For this purpose, Penicillin G potassium salt (Code: 46609, Lot4016X) and Ampicillin trihydrate (Code: 46061, Lot 2316X) (Vetranal analytical standard Sigma-Aldrich GmbH Quality Assurance) were chosen as target antibiotics. Raw milk and UHT milk samples were spiked with Penicillin G and Ampicillin prepared in the concentration of 2, 4, 8 ppb. Copper II Chloride Dihydrate ($CuCl_2 \cdot 2H_2O$) (Code: 102733, Merck) were used for biocrystallization of spiked and antibiotic free milk samples. An incubator (Memmert-HCP 108, Berlin, Germany) adjusted to certain temperature and relative humidity was used for crystallization of the copper chloride added samples. Dendritic patterns were formed during crystallization from an aqueous solution containing milk and $CuCl_2$.

After crystallization process, all of the biocrystallograms were evaluated by using a computerized image processing technique and a discrimination test.

3.2. Methods

3.2.1. New SNAP* Beta-Lactam Test

3.2.1.1. Procedure

The New SNAP* Beta-Lactam Test is an enzyme-linked receptor binding assay for rapid determination of penicillin G, amoxicillin, ampicillin, ceftiofur and cephapirin residues in raw, commingled cow milk at or below established tolerance and/or safe levels at the EU/Codex maximum residue limits (MRLs) (Table 3.1).

Table 3.1.Detection levels and MRL's for New SNAP* Beta-Lactam Test (Source: Idexx, 2011)

Antibiotics	**New SNAP* Beta-Lactam Test Detection Level Range (ppb)**	**EU/Codex MRL (ppb)**
Penicillin G	< 2-5	4
Ampicillin	2-10	4

450 µL milk sample is added carefully to a sample tube. (Figure 3.1) After that the sample tube is shaked to dissolve the reagent pellet and incubated at 45°C for five minutes. Secondly, the entire contents of the sample tube is poured into the sample well of the SNAP device.

When the blue activation circle begins to disappear, the activator is pushed FIRMLY until it snaps flush with the body of the SNAP device. After waiting four minutes for second incubation, the sample spot is examined. If the sample spot is darker or equal to control spot, this means negative sample (Figure 3.2). If the sample spot is lighter than control spot, this results indicates positive sample.

- SNAP device
- Sample tube and cap
- Reagent pellet
- Pipette†

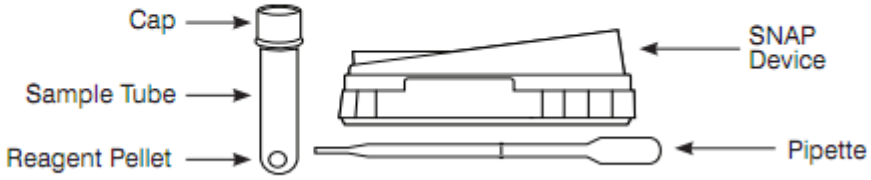


Figure 3.1.SNAP* Beta-Lactam Test design

(Source: Idexx, 2011)

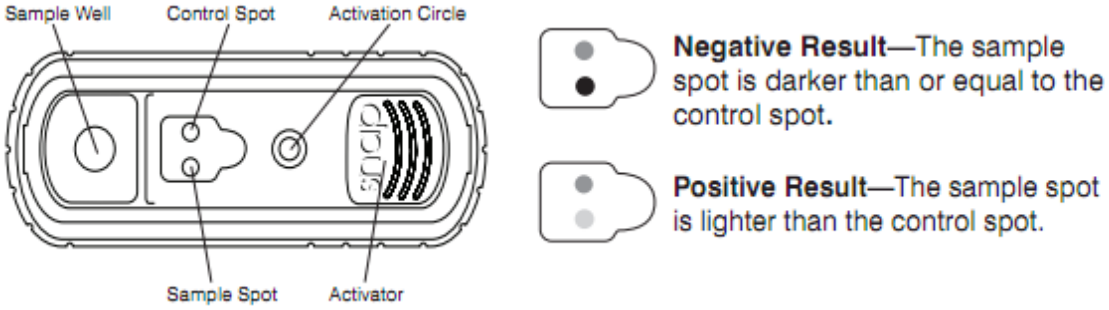


Figure 3.2. SNAP* Beta-Lactam Test results
(Source: Idexx, 2011)

3.2.1.2. Preparation of Antibiotic Standard Solution

Penicillin G ($C_{16}H_{17}KN_2O_4S$, 99.4%, 372.48 g/mol) and Ampicillin trihydrate ($C_{16}H_{19}N_3O_4S.3H_2O$, 98.1%, 403.45 g/mol) were used to prepare standard solution. Working standard solutions of Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb) concentrations were prepared by diluting the stock standard solution with water (Sivakesava and Irudayaraj, 2002).

3.2.2. Determination of Fat, Protein, Lactose and SNF (Fat-Free Dry Matter)

The percent of protein, fat, lactose, minerals and fat-free dry matter (SNF) of milk samples are determined by using Funke Gerber 3510 Lactostar milk analysis device (Funke Gerber, Berlin, Germany) supplied with fully automatic cleaning and rinsing system and zero point calibration for the fast and accurate testing of milk. Lactostar has been used for the routine testing of milk content. It is composed of four cells allocated in two measurement units' i.e. optical unit (blue box) and thermal unit (red box) (Figure 3.3).

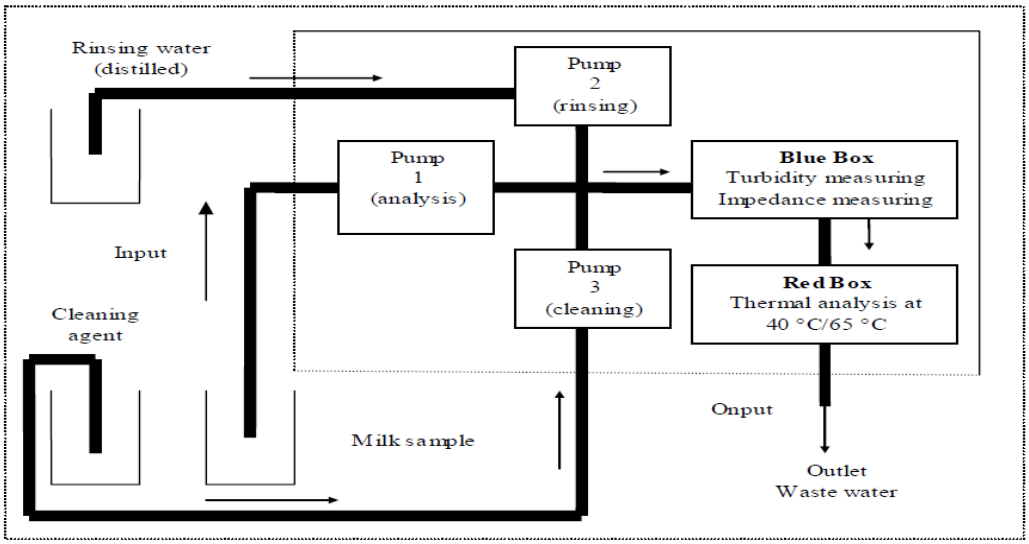


Figure 3.3. Lactostar mechanism
(Source: Bentleyczech 2008)

The milk sample (12 to 20 ml) pumped into different cells is analyzed by using a technique combining both thermal and optical measurement methods in these two measurement units. The blue box measures the turbidity to determine the amount of undissolved substances e.g. fat and protein content and employs impedance or conductance methods. Red box contains two thermo analytical measurement cells. In these cells, measurements are carried out at two different temperatures (40.00 °C / 65.00 °C). The fat content and the fat-free dry matter are measured through thermal effects at different measuring temperatures.

The content of antibiotic free raw and UHT milk samples and milk samples spiked with Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb) were determined at

60 sec tempering and 15 sec measurement time. All measurements were performed three times.

3.2.3. Somatic Cell Count

The flow cytometer (FACSCanto^{TM}, BD Bioscience, San Jose, USA) equipped with a 15-mW argon laser emitting light at 488 nm was used for somatic cell analyses. The instrument was equipped with forward-angle light scatter (<15°), side-angle light scatter (>15°), and three fluorescence detectors: FL1 (515 to 565 nm), FL2 (565 to 605 nm), and FL3 (>605 nm). Somatic cells were also counted by means of a light microscope (OLYMPUS-CX31, JAPAN) and hemocytometer to confirm the results obtained from flow cytometer measurement.

Flow cytometer (FCM) is a very sensitive instrument used for a rapid analysis of somatic and microbial cell count in milk. FCM is combined with fluorescent stains or fluorogenic substrates for detection and discrimination of viable and nonviable organisms (Gunasekera, et al. 2003). Prior to measurement, it is waited for the fluorescent dye to penetrate into the cell and interact with nuclear DNA (Sierra, et al. 2006). Then the sample is pumped through a flow cell having a very small diameter which allows only one cell to pass at a time (Feng and Zheng, 2004 and Gonzola, et al. 2004**)**. Data acquired from FCM was converted and analysed with a software as Fax Diva Version; 5.0.3 software (FACSCANTO, BD Bioscience, San Jose, USA).

Before the measurement, milk samples were centrifuged at 180×g for 10 min to remove lipids and thereby allow distinction of somatic cells by flow cytometry (Dosogne, et al. 2003). The lipid layer collected on top of the samples and adhered to the tube wall was drawn off with a micropipette and a soft swab without disturbing the pelleted material, which contained somatic cells. Cleared milk pellets were suspended in 10 ml. phosphate-buffered saline (PBS). After this process the pellets were centrifuged at 180×g for 10 min and then resuspended again in PBS and 0,1% Triton X-100 (trit-X) (Code: 0694, Amresco).

Two different dyes, i.e. acridine orange AO (Code: 158550, Sigma-Aldrich) and ethidium bromide (EtBr) (Code: 32221, Sigma-Aldrich) were used .for staining the pellets. Stock solutions of AO (0.1 mg/ml) and EtBr (5 mg/ml) were prepared in pure water. The cell pellets suspended in both PBS and Triton X-100 were stained with AO.

However, only Triton X-100 solution was used to resuspend the pellets in the staining with EtBr. Polystyrene flow cytometry tubes in the size of 12×75 mm prepared according to BD Cell Viability Kit and filled with 850 µl of pellet, 50 µl of bead (Code: 349480 with BD Liquid Counting Beads) and 100 µl of stock dye solution. This mixture was stored for 15 min at room temperature to let the dyes penetrate into the cells.

The stained samples were analyzed with a flow cytometer. For counting the AO stained cells, excitation and emission wavelength of the argon laser was adjusted to 488nm and 530 ± 20 nm respectively in Fluorescein isothiocyanate (FITS) channel. On the other hand, the cells stained with EtBr, the excitation wavelength was kept at 488nm and the emission wavelength was set to 585± 20 nm in Phycoerythrin (PE) channel. The experiments were carried out in duplicate. Data plotted interms of FITS-H and PE-H versus SSC-H was obtained by a computer program (Fax Diva, Version 5.0.3, FACSCANTO BD Bioscience, San Jose, USA). Total number of somatic cells was determined using Equation (3.1).

$$\frac{\frac{number\ \ of}{cell\ \ events}}{\frac{number\ \ of}{bead\ \ events}} \times \frac{assigned\ \ bead\ \ count\ \ of\ \ the\ lot\ \left(\frac{bead}{50}\mu l\right)}{volume\ \ of\ \ sample} = concentration\ of\ sample\ as\left(\frac{cells}{\mu l}\right) \quad (3.1)$$

For direct microscopic count, 100 µl cleared cells and 100 µl of 0.5% trypan blue solution (Code: 03-102, Biorad Lab.) were mixed and spread uniformly onto a slide and counted using a hemocytometer.. Somatic cells possessing dark blue nucleus were counted by means of a light microscope (OLYMPUS-CX31, JAPAN) fitted with a 40X objective. The number of somatic cells screened in 30 different areas on the slide was recorded and reported as the total number per milliliter. The experiment was repeated two times. Correlations between flow cytometric method and total microscopic counts were calculated with statistical software (Minitab Statistical Software 14 Trial version (Minitab Inc., State College, PA, USA).

3.2.4. Biocrystallization Method

The biocrystallization method comprises of different steps including sample preparation, mixing aqueous solution of sample extract and $CuCl_2.2H_2O$ in a glass petri at a defined ratio, drying of glass petries in an air climate cabinet at a constant

temperature and humidity to obtain clear biocrystalograms, taking the pictures in a dark illumination and evaluation of the pictures of biocrystallograms by means of computerized image processing method and visual inspection by trained people (Figure 3.4). Computerized image analysis was not in the focus of this study. It was carried out by another work group in University of Economics, İzmir, Turkey.

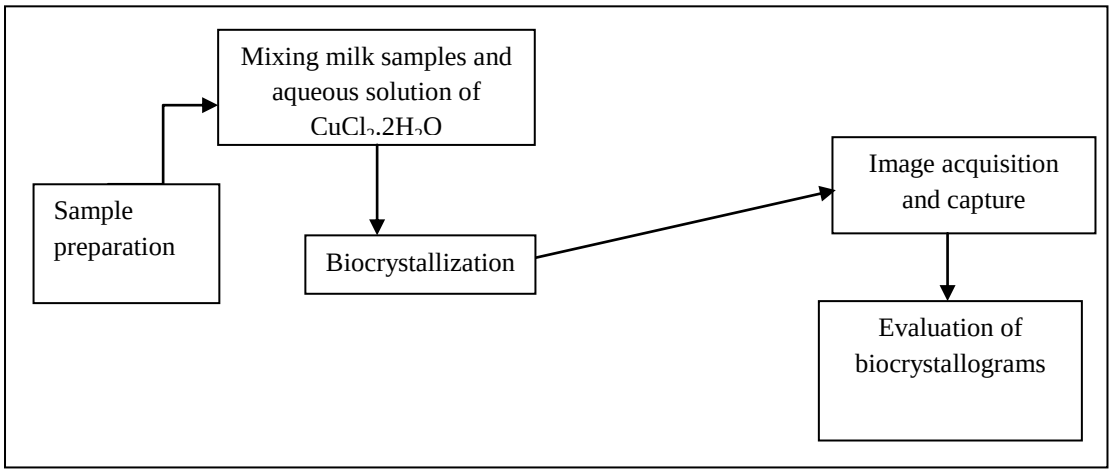


Figure 3.4. Details of biocrystallization method

3.2.4.1. Sample Preparation, Biocrystallization and Image Acquisition

Raw milk samples obtained from a selected cow housed in a local farm were preserved by adding 0.02% bronopol until used. The content of antibiotic free raw and UHT milk samples and samples spiked with Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb) were diluted with milli-Q-water in different concentrations in the range of 0.02% to 0.5 %.

Aqueous solution of $CuCl_2.2H_2O$ was prepared with milli-Q-water in different concentrations changing from 5% to 15%.

Circular glass petri dishes with a diameter of 100 mm and a thickness of 2 mm were washed with a regular dish detergent and rinsed with de-ionized water at 98°C. After that the petries were cleaned with 99.5% Ethanol (Merck) and dried in an oven (Nüve EN-050, TURKEY) at 50°C for 1 hour.

In the crystallization step, the known amount of milk sample (0.02 – 0.5%) and aqueous $CuCl_2.2H_2O$ solution (5 – 15%) were mixed in a cleaned glass petri dish at a defined mixing ratio (milk/$CuCl_2.2H_2O$) and dried in an air climate cabinet (Memmert-HCP108, Germany) adjusted to certain temperature and relative humidity. Factors

including the amount of mixture (4-8 ml) and mixing ratio (1/1-3/1), drying temperature (25°C - 40°C) and relative humidity (40-60%) affect the ramification patterns of biocrystallograms. The experiments were designed to optimize these factors in order to generate biocrystallograms having the best observable dendiritic pattern during crystallization i.e. drying period.

After crystallization step, digital images of the pictures were captured by means of a Nikon D90 camera fitted with a 60 mm f/2.8D AF Micro-Nikkor lens with the following mode specifications: ISO 250, record mode HQ (jpeg file format 1/8 compression), aperture priority F 5.6, exposure -0.3EV and focusing mode single AF. The camera was positioned vertically over the sample at a certain distance. The angle between the camera lens, the lighting source, and illumination were fixed and kept the same for all the sample pictures. For this purpose, a wooden lightbox (dimensions 390×390×160 mm, l×w×h) providing a dark illumination was constructed according to instructions described by Anderson et., al, 2003. After that, the images were transferred and stored in a PC as a JPEG format of "high resolution" and "superfine quality".

3.2.4.2. Evaluation of Biocrystallograms

Images of biocrystallograms were evaluated visually by trained people using defined criteria (Huber, et al. 2010).

