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Original article

Differential scanning calorimetry as a tool to detect antibiotic residues in ultra high temperature whole milk

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Summary Detection of penicillin G, ampicillin and tetracycline in ultra high temperature whole milk was studied by differential scanning calorimetry (DSC). Thermal parameters including the heat of fusion, the evaporation temperature, the heat of evaporation and the melting temperature obtained from DSC analysis were used to characterise thermal behaviour of antibiotic free milk samples and milk samples fortified with Penicillin G, Ampicillin and Tetracycline. DSC curves of these antibiotics at selected concentrations (0, 2, 4, 8 ppb for Penicillin G and Ampicillin; 0, 100, 250, 500 ppb for Tetracycline) show big endothermic peaks in the temperature range of −30 °C and 200 °C. It was concluded that the antibiotic concentration had a significant effect on the thermal parameters at a 95% confidence level. The differences between the melting temperatures and the peak areas in heat flow curves provided a basis for detection of antibiotic residues in UHT whole milk.

Keywords Animal drug residues, antibiotics, differential scanning calorimetry, food safety, milk/milk products.

Introduction

Milk is wholesome food for people of all ages, particularly infants, growing children, pregnant and lactating mothers, diseased and old age persons, etc. The milk is converted into products and subsidiary products. The necessity of use of veterinary drugs (Antibiotics) lies under the following situations: drugs applied or administered to lactating cows for therapeutic, prophylactic or diagnostic purposes or for modification of physiological functions. All the antibiotics administered to animal enter milk to some degree, hence residue may occur in milk and milk products. The antibiotics residue in milk/milk products should be addressed for following health aspects: (i) possible impact on emergence of antimicrobial resistance for antimicrobials administered in human therapy, (ii) possible occurrence of allergic symptoms. From the technological point of view, residue causes economical losses in fermented milk products as it slows down or inhibits acid formation and decreases aroma formation, causes defects in cheeses and the residue is also weighed for payment of milk on the basis of quality in terms of inhibitors (Honkanen & Reybroeckm, 1997 and Pedersen & Suhren, 2000). Milk producers must protect their milk from contamination by any antibiotic residues not allowed to use at a level higher than the Maximum Residue Limits (MRLs) (Zvirdauskiene & Salomskiene, 2007). O'Keeffe & Kennedy (1998) reported that some of the more important classes of veterinary