There is so far only one published standardized evaluation method for biocrystallization (Huber et al, 2010). In this study, dendirict patterns of biocrystallagroms were assessed by a simple descriptive test adapted and modified from ISO-Norm 11035, 1994 which is used for sensory analysis to identify and select descriptors for establishing a sensory profile by a multidimensional approach (ISO, 1994; Huber, et al. 2010). It states a maximum of 15 descriptors (criteria). In this work, panelists assessed the pictures of biocrystallograms based on seven descriptors in the screening step (Table 3.5). In the case of optimization step, five factors were evaluated and depicted in Table 3.3. According to this norm, a panel must be constituted by a minimum of six people. A panel was formed from members of the experienced and inexperienced people at the start. All panelists were trained by the panel leader. The different series of pictures were produced from raw milk, UHT milk and milk samples spiked with Penicillin and Ampicillin (Table 3.2). Each series was composed of

minimum 60 biocrystallograms referring to 3 replicate pictures per sample preparation. Multi-centered pictures were discarded and not used for visual inspection. Thus, only four patterns per sample were evaluated simultaneously. They scored these criteria using a ranking scale of intensity, ranging from 1 to 9 (Table 3.3). For all criteria, reference pictures were chosen which connected to the scale intensities 1-4-7-9 (Appendix A.1).

Table 3.2. The tests performed and number of panel members per test

Series	**Samples Compared**	**Panelist Number**
1	Raw milk-UHT milk	6
2	Raw milk- raw milk adding with penicillin G (2, 4, 8 ppb)	6
3	Raw milk- raw milk adding with ampicillin (2, 4, 8 ppb)	6
4	UHT milk- UHT milk adding with penicillin G (2, 4, 8 ppb)	6
5	UHT milk- UHT milk adding with ampicillin (2, 4, 8 ppb)	6

Table 3.3. Overview of the 5 descriptive criteria used in panel judgements to evaluate the biocrystallograms

1) Centre co-ordination: Centre of biocrystallograms located near the side of the petri not mid-point or quite side of petri.
2) Regulation of branches: Branches show symmetrical distribution according to centre.
3) Length of the branches: Branches must be long and entire not short or discrete, distribute in whole petri.
4) Density of branches: They must be loud and clear, not intense or penetrating.
5) Lemniscate form: The eight forms composed at the centre of image do not be empty.

Table 3.4. Overview of the 7 descriptive criteria used in panel judgements of evaluating optimized biocrystallogram images

1) Integration: Distribution of the biocrystallograms on whole glass petri.
2) Centre number: It is needed one centre point on the petri if more, it is not desirable.
3) Centre co-ordination: Centre of biocrystallograms located near the side of the petri not mid-point or quite side of petri.
4) Regulation of branches: Branches show symmetrical distribution according to centre.
5) Length of the branches: Branches must be long and entire not short or discrete, distribute in whole petri.
6) Density of branches: They must be loud and clear, not intense or penetrating.
7) Lemniscate form: The eight forms composed at the centre of image do not be empty.

3.2.5. Optimization of Biocrystallization Method

3.2.5.1. Screening the Important Factors and Optimization of Biocrystallization Process

Biocrystallization is a very sensitive method affected by both physical conditions of drying (crystallization) chamber and concentration matrix. The most important physical conditions in the crystallization chamber that influence the biocrystallogram are drying temperature, relative humidity and drying time, and the others are mechanical vibrations and air movement in the chamber. In the case of the solution concentration matrix, the milk concentration, $CuCl_2.2H_2O$ concentration, optimal mixing ratio between milk and copper chloride (volume of milk/ volume of $CuCl_2.2H_2O$) and volume of mixture are very important factors influencing the crystallization process. Therefore, in order to obtain the best biocrystallogram images, experiments were started with the screening and optimization of the factors and then continued with the validation experiments. First of all, the factors and their levels were screened and three different designs were employed for this purpose. For each experimental design, different ranges of levels were chosen. These levels are depicted in Table 3.4.

For the first design, only three factors including the milk concentration, $CuCl_2.2H_2O$ concentration and their mixing ratio were tried to be optimized. The other factors were held constant. In the second design, the amount of mixture, drying temperature, the relative humidity and the drying time were added as new factors. In the third design, according to the results of previous screening experiments the levels of the factors were optimized. Each design was repeated two times. In the first design, total 16 (2^3) plates were hold, in the second one the total of 256 (2^7) plate and in the last design 128 (2^6) plates were hold.

Table 3.5. Experiment factors and their low-high levels

Factors	**Low-high levels of design experiment 1**	**Low-high levels of design experiment 2**	**Low-high levels of design experiment 3**
Milk Concentration (%)	0.04 – 0.4	0.04 – 0.4	0.2 – 0.5
$CuCl_2.2H_2O$ Concentration (%)	5 – 15	5 – 15	5 – 15
Mixing Ratio (Milk/ $CuCl_2.2H_2O$)	1/1– 3/1	1/1– 3/1	1/1 – 3/1
Amount of Mixture (ml)	8	4 – 8	4 – 6
Drying Temperature (°C)	30	25 – 40	30 – 40
Drying Humidity (RH %)	53	45 – 60	50 – 60
Drying Time (h)	18	16 – 24	22

3.2.5.2. Validation of Biocrystallization

After the screening and optimization step, the optimum value of each factor was determined as shown in Table 3.6. They are validated at their optimum values using raw milk and milk samples spiked with Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb), UHT milk and UHT milk samples containing Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb). From each sample, 60 biocrystallogram images were prepared and evaluated both visually by trained panelists.

Table 3.6. Optimum values of factors

Factor	Optimum Values
Milk Concentration (%)	0.5
$CuCl_2.2H_2O$ Concentration (%)	5
Mixing Ratio (Milk/ $CuCl_2.2H_2O$)	3/1
Amount of Mixture (ml)	6
Drying Temperature (°C)	40
Drying Humidity (RH %)	60
Drying Time (h)	22

3.2.6. Statistical Analysis

The statistical analyses were performed using the statistical software Minitab Statistical Software 14 Trial version (Minitab Inc., State College, PA, USA) and State-Ease Design Expert Software 7.1 Trial version (Stat-Ease, Inc., Minneapolis, MN, USA). Somatic cell count results of raw milk samples were expressed with one-way analysis of variance (ANOVA) with Fisher's, individual error rate. The test was carried out in order to evaluate the effect of AO and EtBr and Trit-X-100 and PBS solutions on somatic cell number of raw milk samples at the level of $p < 0.05$. In other words, the ANOVA test was performed for all experimental runs at 95 percent confidence interval.

The p-value is the smallest level of significance. It would lead to rejection of the null hypothesis H_0. The ANOVA is a general and one of the most powerful statistical methods that can be used to test the hypothesis that means among two or more groups are equal under the assumption that sampled populations are normally distributed. The reason for applying an ANOVA is to see if there is any difference between groups on the same variable. In one-way or one-factor ANOVA, there is only one factor, and the analysis of variance is used to analyze the effect of one factor. The ANOVA table includes the sum of squares, the mean square and an F distribution with degrees of freedom.

For the visual evaluations of the optimized biocrystallograms, 2^k factorial design was applied to determine the effects and interactions of the factors and determine the optimum conditions with using ANOVA. Significant factors and interaction between factors were determined according to the p-value prob> F value which is smallest than 0.0001. The results were presented with graphics such as main effect, interaction plots and contour plots.

The method means a design with *k* factors at two levels. The statistical model for a 2^k design include k main effects, $\binom{k}{2}$ two-factor interactions, $\binom{k}{3}$ three-factor interactions,..., and one k-factor interaction. The method is consisted of 6 steps. The first step provides to estimate factor effects and examine their signs and magnitudes. In the second step i.e. in forming initial model for experiment, the full model was chosen with levels. In the third step, ANOVA were used for significance of main effects and interactions. In step 4, the model refined, usually removed nonsignificant variables from the full model. A residual analysis was performed to check the model adequacy and check the assumptions in the fifth step. In the final step, a graphical analysis was applied to main effect to draw interaction plots or response surface and contour plots (Montgomery, 2001).

CHAPTER 4

RESULT AND DISCUSSIONS

4.1. Fat, Protein, Lactose, SNF Results of Milk Samples

Lactostar is a new developed machine for the routine testing of milk. The measurement is based on a thermo-optical procedure combination. In opto-unit, the undissolved (visible) substances are analyzed such as the sum of fat and protein. It also contains impedance or conductance measurement. In thermal unit, the fat content and the fat-free dry matter are measured.

In this study, this experiment was aimed to investigate the effects of seasons and antibiotics on the composition of raw milk samples. The experimental data obtained for raw milk samples as fat%, protein%, lactose% and SNF%. By using Minitab 14 trial version, the data were evaluated statistically. The statistical testing showed that seasons had a significant effect on the fat%, protein%, lactose% and SNF% content of raw milk. The clear differences were observed on the fat content of raw milk. In summer season, fat content was at the lowest value which was % 2.56; the highest value % 8.058571 was obtained in autumn season. The highest protein, lactose and SNF content of raw milk were obtained in summer season; the lowest ones were measured in winter season (Table 4.1).

The raw milk which is naturally contaminated with antibiotic (Penicillin G) was obtained in autumn season. The statistical testing showed no significant difference among the values of protein%, lactose% and SNF% at a 95% confidence level. In contrast, fat% content of antibiotic free and naturally contaminated with antibiotic of raw milk samples significantly different from each other ($p < 0.05$) (Table 4.2).

Table 4.1. Effect of seasons on the Lactostar results of raw milk samples with using Fisher Test.

Raw milk (antibiotic free)	Fat (%)	Protein (%)	Lactose (%)	SNF (%)
Spring	8.016667 ±0.217777[c]	7.248333 ±0.038687[b]	7.04 ±0.044721[b]	7.135 ±0.042308[b]
Summer	2.562857 ±0.16769[a]	5.931429 ±0.192293[ab]	5.711429 ±0.476966[ab]	5.718571 ±0.476099[ab]
Autumn	8.058571 ±0.080911[c]	6.654286 ±0.736713[b]	6.45 ±0.717068[b]	6.541429 ±0.72447[b]
Winter	4.95 ±0.311234[b]	5.67 ±0.700219[a]	5.571429 ±0.733994[a]	5.585714 ±0.758129[a]

[a-c]values in a column with the same superscript are not significantly different by Fisher's test ($p<0.05$)

Table 4.2. Effect of naturally contaminated with antibiotic on the Lactostar results of raw milk using Fisher Test

Autumn Season	Fat (%)	Protein (%)	Lactose (%)	SNF (%)
Raw milk (naturally contaminated with antibiotic)	8.77 ±0.042436[b]	6.93 ± 0.098995[a]	6.775 ± 0.106066[a]	6.845 ± 0.106066[a]
Raw milk (antibiotic free)	8.058571 ±0.080911[a]	6.654286 ±0.736713[a]	6.45 ±0.717068[a]	6.541429 ±0.72447[a]

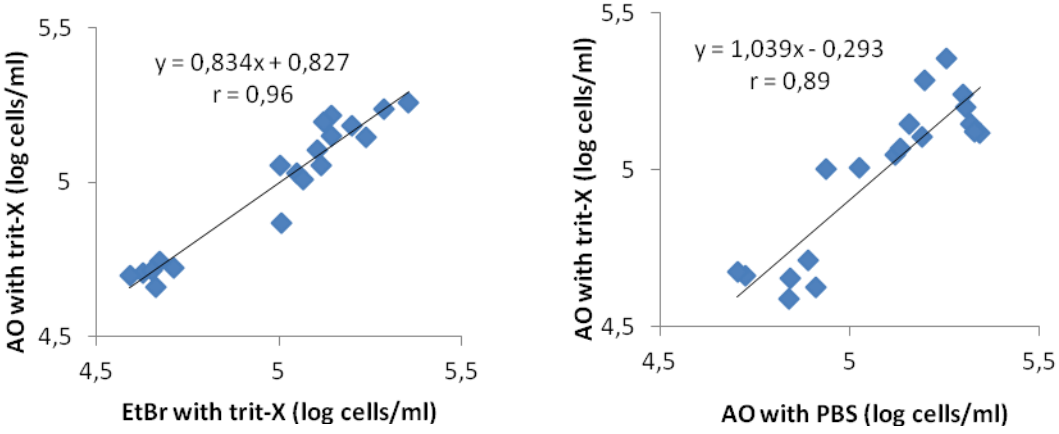
[a-b]values in a column with the same superscript are not significantly different by Fisher's test ($p<0.05$)

4.2. Somatic Cell Count Results

Somatic cell analysis is important with regard to raw milk quality and identification of potential dairy cow infections. This is because high number of somatic cell can result from disease such as mastitis (Kehrli, et al. 1994). In addition, high

number of somatic cells in milk affects negatively the commercial antibiotic screening test kits which result in false positive.

To determine the feasibility of using flow cytometry for somatic cell count, we first remove the lipids in raw milk. Presence of lipid particles in milk increase the amount of debris causeing limited somatic cell counting. Two different DNA-binding fluorescent dyes acridine orange (AO) and ethidium bromide (EtBr) were used to identify the types of inflammatory cells present in milk. Since AO can be penetrating either live or death cell's DNA, cells were suspended in PBS solution before staining. On the other hand, EtBr advised by AOAC 978.26 method to determine the SCC with optical somatic cell count method can penetrate only death cells. In order to stain the live cells with EtBr, cells were suspended in Triton-X-100 solution to create the pore on their cell wall. Also in order to evaluate the effect of triton-X-100 with AO, again cells were suspended in Trit-X-100 than stained with AO. All flow cytometric results were confirmed with direct microscopic results.



(a) (b)

Figure 4.1. Correlation between AO and EtBr staining of somatic cells, (a) the measurement performed with flow cytometer by using Trit-X-100 surfactant, (b) correlation between Triton-X-100 and PBS in somatic cell count with flow cytometer by stained cell with AO

There was a good correlation between AO and EtBr staining with using the same surfactant in flow cytometric somatic cell counting (r=0.96) (Figure 4.1.a). One-way ANOVA with Tukey's test, individual error rate were performed to evaluate the counting results carried out with these two dyes. ANOVA results addressed that the means of somatic cell number were not statistically different each other ($p>0.05$) (Appendix B.1). Moreover the same analyses were done with AO with trit-X-100 and AO with PBS to perform the effect of surfactant on the somatic cell counting by flow

cytometry (Figure 4.1.b). The results showed that there was a good correlation and statistically no significant differences between two surfactant (r=0.89, p>0,05) (Appendix B.1).

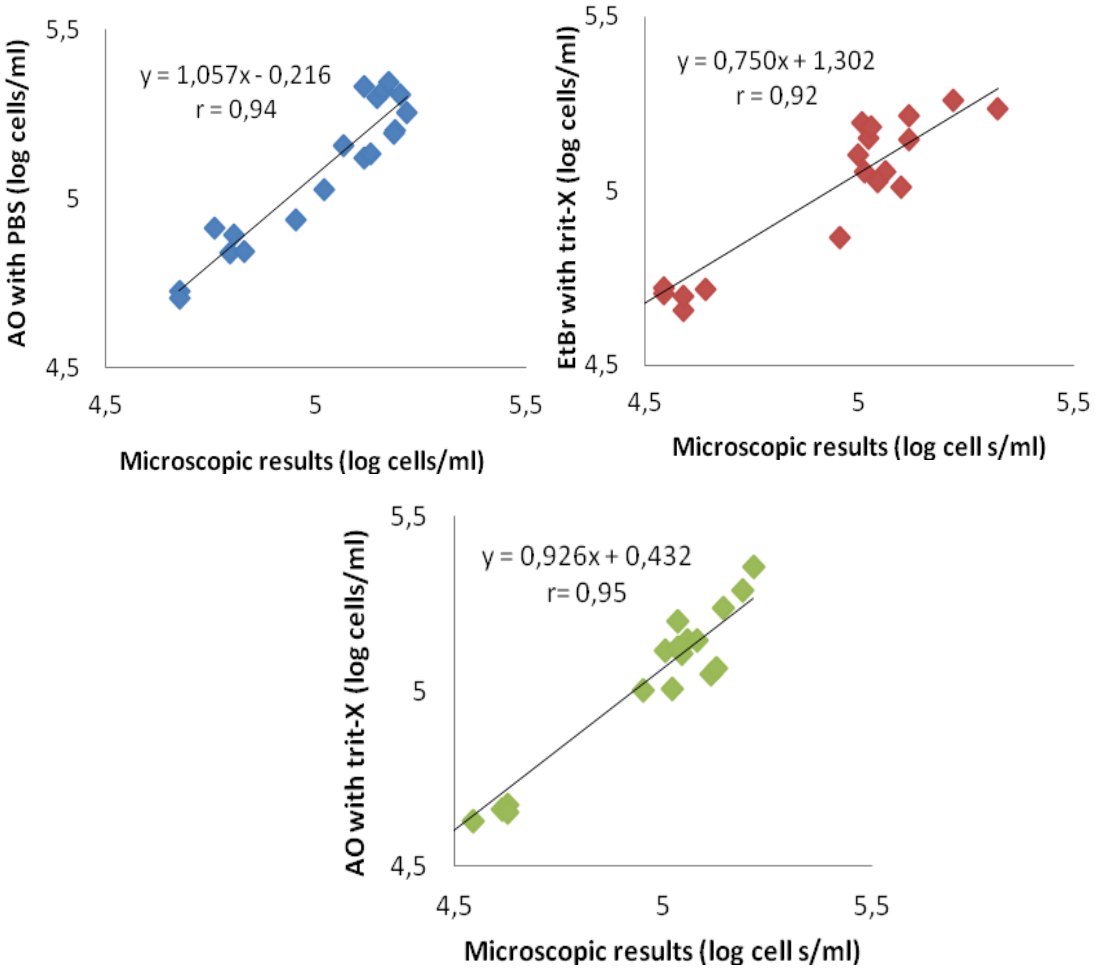


Figure 4.2. Correlation between somatic cell counting in raw milk samples by direct microscopy and AO with trit-X-100, EtBr with trit-X-100 and AO with PBS by flow cytometry methods

Finally all flow cytometry counts were compared with direct microscopy results. The data given in Figure 4.2 showed that there were no significant differences (p>0.05) between all methods (Appendix B.2). Furthermore, there was a better correlation between the method which uses AO dye with the surfactant trit-X-100 and direct microscopic results (r=0.95, n=30) compared to the other methods employing different dye combinations for somatic cell counting in raw milk samples.

The somatic cell number in milk was evaluated in the range of 50×10^3 and 135×10^3 cells/ml. According to Bergonier, et al. (2003), non-pathological factors are responsible for variation of SCC in cow milk between 40×10^3 and 100×10^3 cells/ml.

This variety in SCC did not depend on directly seasons. High number of somatic cell can fluctuate from one day to another particularly in the late lactation and even within days. Influence of sample collection during milking or after milking was reported to change SCC levels (Raynal-Ljutovac, et al. 2007).

4.3. Screening and Optimization of Biocrystallization

In order to obtain the best biocrystallogram images, the screening was first carried out to determine the important factors and their levels. In the optimization step, according to the results of screening experiments the levels of the factors were optimized. Totally, three different designs of experiments were employed.

In the first design, only milk and $CuCl_2.2H_2O$ concentration, their mixing ratio and their low and high levels were determined and tried to be optimized (Table 4.3), the other factors hold constant. 8 ml milk and $CuCl_2.2H_2O$ mixture were dried at 30°C, 53% RH for 18h.

Table 4.3. Factors of first design at low-high level

Factors	**Low Level**	**High Level**
Milk concentration	0.04%	0.4%
$CuCl_2.2H_2O$	5%	15%
Mixing ratio (Milk/ $CuCl_2.2H_2O$)	3/1	1/1

After holding black-white 16 biocrystallogram images via gel image analysis system (VILBER LOURMAT, CN3000WL, France), a panel was performed according to 7 descriptive criteria by trained panelists to evaluate the images visually. The panel scores were performed with using the statistical software Design-Expert 7.1 to evaluate the efficiency and acceptability of the model and determine the significant factors on the biocrystallograms.

By using ANOVA, the results were obtained (Appendix C.1.). The model F-value is 18.49 that implied the model was significant. R-squred 0.9418 was good for designing our model. However milk concentration was the most significant because of high % contribution, the interaction between milk concentration and other factors were

also found significant in affecting the biocrystallogram images. Thus, $CuCl_2.2H_2O$ concentration and mixing ratio were significant due to hierarchy of each other.

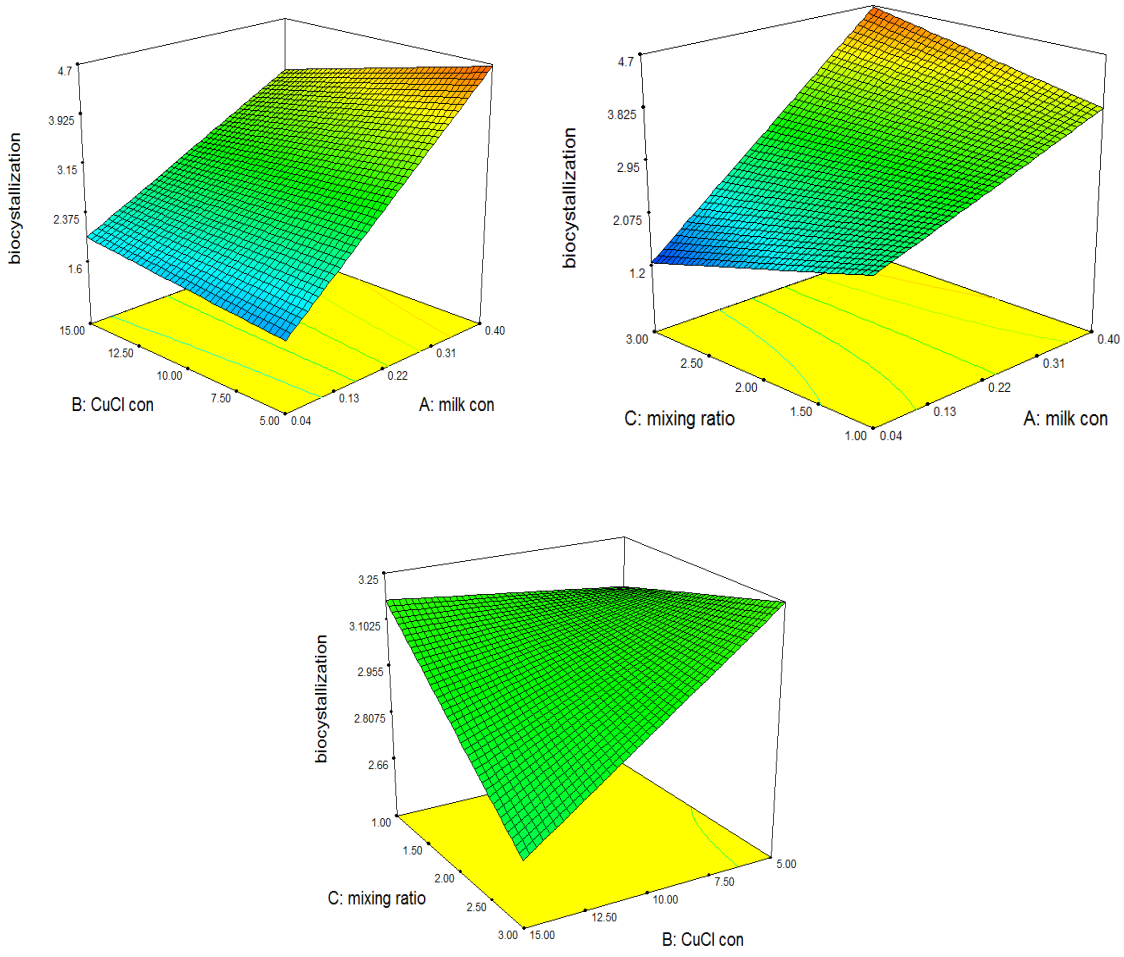
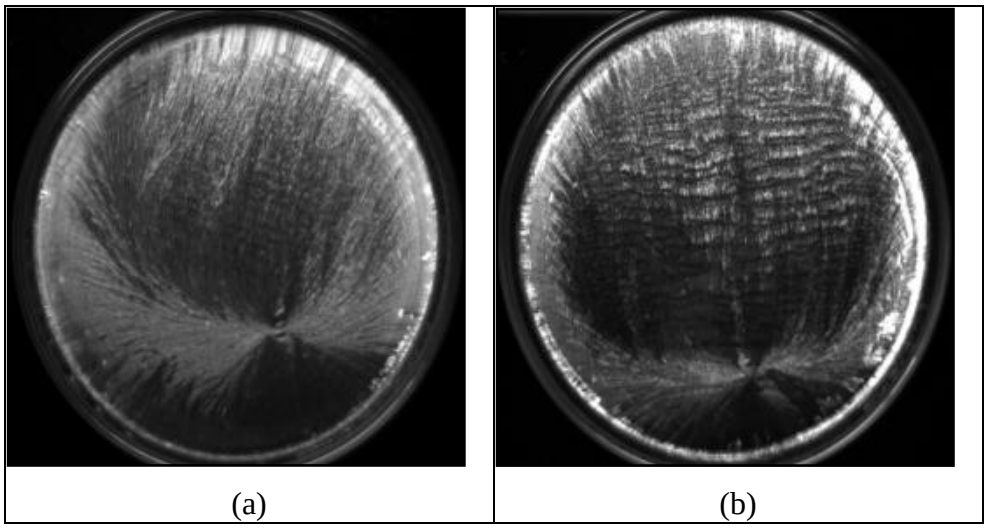


Figure 4.3. 3D surface plot effect of milk and $CuCl_2.2H_2O$ concentration and mixing ratio in biocrystallogram images (biocrystallization on the vertical axis refers to total scores given to the images by panelists)

3D surface plots show the effect of milk and $CuCl_2.2H_2O$ concentration and mixing ratio on biocrystallogram images in Figure 4.3. Increase of milk concentration affected the biocrystallogram images positively. On the other hand, increase of $CuCl_2.2H_2O$ concentration and mixed ratio influence were negative on biocrystallogram images. 3D surface plots indicated that milk and $CuCl_2.2H_2O$ concentrations could be changed in a range between 0.04% and 0.4%, and between 5% and 15%, respectively. On the other hand, the results for mixed ratio were not clear as it was obvious in Figure 4.4.



(a) (b)

Figure 4.4. Inadequate biocrystallograms images from the 1st design of experiment. ((a) 0.4% milk, 5% $CuCl_2.2H_2O$ concentration, 3/1 mixing ratio (b) 0.4% milk, 5% $CuCl_2.2H_2O$ concentration, 1/1 mixing ratio)

Poor and inadequate crystal structure was observed on this biocrystallograms images. The distributions of needles were not clear and the needles exhibit interpenetrating structure (Figure 4.4). Therefore, a new experiment design was applied including other factors.

Table 4.4. Factors of 2^{nd} design at low-high level

Factors	**Low levels**	**High levels**
Milk Concentration (%)	0.04	0.4
$CuCl_2.2H_2O$ Concentration (%)	5	15
Mixing Ratio (Milk/ $CuCl_2.2H_2O$)	1/1	3/1
Amount of Mixture (ml)	4	8
Drying Temperature (°C)	25	40
Drying Humidity (RH %)	45	60
Drying Time (h)	16	24

In second design, 2^7 general factorial design were applied with two replicate. High and low levels of these factors were given in Table 4.4. The ANOVA results for the second design gave the model F- value as 15.71 indicating that the model was significant (Appendix C.2). On the other hand, the "Lack of Fit F-value" was calculated as 2.87 implying that the Lack of Fit was significant. Therefore it was concluded that

the model for the second design of experiment was inadequate to fit the data well, so the model was needed to refine with changing the levels of factors.

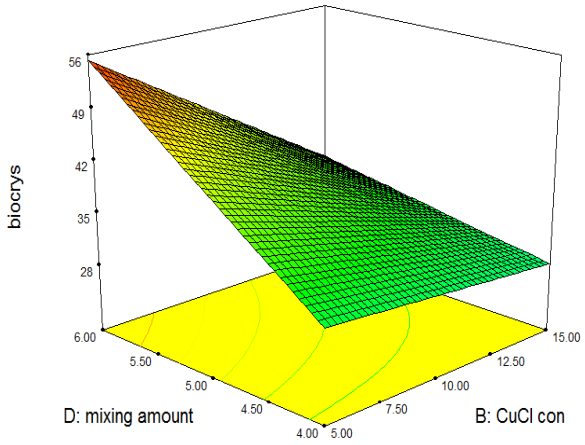
In third design, 2^6 general factorial design was performed with two replicate to optimize the levels of the factors. Design factors and levels are shown as Table 4.5.

Table 4.5. Factors of 3rd design at low-high level

Factors	**Levels**
Milk Concentration	0.2%-0.5%
$CuCl_2.2H_2O$ Concentration	5%-15%
Mixture Ratio (milk/ $CuCl_2.2H_2O$)	1/1-3/1
Amount of Mixture	4ml-6ml
Drying Temperature	30°C-40°C
Drying Humidity	50%-60%

The petries were dried, in the humidity controlled air cabinets for 24 h, 256 biocrystallograms were generated. Photograph of the images were taken. In order to determine the significant factors and levels of this experiment, a panel was organized. The 6 panelist enumerate 7 descriptive criteria shown as Table 3.5 according to scores ranging from 1 to 9.

According to ANOVA results (Appendix C.3), the Model F-value of 9.88 implies the model was significant. Also "Lack of Fit" value was not significant. Non-significant lack of fit is good for the model. $CuCl_2.2H_2O$ concentration , mixing ratio, temperature and humidity were determined to be significant factors. Interaction of $CuCl_2.2H_2O$ and amount of mixture and interaction of $CuCl_2.2H_2O$, mixing ratio, amount of mixture, temperature and humidity terms are also found significant. The other terms took a part in the model because of hierarchy between significant terms.



Actual Factors	Levels
Milk Con.	0,2%
Mixing Ratio	1/1
Temperature	40°C
Humidity	60%

Figure 4.5. 3D surface plot effect of interaction between mixing amount and $CuCl_2.2H_2O$ on biocrystallograms score with as given actual factors values.

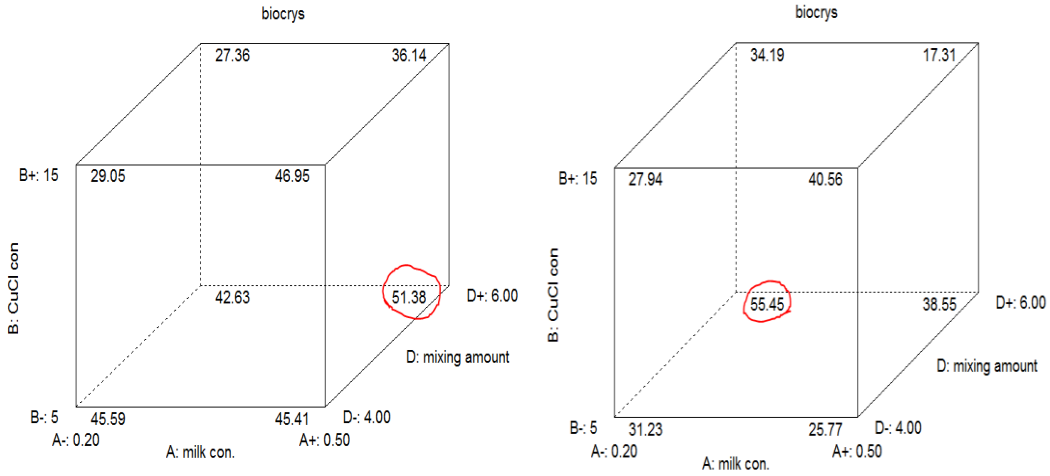


Figure 4.6. Cube plot of effect the milk concentration, mixing amount and $CuCl_2.2H_2O$ concentration on biocrystallograms with actual values for 6 ml mixing volume, 40°C and 60 %RH.

Interaction between mixing amount and $CuCl_2.2H_2O$ concentration prove that 5% $CuCl_2.2H_2O$ concentration, 1/1 mixing ratio, 6 ml amount of mixing, 40°C temperature and 60% humidity are the possible optimum conditions for the experiment. However the level of milk concentration could not be certainly decided because it is an insignificant factor of this design.

The cube plots (Figure 4.6) were generated for biocrystallograms obtained under the conditions where 5% $CuCl_2.2H_2O$ concentration, 6 ml mixing amount were used and, 40°C and 60 %RH drying conditions were applied. According to these plots, different mixing ratio values changed the biocrystallogram scores at different milk

concentration. But these values are very close to each other. If mixing ratio value is 1/1, biocrystallogram score was 55.45 at 0.2% milk concentration. If mixing ratio value was 3/1, biocrystallogram score was changed to 51.38 when milk concentration was 0.5%. In conclusion, 5% $CuCl_2.2H_2O$ concentration, 6 ml amount of mixing at 40°C, 60% RH were selected as the optimum value of experimental condition. On the other hand inorder to decide on the mixing ratio and milk concentration, the experiment was repeated with selecting milk concentration and mixing ratio in range of 0.2% to 0.5 % and 1/1 to 3/1, respectively.

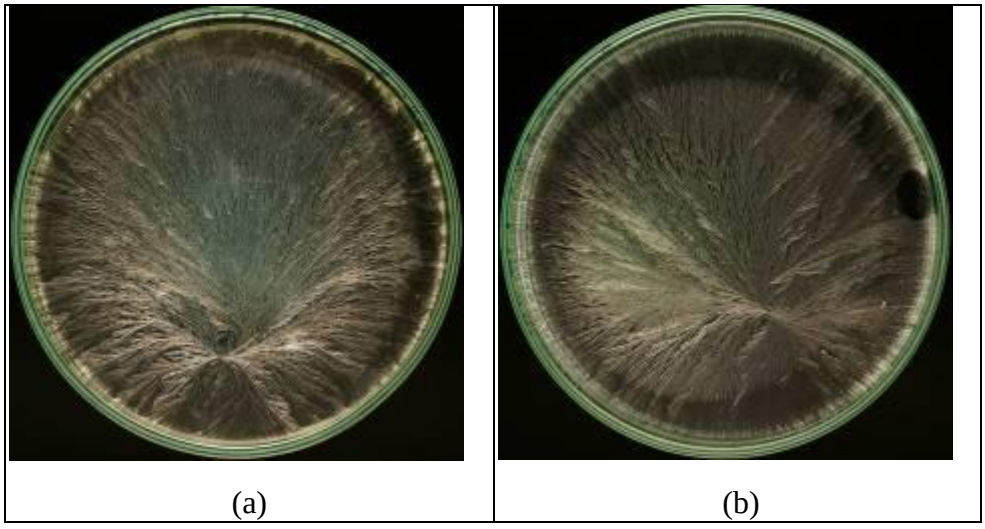


Figure 4.7. Biocrystallograms images from the 3rd design of experiment. ((a) 0.5% milk, 5% $CuCl_2.2H_2O$ concentration, 3/1 mixing ratio, 6 ml mixing amount, 40°C, 60% RH (b) 0.5 milk, 5% $CuCl_2.2H_2O$ concentration, 1/1 mixing ratio, 6 ml mixing amount, 40°C, 60% RH).

4.4. Validation of Biocrystallization

After the screening and optimization step, the optimum value of each factor was determined as shown in Table 4.6. They are validated at their optimum values using raw milk and milk samples spiked with Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb), UHT milk and UHT milk samples containing Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb). From each sample, 60 biocrystallogram images were prepared.

Table 4.6. Optimum values of factors

Factor	Optimum Values
Milk Concentration (%)	0.5
$CuCl_2.2H_2O$ Concentration (%)	5
Mixing Ratio (Milk/ $CuCl_2.2H_2O$)	3/1
Amount of Mixture (ml)	6
Drying Temperature (°C)	40
Drying Humidity (RH %)	60
Drying Time (h)	22

4.4.1. Visual Evaluation of Valid Biocrystallogram Images

In this study, the descriptive test was adapted and modified from ISO-Norm 11035, 1994 (Huber. et al., 2010). Among 60 biocrystallogram images per samples, multi-centered and failed integrated biocrystallogram images were discarded. Only four biocrystallograms per sample were randomly selected among 60 biocrystallogram images. Panelists scored raw milk and raw milk samples spiked with Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb), UHT milk and UHT milk samples containing Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb) from 1 to 9 according to 5 criteria including centre co-ordination, regulation of the branches, length of the branches, density of the branches, lemniscate form and overall these criteria. The results of visual analyses were given in Appendix D Table D.1, D.2, D.3, D.4 and D.5.

According to Figure 4.8 in the visual inspection and evaluation of the raw milk samples according to the centre co-ordination criteria was scored as 6.83. Besides, raw milk spiked with penicillin G 2, 4 and 8 ppb were scored 7.91, 7.75 and 7.83. Statistically, significant differences were observed in between raw milk and the one spiked with penicillin G 2, 4 and 8 ppb samples ($p< 0.05$) (Appendix D.1). On the other hand, no significant differences were determined among the samples containing different amount of penicillin G ($p> 0.05$) (Appendix D.1).

In the raw milk samples, the criteria for regulation of the branches was scored 7.75. Besides, raw milk samples spiked with penicillin G in the amount of 2, 4 and 8 ppb were scored as 7.5, 7.08 and 7.25. The scores of raw milk and raw milk spiked with penicillin G in the amount of 2 ppb showed similarity. Statistically there were no significant differences between each other ($p > 0.05$) (Appendix D.1). Moreover, there were no significant differences observed between the scores for raw milk spiked with 4 ppb and 8 ppb ($p > 0.05$). On the other hand, compelling differences were observed in the scores of raw milk and raw milk spiked with penicillin G in the amount of 4 ppb and 8 ppb samples ($p < 0.05$).

The scores of the criteria for the length of the branches were significantly different for raw milk and raw milk containing 2 ppb penicillin G ($p < 0.05$). But the scores for 2 and 4 ppb Penicillin G samples were statistically not different from each other ($p > 0.05$) (Appendix D.1).

The score for the density of the branches of raw milk was determined as 7.91. The raw milk samples spiked with 2, 4 and 8 ppb scored as 6.91, 6.5 and 7.16. As a result, the raw milk samples were successfully discriminated from the spiked samples conatining penicillin G (2, 4 and 8 ppb) according to this criteria ($p<0.05$) (Appendix D.1).

Lemniscate form of the raw milk samples scored 8.0, and the raw milk spiked with 2, 4 and 8 ppb were 8.33, 8.91 and 5.75. Lemniscates form of the raw milk and samples spiked with different concentration of penicillin G were significantly different from each other ($p<0.05$) (Appendix D.1).

Overall scores of raw milk and raw milk spiked with penicillin g (2, 4 and 8 ppb) were 7.6, 7.4, 7.32 and 7.03 respectively. Statistically there were significant differences between raw milk and samples spiked with different concentration of penicillin G ($p<0.05$) (Appendix D.1).

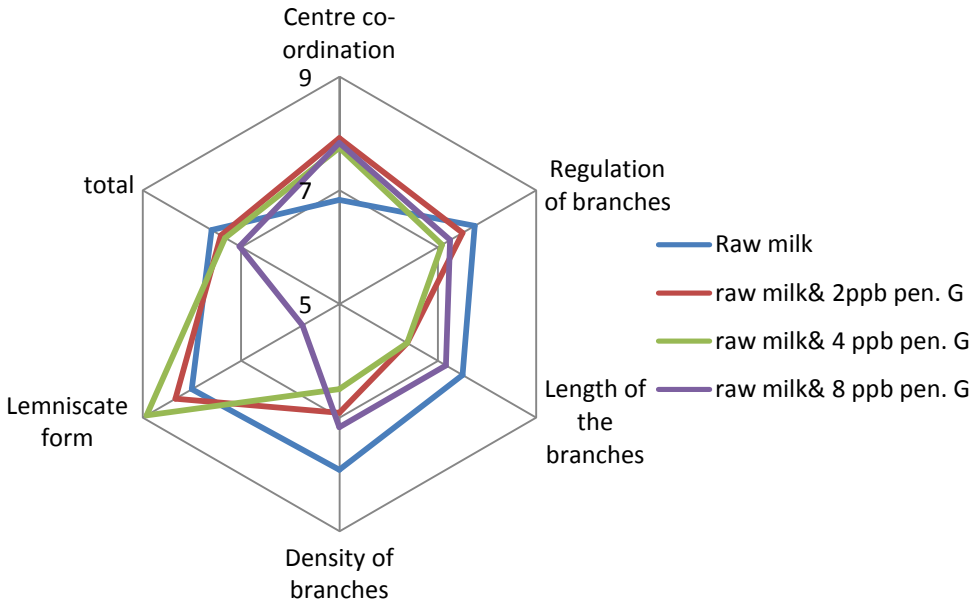
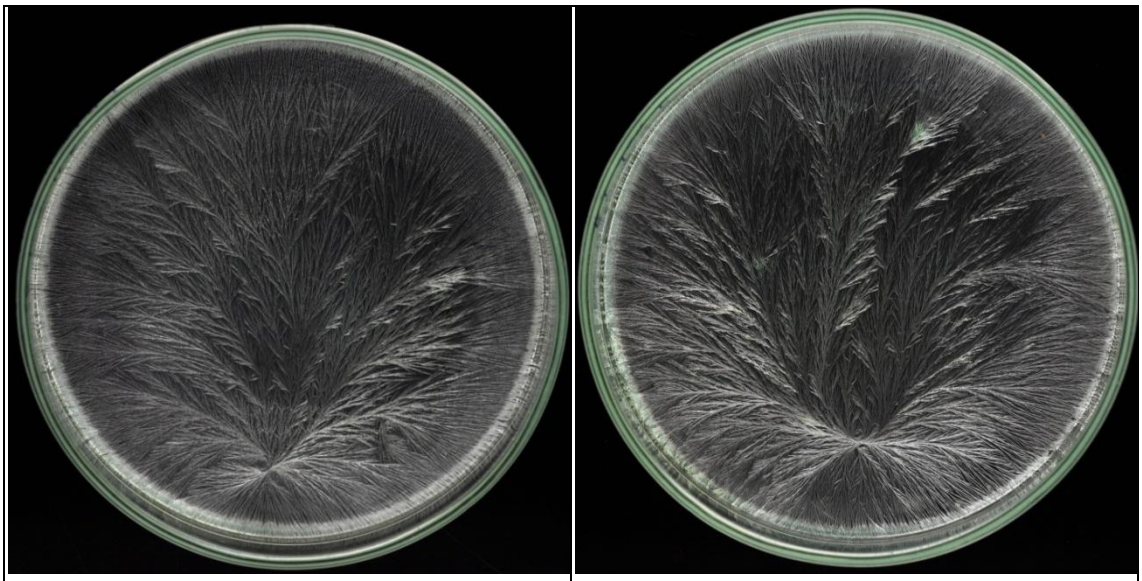
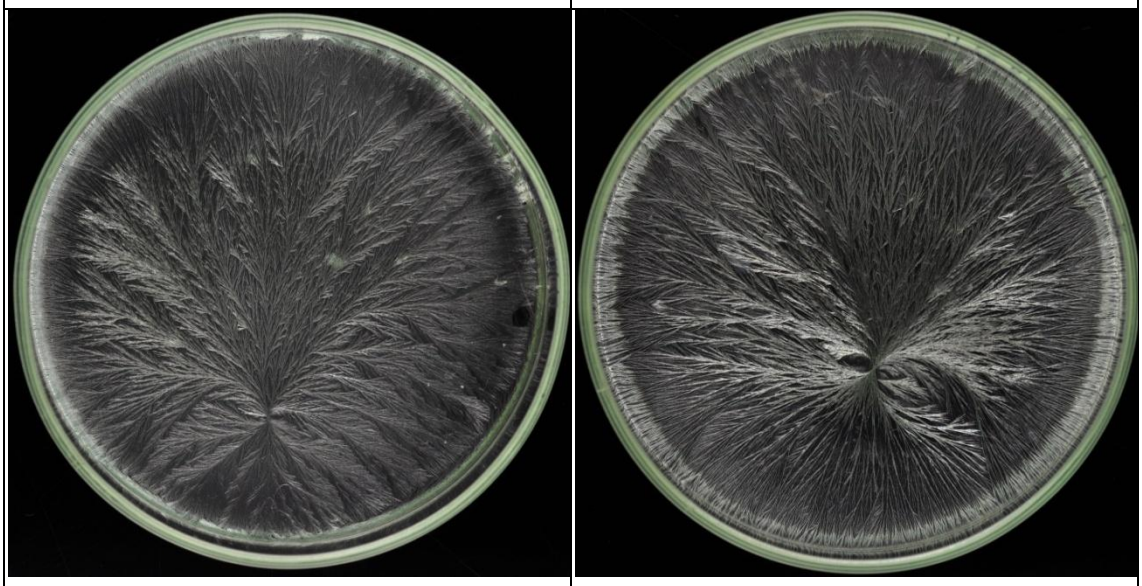


Figure 4.8. Graphical presentation of the mean panel scores, with criteria between raw milk and spiked with Penicillin G (2, 4, 8 ppb).



a) Raw milk

b) Raw milk spiked with Penicillin G (2 ppb)



c) Raw milk spiked with Penicillin G (4 ppb)

d) Raw milk spiked with Penicillin G (8 ppb)

Figure 4.9. Example biocrystallograms images raw milk and raw milk spiked with Penicillin G (2, 4, and 8 ppb)

According to Figure 4.10 and 11, the score for the centre co-ordination of raw milk was determined as 6.83. Besides, raw milk spiked with Ampicillin 2, 4 and 8 ppb were scored as 7.66, 7.25 and 8.0. Statistically, significant differences were observed among the raw milk and samples spiked with penicillin G 2, 4 and 8 ppb ($p < 0.05$) (Appendix D.2).

The score for regulation of the branches of raw milk was 7.75 and the raw milk spiked with different amount of Ampicillin (2, 4 and 8 ppb) were scored as 6.66, 6.75 and 7.58. The scores for raw milk and samples containing 2 and 4 ppb Ampicillin were

statistically different ($p<0.05$) (Appendix D.2). But the method was failed to discriminate the samples spiked with 8 ppb ampicillin ($p> 0.05$). Moreover, no significant differences were observed among the raw milk samples spiked with 2 and 4 ppb Ampicillin ($p> 0.05$) (Appendix D.2). The criteria of length of the branches successfully discriminated the raw milk and samples containing Ampicillin in the amount of 2, 4 and 8 ppb ($p< 0.05$) (Appendix D.2).

Density of the branches of raw milk was scored as 7.91, raw milk spiked with 2, 4 and 8 ppb samples were scored as 7.25, 7.33 and 7.83. The scores for raw milk and samples spiked with Ampicillin 2 and 4 ppb were statistically different ($p< 0.05$). But this criteria was not succeded in discrimination of the raw milk samples spiked with 8 ppb Ampicillin ($p> 0.05$) (Appendix D.2).

Lemniscate form of the raw milk samples scored 8.0; raw milk samples spiked with 2, 4 and 8 ppb Ampicillin had 8.5, 7.08 and 6.75 points. Lemniscates form of raw milk and spiked with different concentration of Ampicillin samples were significantly different from each other ($p<0.05$) (Appendix D.2).

Overall score of the raw milk and samples spiked with 2, 4 and 8 ppb Ampicillin scored as 7.6, 7.4, 7.03 and 7.58. A clear discrimination could not be obtained for the raw milk and spiked with different concentration of Ampicillin samples (Appendix D.2).

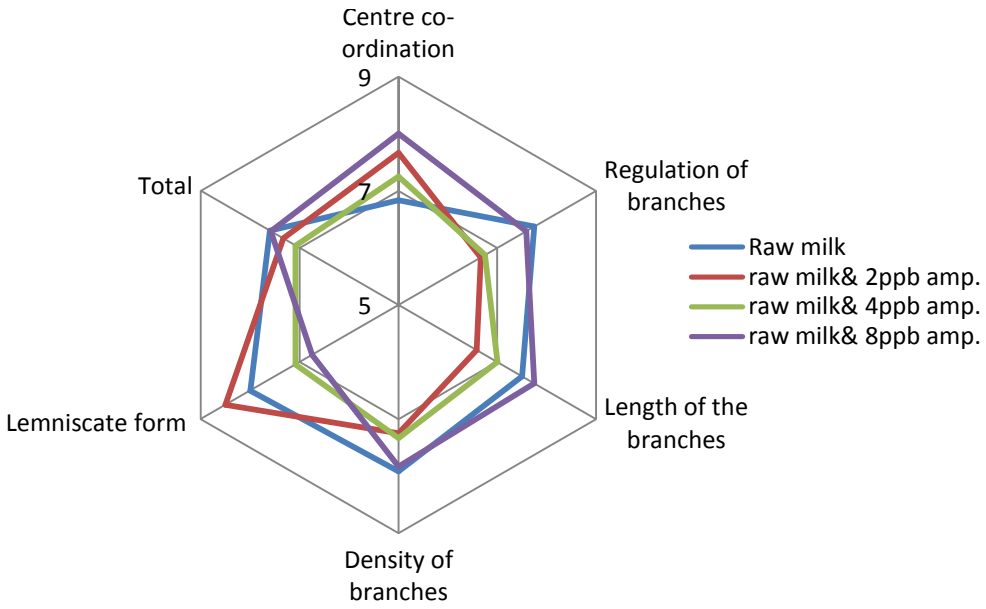
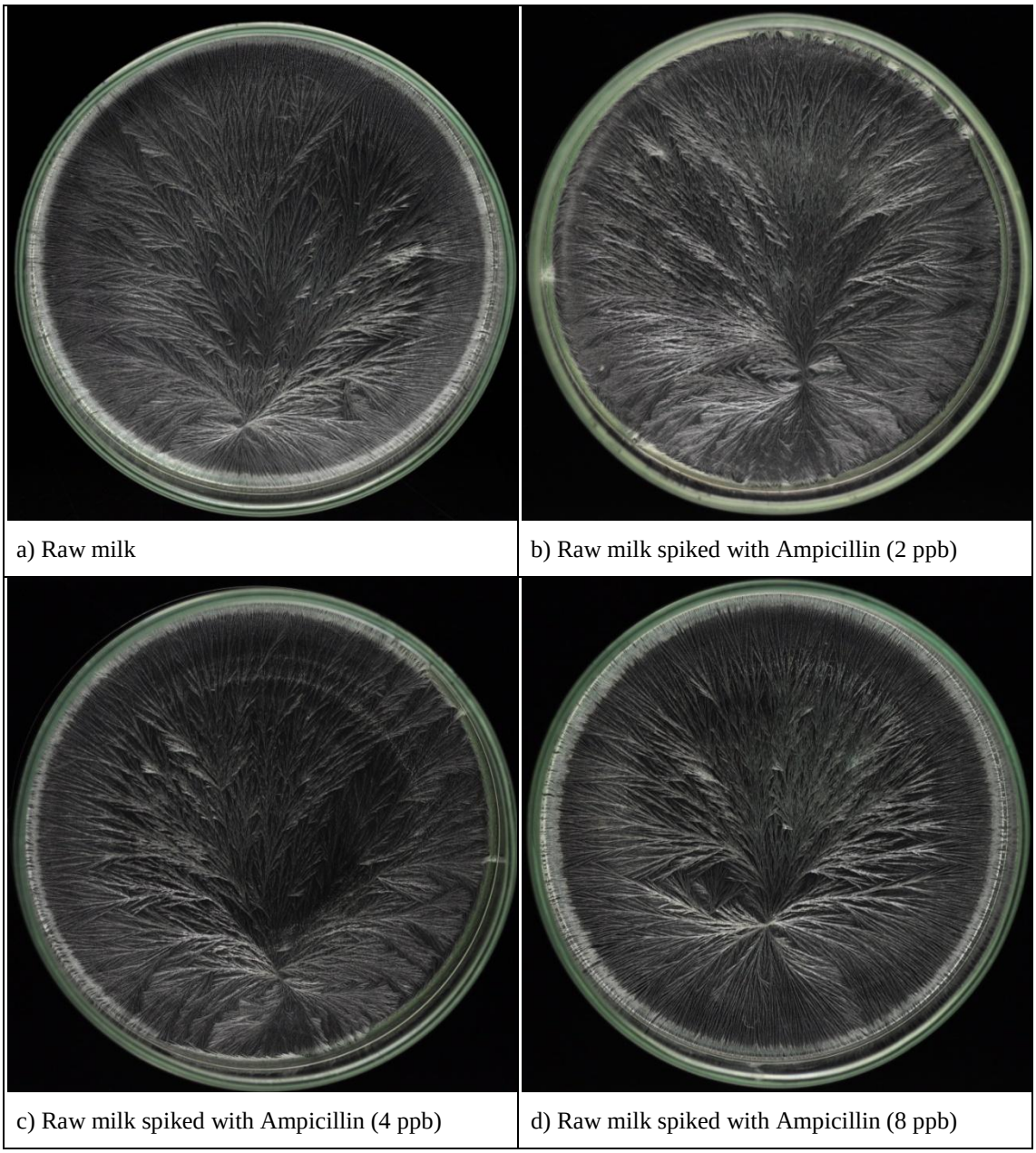


Figure 4.10. Graphical presentation of the mean panel scores, with criteria between raw milk and spiked with Ampicillin (2, 4, 8 ppb)



a) Raw milk

b) Raw milk spiked with Ampicillin (2 ppb)

c) Raw milk spiked with Ampicillin (4 ppb)

d) Raw milk spiked with Ampicillin (8 ppb)

Figure 4.11. Example biocrystallograms images raw milk and raw milk spiked with Ampicillin (2, 4, and 8 ppb)

The scores of visual evaluation of UHT milk and UHT milk spiked with Penicillin G were shown in Figure 4.12 and 4.13. Centre co-ordination of UHT milk had 6.83 points and UHT milk spiked with Penicillin G 2, 4 and 8 ppb were scored 7.91, 7.75 and 7.83. Statistically, there were significant differences between UHT milk and samples spiked with penicillin ($p< 0.05$). On the other hand, the same differences could not be obtained between UHT milk containing different concentration of Penicillin G ($p> 0.05$) (Appendix D.3).

The criteria of regulation of the branches of UHT milk had 7.75 points and showed similarity with UHT milk spiked with 2 ppb Penicillin G samples. The same trend was observed in samples containing 4 and 8 ppb penicillin G ($p > 0.05$). Statistical differences were obtained for only these two groups ($p < 0.05$) (Appendix D.3).

Discrimination of biocrystallograms based on the criteria of length of the branches could not be done clearly. There was a significant differences observed in between only UHT milk and UHT milk sample spiked with 2 ppb Penicillin G ($p < 0.05$) (Appendix D.3).

Density of the branches of UHT milk was scored 8.0, UHT milk spiked with 2, 4 and 8 ppb samples had 8.33, 8.91 and 5.75 points. UHT milk and UHT milk samples spiked with different concentration of Penicillin G were significantly different ($p < 0.05$). However we could not see any difference among UHT milk samples spiked with Penicillin G 2, 4 and 8 ppb ($p > 0.05$) (Appendix D.3).

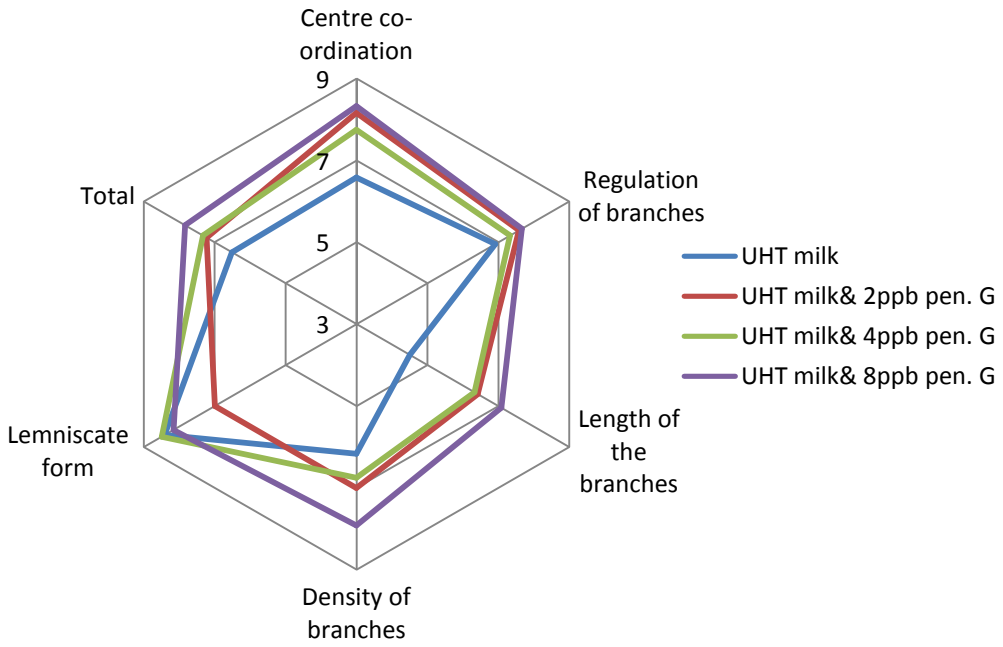
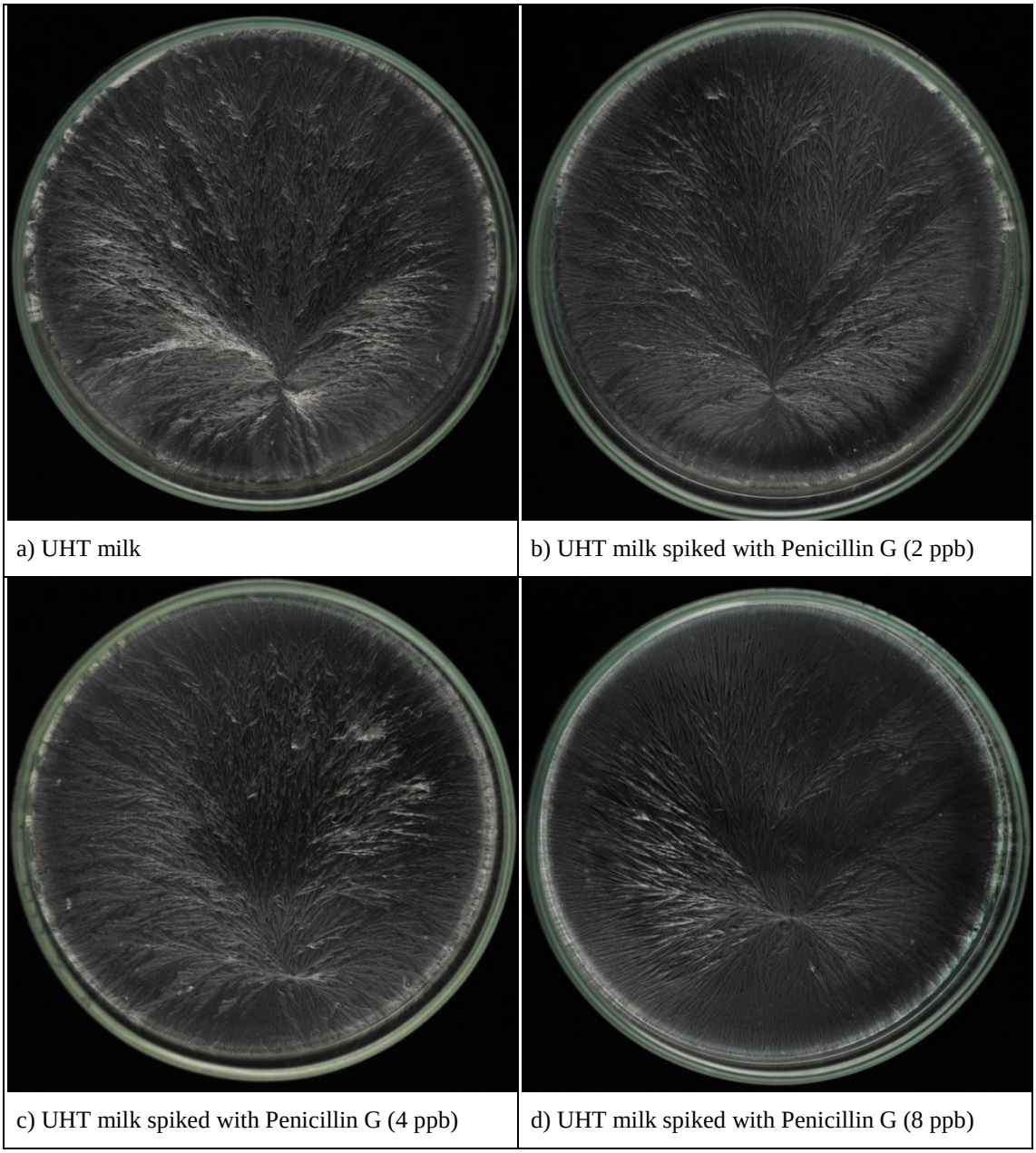


Figure 4.12. Graphical presentation of the mean panel scores, with criteria between UHT milk and with containing Penicillin G (2, 4, 8 ppb)

Lemniscate form of the UHT milk samples scored 8.41; UHT milk spiked with 2, 4 and 8 ppb samples received 7.0, 8.5 and 8.16 points. Lemniscates form of UHTmilk

and samples spiked with different concentration of Penicillin G were not regularly distinguished (Appendix D.3).

Overall scores of UHT milk and UHT milk having 2, 4 and 8 ppb penicillin G scored as 6.51, 7.23, 7.33 and 7.83, respectively. A clear discrimination was observed between UHT milk and spiked samples ($p < 0.05$) (Appendix D.3). But we could not observe any differences among the spiked samples.



a) UHT milk | b) UHT milk spiked with Penicillin G (2 ppb)

c) UHT milk spiked with Penicillin G (4 ppb) | d) UHT milk spiked with Penicillin G (8 ppb)

Figure 4.13. Example biocrystallograms images UHT milk and UHT milk spiked with Penicillin G (2, 4, and 8 ppb)

The scores of visual evaluation of UHT milk and UHT milk spiked with Ampicillin were shown in Figure 4.14 and Figure 4.15. Centre co-ordination criteria for UHT milk was determined as 6.58 and UHT milk spiked with Ampicillin 2, 4 and 8 ppb were scored 8.66, 8.58 and 8.50. Statistically, significant differences were observed between UHT milk and samples spiked with Ampicillin ($p< 0.05$). On the other hand, the same differences could not be obtained among the spiked samples ($p> 0.05$) (Appendix D.4).

Regulation of the branches of UHT milk was scored as 6.91 and samples spiked with Ampicillin in the amount of 2, 4 and 8 ppb scored as 8.0, 7.75 and 7.66. Regulation of the branches of UHT milk biocrystallograms were statistically different from spiked samples ($p< 0.05$). Although 2 ppb Ampicillin containing UHT milk sample could be distinguished from the one having 4 ppb, samples with 4 and 8 ppb Ampicillin could not be discriminated statistically ($p> 0.05$) (Appendix D.4).

The criteria of length of the branches of UHT milk scored 4.5 and UHT milk spiked with Ampicillin samples 2, 4 and 8 ppb scores were 8.5, 8.0 and 8.12. Length of the branches of UHT milk biocrystallograms were statistically different from UHT milk spiked with Ampicillin (2, 4 and 8 ppb) ($p< 0.05$). Although UHT milk spiked with 2 ppb Ampicillin samples could be distinguished from the one containing 4 ppb, UHT milk spiked with 4 and 8 ppb Ampicillin could not be discriminated statistically ($p> 0.05$) (Appendix D.4).

Density of the branches of UHT milk was determined as 6.16, UHT milk spiked with 2, 4 and 8 ppb samples scored as 8.33, 7.91 and 7.75. Density of branches of UHT milk and UHT milk spiked with Ampicillin samples were significantly different ($p< 0.05$). However there were no significant differences between density of branches of raw milk spiked with different levels of Ampicillin (2, 4 and 8 ppb) ($p> 0.05$) (Appendix D.4).

Lemniscate form of the UHT milk samples scored 8.41, UHT milk spiked with 2, 4 and 8 ppb samples were 7.08, 8.58 and 7.75. Lemniscates form of UHT milk spiked with different concentration of Ampicillin samples was not regularly distinguished.

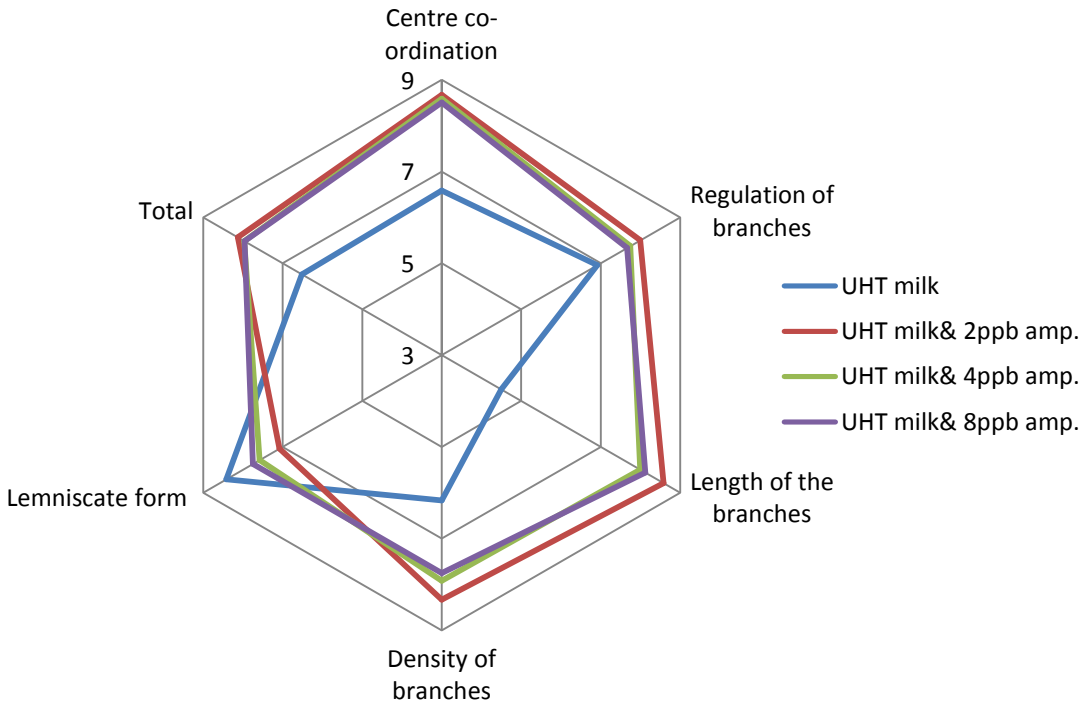
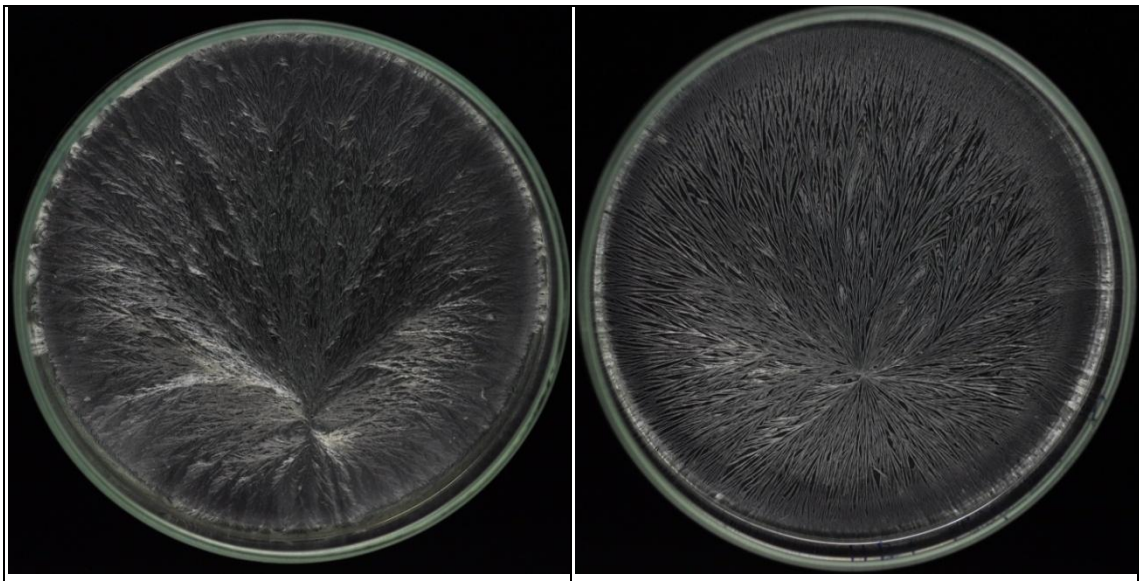


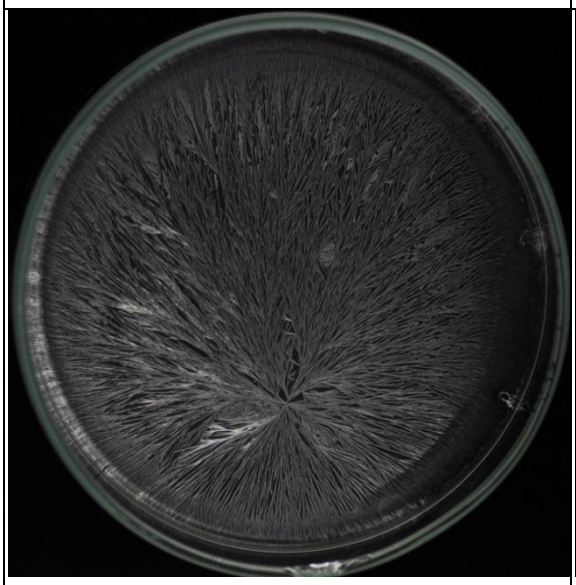
Figure 4.14. Graphical presentation of the mean panel scores, with criteria between UHT milk and with containing Ampicillin (2, 4, 8 ppb)

Overall scores of UHT milk and samples containing Ampicillin in the amount of 2, 4 and 8 ppb scored as 6.51, 8.13, 7.96 and 7.95, respectively. A clear discrimination was observed between UHT milk and spiked samples ($p < 0.05$). The same discrimination could not be observed among the samples spiked with Ampicillin ($p > 0.05$) (Appendix D.4).

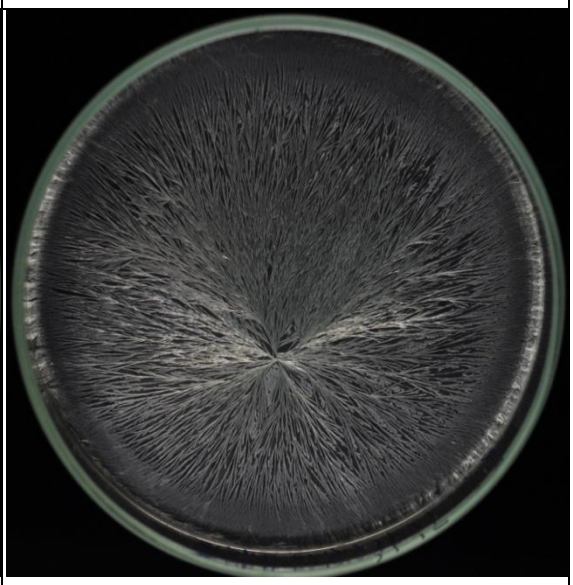


a) UHT milk

b) UHT milk spiked with Ampicillin (2 ppb)



c) UHT milk spiked with Ampicillin (4 ppb)



d) UHT milk spiked with Ampicillin (8 ppb)

Figure 4.15. Example biocrystallograms images UHT milk and UHT milk spiked with Ampicillin (2, 4, and 8 ppb)

4.5. Confirmation of Biocrystallization

Confirmation of this method was done by means of naturally contaminated raw milk taken from the cow treated with Penicillin G. Each biocrystallogram images were obtained using the same optimum conditions given in Table 4.7. Again failed integrated

and multi-centered images were discarded and only four images were selected randomly and evaluated visually.

4.5.1. Visual Evaluation of Confirmed Biocrystallization Images

Visual evaluation results of raw milk and naturally contaminated raw milk were shown in the Figure 4.16 and 4.17. Five discriptive criteria including centre co-ordination, regulation of branches, length of the branches, density of the branches and lemniscate form for raw milk biocrystallograms scored as 6.83, 7.75, 7.5, 7.91 and 8.0, respectively.

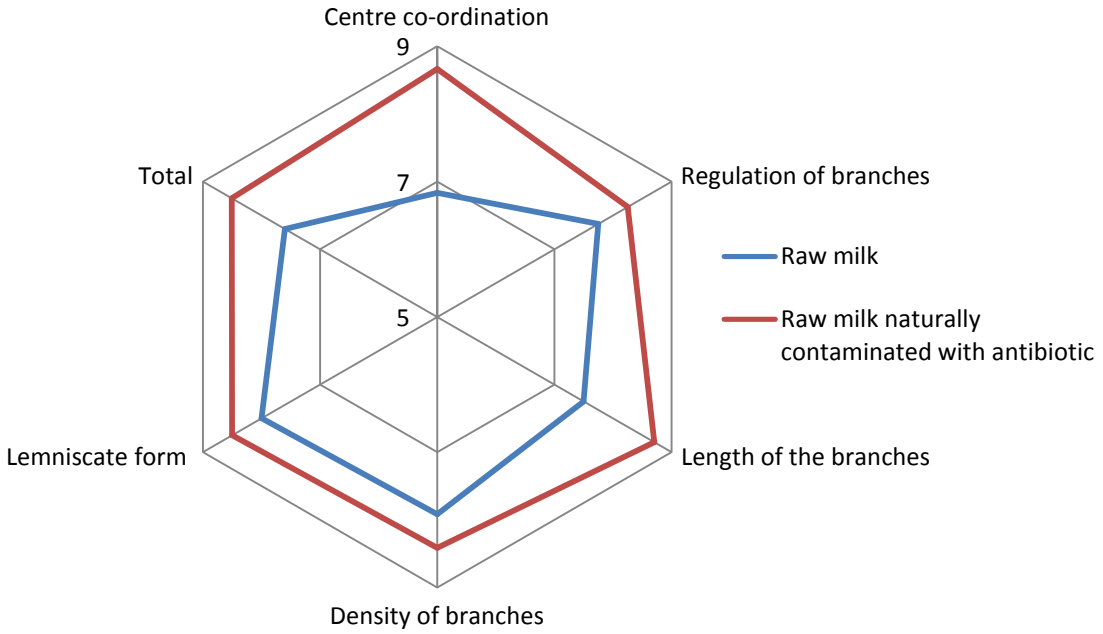
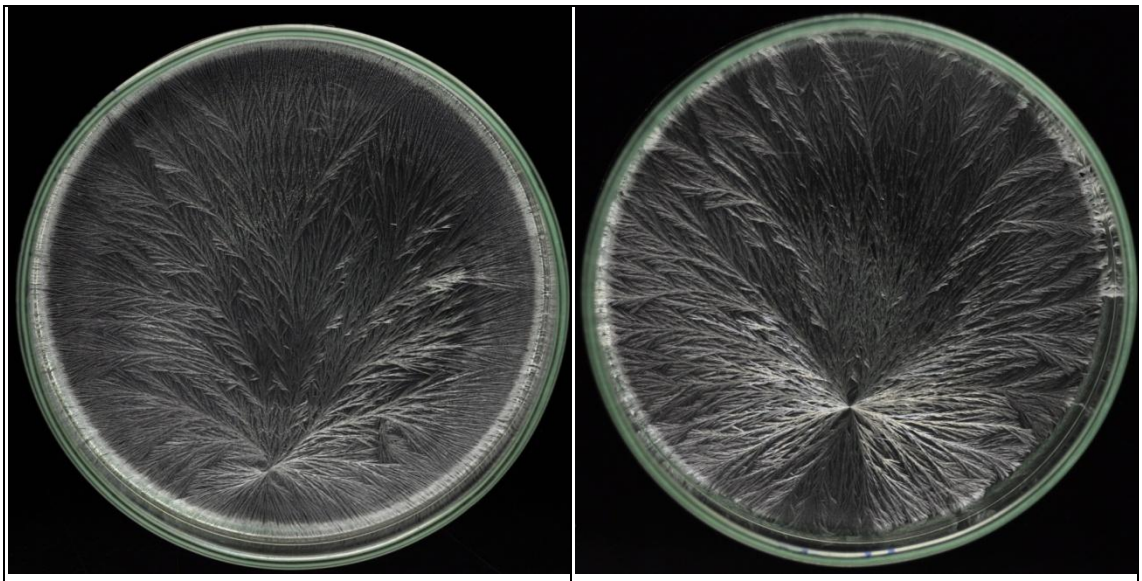


Figure 4.16. Graphical presentation of the mean panel scores, with criteria between raw milk and with naturally containing antibiotic



a) Raw milk

b)Raw milk naturally contaminated with antibiotic

Figure 4.17. Example biocrystallograms images raw milk and raw milk naturally contaminated with antibiotic

On the other hand, the same criteria for naturally contaminated raw milk sample were found to be 8.66, 8.25, 8.7 and 8.5 respectively. Overall scores of biocrystallograms of raw milk and naturally contaminated sample were 7.6 and 8.5. Statistically, antibiotic residues in raw milk samples were significantly different from antibiotic free raw milk samples according to all criteria ($p<0.05$) (Appendix D.5).

Huber, et al. (2010) tried to standardize visual evaluation biocrystallization. For this purpose, they optimized the visual analyses norms and validated the description criteria to discriminate the organic and the conventional carrot samples. Before this standardization there are few studies where biocrystallograms were evaluated via computerized analyses (Andersen, et al. 1999; Meelursarn, 2007; Unluturk et. al, 2011). Kuscu, (2008) tried visually to evaluate the peppers grown both organically and conventionally using a statistical approach. Our results showed that biocrystallization process and the visual evaluation of the biocrystallogram images by using five descriptive criteria including centre co-ordination, regulation, length and density of the branches and lemniscate form of biocrystallograms that were chosen according to the study of Huber, et al. (2010) successfully used to distinguish the naturally contaminated

raw milk from the antibiotic free sample. Therefore, biocrystallization method was found to be efficacious for determination of antibiotic residues in raw milk.

CHAPTER 5

CONCLUSION

The origin of the idea of this study is based on an interest to develop a new method to detect the existence of antibiotic residues in milk. For this purpose "biocrystallization" method was decided to be investigated for determination of residues of beta- lactam groups of antibiotics (Penicillin G and Ampicillin) used in dairy industry commonly.

Since this method is very sensitive, an optimization study was nesessary to be carried out to generate the best biocrystallogram images. For this goal, the most important factors effecting the development of biocrystallograms were determined. These are milk and $CuCl_2.2H_2O$ concentration, their mixing amount and ratio, drying temperature, relative humidity and time. The best biocrystallograms images were tried to be developed based on three experimental design applied for screening and optimization purposes. The first two designs were inadequate to obtain the best biocrystallograms images. The third design gave satisfactory results. The optimum levels of these important factors were determined as 0.5% milk concentration, 5% $CuCl_2.2H_2O$ concentration, 3/1 mixing ratio (milk/ $CuCl_2.2H_2O$), 6 ml mixing amount, 30°C drying temperature, 60% drying relative humidity and 22 h drying time.

In the second step, biocrystallogram images using raw and UHT milk samples spiked with Penicillin G (2, 4, 8 ppb) and Ampicillin (2, 4, 8 ppb) were generated at the optimized conditions and evaluated with a visual inspection. Biocrystallization method was successfully used to distinguish raw milk and UHT milk from the samples spiked with different concentration of Penicillin G. On the other hand the results were inconclusive for discrimination of the raw milk from the milk samples containing Ampicillin. However, the method was able to distinguish UHT milk from the one's spiked with Ampicillin. Additionally, validation tests showed that the raw milk can be successfully discriminated from the one which is naturally contaminated with Penicillin G. In summary, it is shown that biocrystallization can be a potential method to be used in detection of antibiotic residues in milk. Especially, the common usage of antibiotic containing raw milk is produced UHT milk because of degradation of antibiotics and

reducing their level under the detection limits at high temperature. In this case, where rapid test kits give false negative result and the chromatographic methods result in low recovery values when the antibiotic residue levels are little over and under their MRLs. However this method still needs to be investigated more with different type of antibiotics. The visual evaluation has to be coupled and supported with computerized image analysis.

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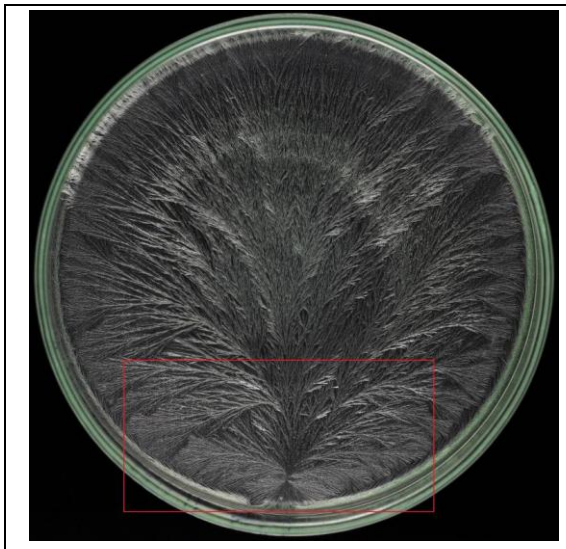
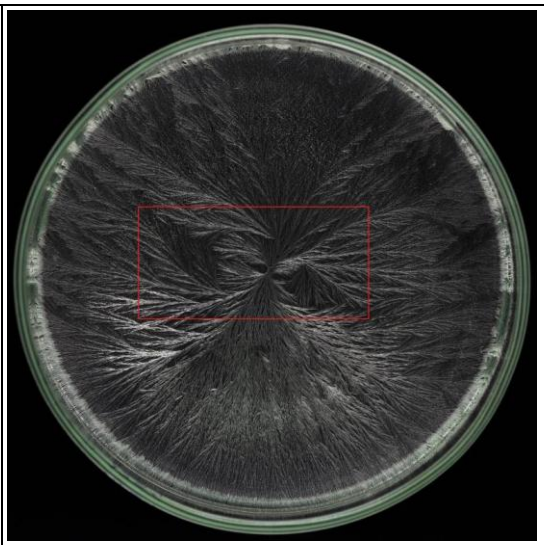
APPENDIX A

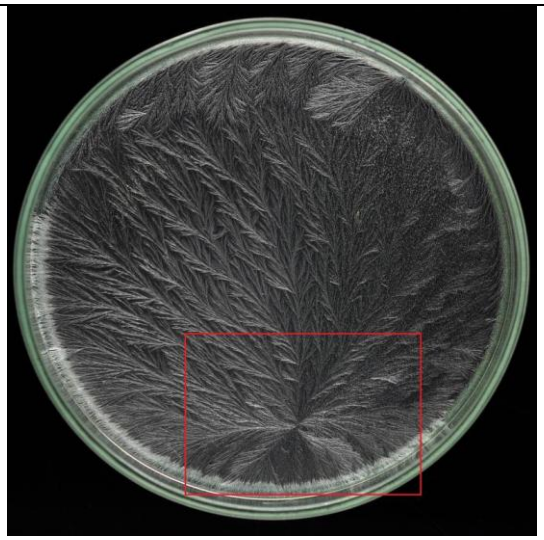
REFERENCE BIOCRYSTALLOGRAM IMAGES SHEET FOR VISUAL EVALUATION



1- point 1



2- point 4



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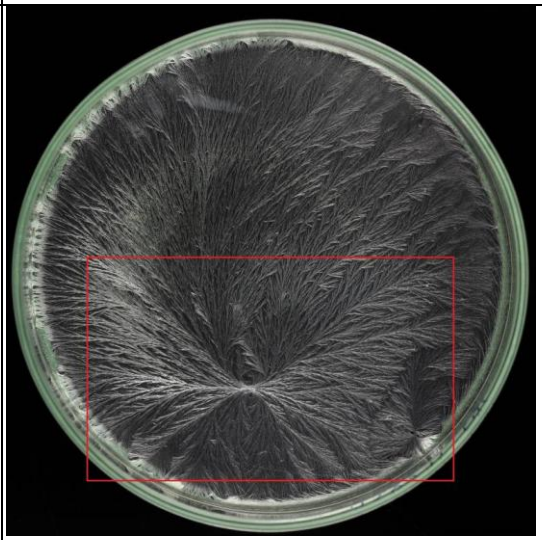
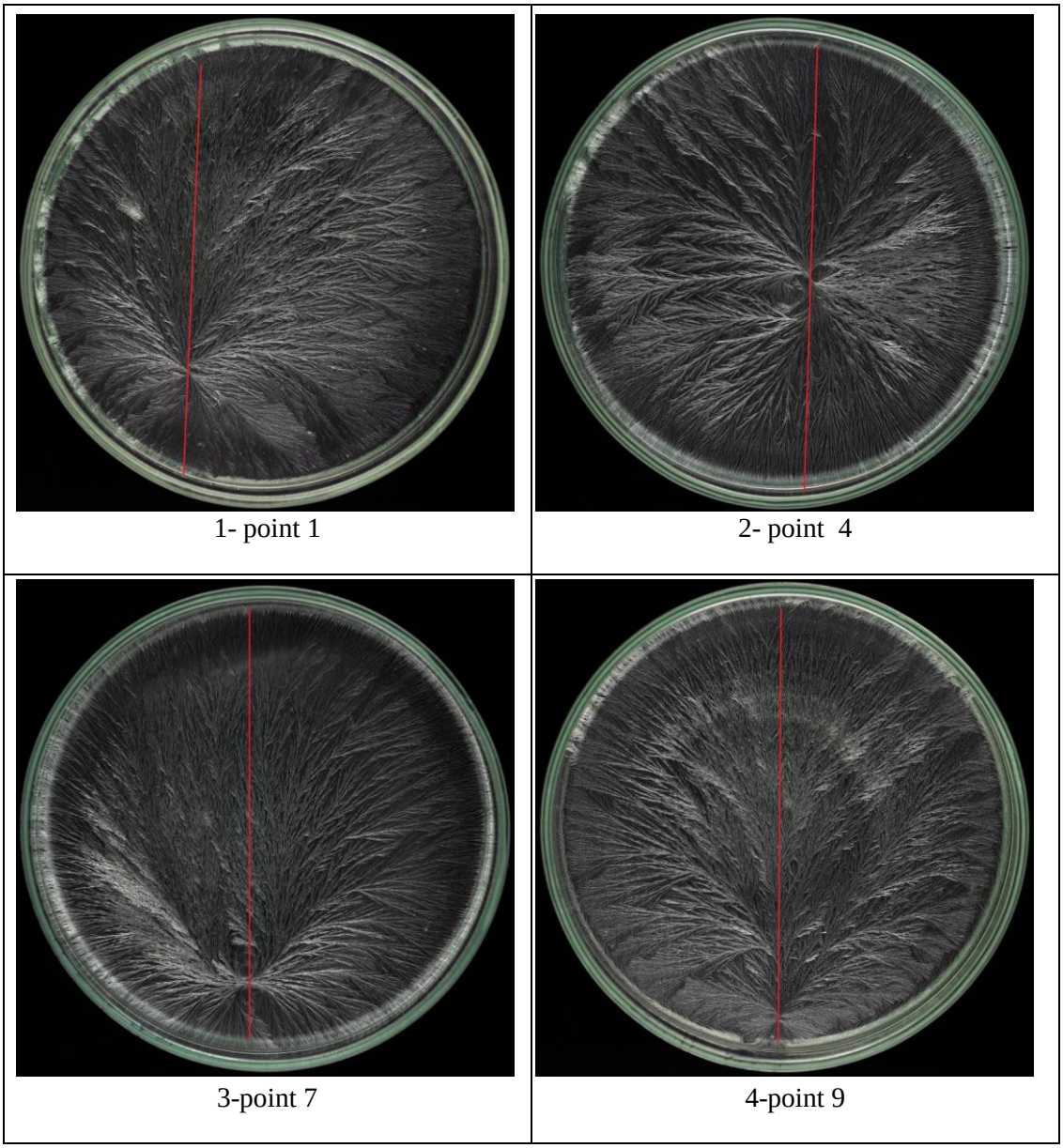


4-point 9

Figure A.1. Centre co-ordination: centre of biocrystallograms located near the side of the petri not mid-point or quite side of petri



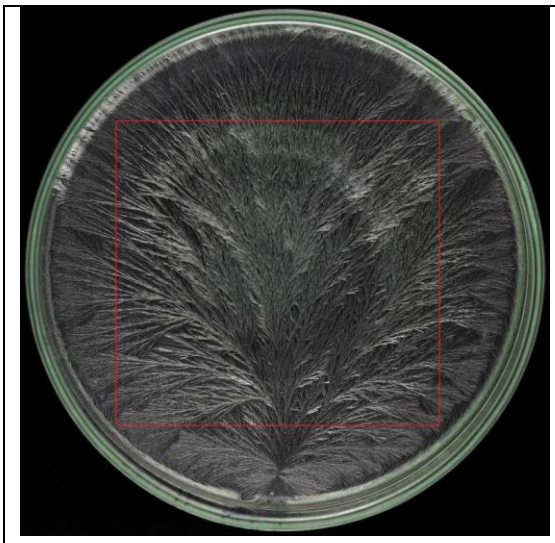
1- point 1

2- point 4

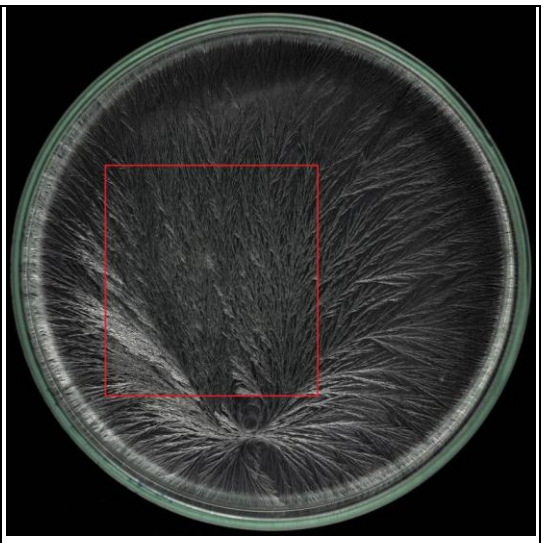
3-point 7

4-point 9

Figure A.2. Regulation of branches: branches show symmetrical distribution according to centre



1- point 1



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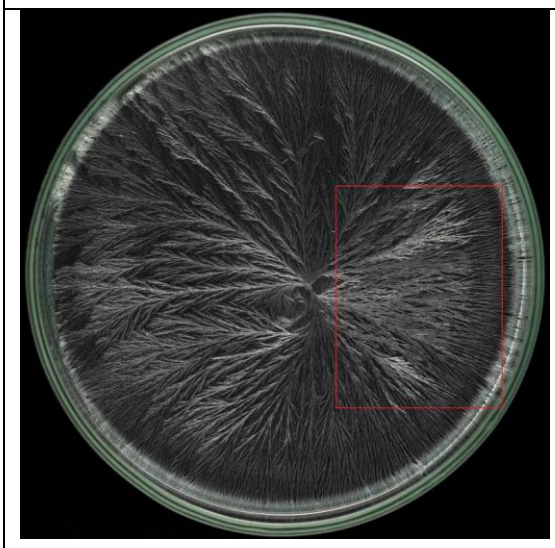

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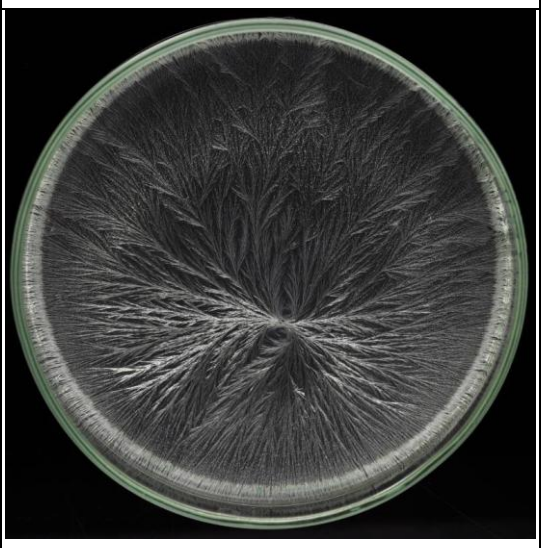
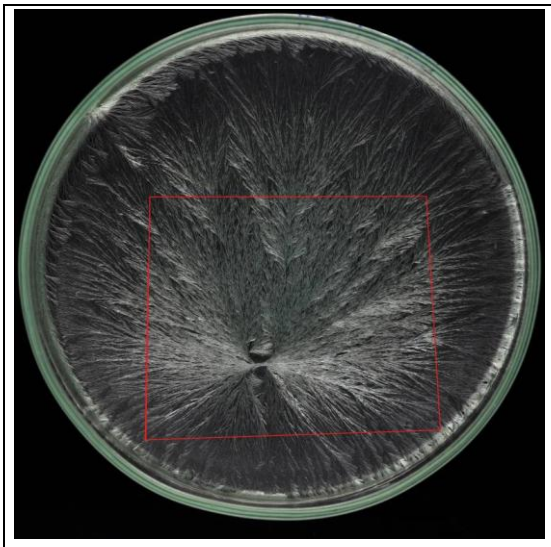
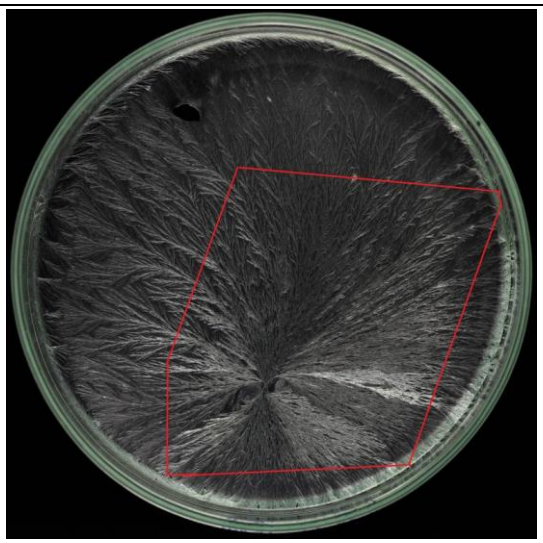


4-point 9

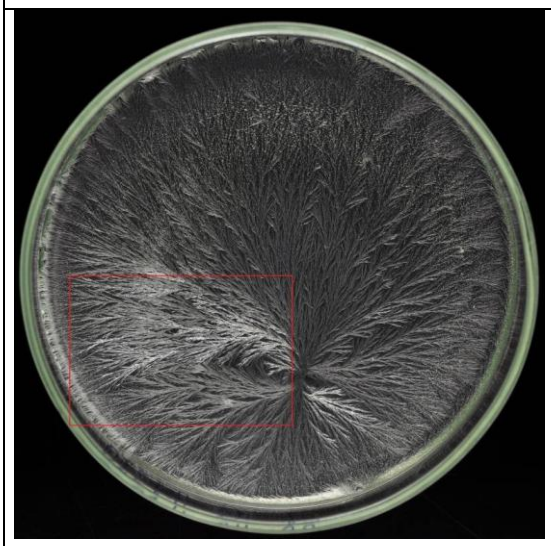
Figure A.3. Length of the branches: Branches must be long and entire not short or discrete, distribute in whole petri



1- point 1



2- point 4



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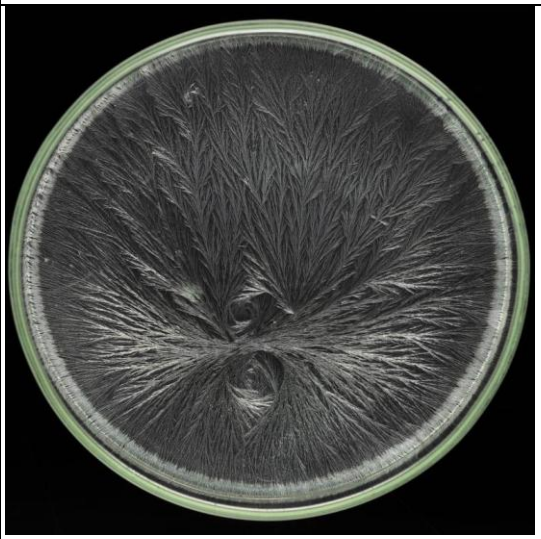
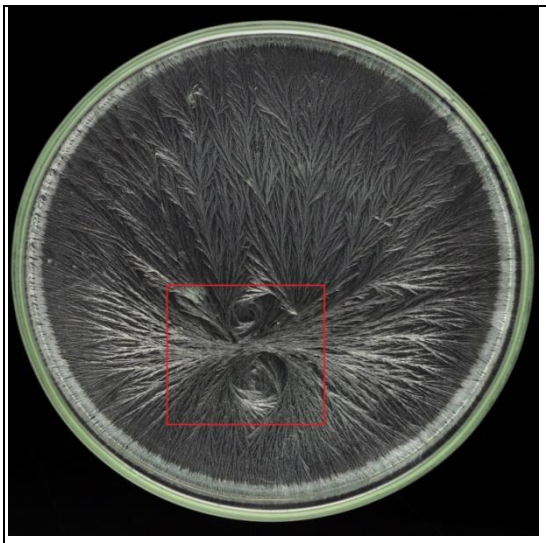


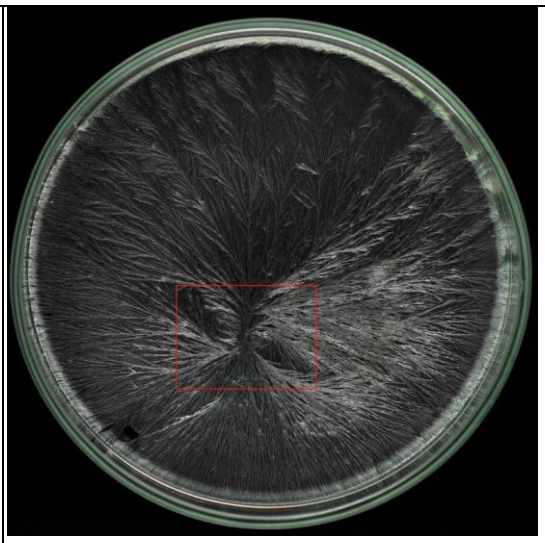
4-point 9

Figure A.4. Density of branches: they must be loud and clear, not intense or penetrating

.



1- point 1



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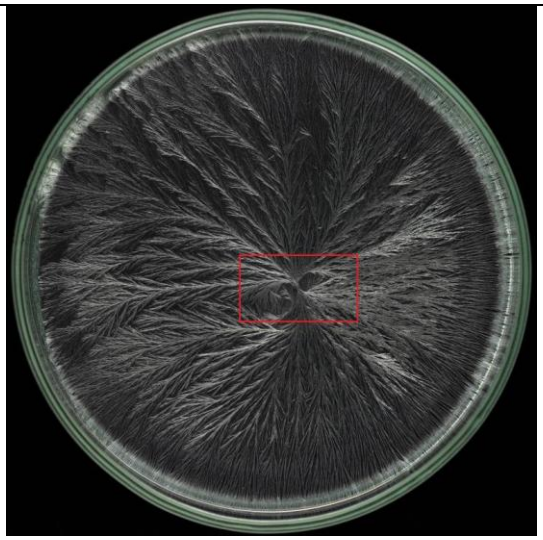
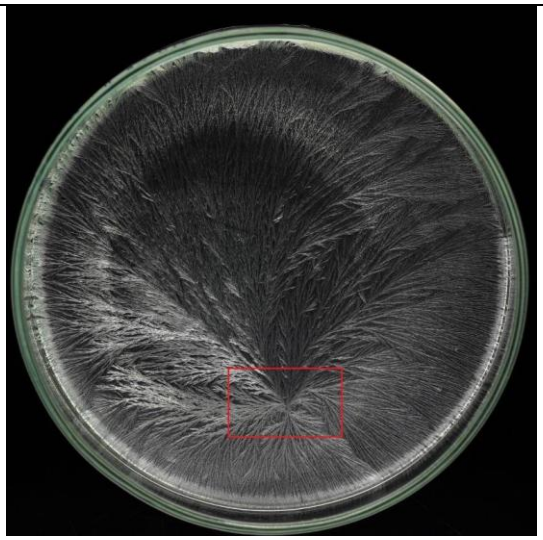


3-point 7



4-point 9

Figure A.5. Lemniscate form: the eight form composed at the centre of image do not be empty

APPENDIX B

SOMATIC CELL COUNT RESULTS

Table B.1. Comparisons between dye and surfactant results on somatic cell counting with flow cytometry

Somatic cell counts with Flow Cytometry	Dye	Surfactant
AO with PBS	-	135 388 [a] ± 6937
AO with Trit-X	139 637[a] ± 10250	-
EtBr with Trit-X	131 986[a] ± 8425	131 986[a] ± 8425

[a]value in a column is not significantly different by Tukey's test ($p<0.05$)

Table B.2. Comparisons between flow Cytometric and microscopic somatic cell counting results

Somatic cell counts	AO with PBS	AO with Trit-X	EtBr with Trit-X
Flow Cytometric Counts	135 388 [a] ± 6937	139 637[a] ± 10250	131 986[a] ± 8425
Microscopic Counts	130 748 ± 7835	136 523 ± 9623	133 214 ± 4867

[a]value in a column is not significantly different by Tukey's test ($p<0.05$)

APPENDIX C

DESIGN OF BIOCRYSTALLIZATION RESULTS

Table C.1. Design Expert output of 1st design of biocrystallization

ANOVA for selected factorial model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	30.54	7	4.36	18.49	0.0002	significant
A-milk concentr	*23.38*	*1*	*23.38*	*99.07*	*< 0.0001*	
B-cucl2 concen	*0.25*	*1*	*0.25*	*1.06*	*0.3335*	
C-mix ratio	*0.11*	*1*	*0.11*	*0.48*	*0.5099*	
AB	*1.36*	*1*	*1.36*	*5.75*	*0.0433*	
AC	*4.00*	*1*	*4.00*	*16.95*	*0.0034*	
BC	*0.44*	*1*	*0.44*	*1.87*	*0.2082*	
ABC	*1.00*	*1*	*1.00*	*4.24*	*0.0735*	
Pure Error	1.89	8	0.24			
Cor Total	32.43	15				

Std. Dev.	0.49	R-Squared	0.9418
Mean	3.04	Adj R-Sqaured	0.8908
C. V. %	15.97	Pred R- Squared	0.7671
PRESS	7.55	Adeq Precision	11.645

Table C.2. Design Expert output of 2nd design of biocrystallization

ANOVA for selected factorial model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	267.56	48	5.57	15.71	< 0.0001
A-milk con.	18.60	1	18.60	52.40	< 0.0001
B-cc con.	13.60	1	13.60	38.31	< 0.0001
C-mixed ratio	3.29	1	3.29	9.26	0.0027
D-mixed amount	5.94	1	5.94	16.74	< 0.0001
E-drying tem	0.32	1	0.32	0.89	0.3462
F-drying humdity	7.22	1	7.22	20.35	< 0.0001
G-drying time	17.54	1	17.54	49.41	< 0.0001
AB	0.66	1	0.66	1.86	0.1741
AC	3.906E-003	1	3.906E-003	0.011	0.9165
AE	14.54	1	14.54	40.96	< 0.0001
AF	3.29	1	3.29	9.26	0.0027
AG	9.38	1	9.38	26.43	< 0.0001
BC	0.035	1	0.035	0.099	0.7533
BE	0.47	1	0.47	1.33	0.2498
BF	1.72	1	1.72	4.85	0.0287
BG	3.75	1	3.75	10.58	0.0013
CE	1.41	1	1.41	3.97	0.0475
CG	1.13	1	1.13	3.18	0.0760
DE	9.38	1	9.38	26.43	< 0.0001
DF	13.60	1	13.60	38.31	< 0.0001
DG	25.63	1	25.63	72.21	< 0.0001
EF	1.72	1	1.72	4.85	0.0287
EG	1.13	1	1.13	3.18	0.0760
FG	16.50	1	16.50	46.50	< 0.0001
ABC	3.906E-003	1	3.906E-003	0.011	0.9165
ABE	0.19	1	0.19	0.54	0.4635
ABG	0.66	1	0.66	1.86	0.1741
ACE	0.66	1	0.66	1.86	0.1741
ACG	0.19	1	0.19	0.54	0.4635
AEF	12.69	1	12.69	35.76	< 0.0001
AEG	2.44	1	2.44	6.88	0.0094
AFG	4.79	1	4.79	13.48	0.0003
BCE	0.32	1	0.32	0.89	0.3462
BCG	0.035	1	0.035	0.099	0.7533
BEF	2.44	1	2.44	6.88	0.0094
BEG	1.13	1	1.13	3.18	0.0760
CEG	0.19	1	0.19	0.54	0.4635
DEF	30.94	1	30.94	87.18	< 0.0001
DEG	29.57	1	29.57	83.31	< 0.0001
DFG	1.13	1	1.13	3.18	0.0760
EFG	3.906E-003	1	3.906E-003	0.011	0.9165
ABCE	3.906E-003	1	3.906E-003	0.011	0.9165
ABCG	0.47	1	0.47	1.33	0.2498
ABEG	0.19	1	0.19	0.54	0.4635
ACEG	0.035	1	0.035	0.099	0.7533
BCEG	3.906E-003	1	3.906E-003	0.011	0.9165
DEFG	5.35	1	5.35	15.07	0.0001
ABCEG	3.29	1	3.29	9.26	0.0027
Residual	73.46	207	0.35		
Lack of Fit	46.96	79	0.59	2.87	< 0.0001
Pure Error	26.50128	0.21			
Cor Tota	341.03255				

Std. Dev.	0.60	R-Squared	0.7846
Mean	1.29	Adj R-Squared	0.7346
C.V. %	46.08	Pred R-Squared	0.6705
PRESS	112.36	Adeq Precision	16.636

Table C.3. Design Expert output of 3rd design of biocrystallization

ANOVA for selected factorial model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	18853.29	45	418.96	9.88	< 0.0001
A-milk con.	39.38	1	39.38	0.93	0.3380
B-CuCl con	825.20	1	825.20	19.47	< 0.0001
C-mixing ratio	1883.45	1	1883.45	44.43	< 0.0001
D-mixing amount	273.20	1	273.20	6.44	0.0130
E-tem	4765.32	1	4765.32	112.41	< 0.0001
F-RH	2252.88	1	2252.88	53.14	< 0.0001
AB	3.45	1	3.45	0.081	0.7763
AC	182.88	1	182.88	4.31	0.0409
AD	297.07	1	297.07	7.01	0.0097
AE	35.07	1	35.07	0.83	0.3657
AF	37.20	1	37.20	0.88	0.3517
BC	20.32	1	20.32	0.48	0.4907
BD	940.70	1	940.70	22.19	< 0.0001
BE	5.70	1	5.70	0.13	0.7149
BF	233.82	1	233.82	5.52	0.0213
CD	106.95	1	106.95	2.52	0.1161
CE	388.51	1	388.51	9.16	0.0033
CF	25.38	1	25.38	0.60	0.4413
DE	8.51	1	8.51	0.20	0.6553
DF	155.32	1	155.32	3.66	0.0591
EF	4.88	1	4.88	0.12	0.7352
ABD	652.51	1	652.51	15.39	0.0002
ABF	565.32	1	565.32	13.34	0.0005
ACD	134.07	1	134.07	3.16	0.0790
ACE	453.76	1	453.76	10.70	0.0016
ACF	79.70	1	79.70	1.88	0.1741
ADE	297.07	1	297.07	7.01	0.0097
ADF	29.07	1	29.07	0.69	0.4100
BCD	354.45	1	354.45	8.36	0.0049
BCE	51.26	1	51.26	1.21	0.2747
BCF	150.95	1	150.95	3.56	0.0627
BDE	239.26	1	239.26	5.64	0.0198
BDF	41.63	1	41.63	0.98	0.3246
BEF	155.32	1	155.32	3.66	0.0591
CDE	402.57	1	402.57	9.50	0.0028
CDF	138.20	1	138.20	3.26	0.0747
CEF	409.70	1	409.70	9.66	0.0026
DEF	39.38	1	39.38	0.93	0.3380
ACDF	297.07	1	297.07	7.01	0.0097
BCDE	10.70	1	10.70	0.25	0.6168
BCDF	416.88	1	416.88	9.83	0.0024
BCEF	0.38	1	0.38	9.030E-003	0.9245
BDEF	89.45	1	89.45	2.11	0.1502
CDEF	524.07	1	524.07	12.36	0.0007
BCDEF	835.38	1	835.38	19.71	< 0.0001
Residual	3476.14	82	42.39		
Lack of Fit	978.64	18	54.37	1.39	0.1661
Pure Error	2497.50	64	39.02		
Cor Total	22329.43	127			

Std. Dev.	6.51	R-Squared	0.8443
Mean	26.73	Adj R-Squared	0.7589
C.V. %	24.36	Pred R-Squared	0.6207
PRESS	8470.12	Adeq Precision	12.050

APPENDIX D

VISUAL EVALUATION RESULTS

Table D.1. Visual evaluation result of raw milk and raw milk spiked with Penicillin G (2, 4 and 8 ppb)

	Centre co-ordination	Regulation of the branches	Length of the branches	Density of the branches	Lemniscate form	Total view of Biocrystallogram
Raw milk	6.8333^{a} ±0.3028	7.7500^{b} ±0.2236	7.5000^{b} ±0.2236	7.9167^{c} ±0.1291	8.0000^{b} ±0.000	7.6000^{c} ±0.1049
Raw milk&2 ppb Penicillin G	7.9167^{b} ±03416	7.5000^{ab} ±0.000	6.3750^{a} ±0.4108	6.9167^{ab} ± 0.2582	8.3333^{c} ±0.1291	7.4083^{bc} ±0.1114
Raw milk&4 ppb Penicillin G	7.7500^{b} ±0.2236	7.0833^{a} ±0.3416	6.3750^{a} ±0.3062	6.5000^{a} ±0.3873	8.9167^{d} ±0.1291	7.3250^{b} ±0.1696
Raw milk&8 ppb Penicillin G	7.8333^{b} ±0.1291	7.2500^{a} ±0.3873	7.1667^{b} ±0.1291	7.1667^{b} ±0.5164	5.7500^{a} ±0.2236	7.0333^{c} ±0.0931

[a-d]values in a column with the same superscript are not significantly different by Fisher's test ($p<0.05$)

Table D.2. Visual evaluation result of raw milk and raw milk spiked with Ampicillin (2, 4 and 8 ppb)

	Centre co-ordination	Regulation of the branches	Length of the branches	Density of the branches	Lemniscate form	Total view of Biocrystallogram
Raw milk	6.8333^{a} ±0.3028	7.7500^{b} ±0.2236	7.5000^{c} ±0.2236	7.9167^{b} ±0.1291	8.0000^{c} ±0.000	7.6000^{c} ±0.1049
Raw milk&2 ppb Ampicillin	7.6667^{c} ±1291	6.6667^{a} ±0.4655	6.5833^{a} ±0.1291	7.2500^{a} ± 0.0000	8.5000^{d} ±0.0000	7.3333^{b} ±0.0931
Raw milk&4 ppb Ampicillin	7.2500^{b} ±0.0000	6.7500^{a} ±0.0000	7.0000^{b} ±0.0000	7.3333^{a} ±0.1291	7.0833^{b} ±0.1291	7.08333^{a} ±0.0258
Raw milk&8 ppb Ampicillin	8.0000^{d} ±0.000	7.5833^{b} ±0.1291	7.7500^{d} ±0.0000	7.8333^{b} ±0.1291	6.7500^{a} ±0.2236	7.5833^{c} ±0.0258

[a-d]values in a column with the same superscript are not significantly different by Fisher's test ($p<0.05$)

Table D.3. Visual evaluation result of UHT milk and UHT milk spiked with Penicillin G (2, 4 and 8 ppb)

	Centre co-ordination	Regulation of the branches	Length of the branches	Density of the branches	Lemniscate form	Total view of Biocrystallogram
UHT milk	6.5833^{a} ±0.2582	6.9167^{a} ±0.1291	4.5000^{a} ±0.0000	6.1667^{a} ±0.1291	8.4167^{c} ±0.1291	6.5167^{a} ±0.0931
UHT milk&2 ppb Penicillin G	8.1667^{c} ±01291	7.5833^{bc} ±0.2582	6.4167^{b} ±0.1291	7.0000^{b} ± 0.4472	7.0000^{a} ±0.0000	7.2333^{b} ±0.1033
UHT milk&4 ppb Penicillin G	7.7500^{b} ±0.3873	7.3333^{b} ±0.1291	6.3333^{b} ±0.3062	6.7500^{b} ±0.0000	8.5000^{c} ±0.0000	7.3333^{b} ±0.0258
UHT milk&8 ppb Penicillin G	8.3333^{c} ±0.1291	7.6667^{c} ±0.1291	7.08333^{c} ±0.2582	7.9167^{c} ±0.1291	8.1667^{b} ±0.1291	7.8333^{c} ±0.0683

[a-c]values in a column with the same superscript are not significantly different by Fisher's test ($p<0.05$)

Table D.4. Visual evaluation result of UHT milk and UHT milk spiked with Ampicillin G (2, 4 and 8 ppb)

	Centre co-ordination	Regulation of the branches	Length of the branches	Density of the branches	Lemniscate form	Total view of Biocrystallogram
UHT milk	6.5833^{a} ±0.2582	6.9167^{a} ±0.1291	4.5000^{a} ±0.2236	6.1667^{a} ±0.1291	8.4167^{c} ±0.1291	6.5167^{a} ±0.0913
UHT milk&2 ppb Ampicillin	8.6667^{b} ±1291	8.0000^{c} ±0.2236	8.5833^{c} ±0.1291	8.3333^{c} ± 0.1291	7.08333^{a} ±0.3416	8.1333^{c} ±0.0931
UHT milk&4 ppb Ampicillin	8.5833^{b} ±0.1291	7.7500^{b} ±0.0000	8.0000^{b} ±0.0000	7.9167^{b} ±0.1291	7.5833^{b} ±0.1291	7.9667^{b} ±0.0258
UHT milk&8 ppb Ampicillin	8.5000^{b} ±0.000	7.6667^{b} ±0.1291	8.1250^{b} ±0.1369	7.7500^{b} ±0.3873	7.7500^{b} ±0.2236	7.9583^{b} ±0.0258

[a-c]values in a column with the same superscript are not significantly different by Fisher's test ($p<0.05$)

Table D.5. Visual evaluation result of raw milk and raw milk naturally contaminated with antibiotic

	Centre co-ordination	Regulation of the branches	Length of the branches	Density of the branches	Lemniscate form	Total view of Biocrystallogram
Raw milk	6.8333[a] ±0.3028	7.7500[a] ±0.2236	7.5000[a] ±0.2236	7.9167[a] ±0.1291	8.0000[a] ±0.0000	7.6000[b] ±0.1049
Raw milk naturally contaminated with antibiotic	8.6667[b] ±01291	8.2500[b] ±0.0000	8.7083[b] ±0.1882	8.4167[b] ± 0.2041	8.5000[b] ±0.0000	7.4083[a] ±0.1114

[a-b]values in a column with the same superscript are not significantly different by Fisher's test ($p<0.05$)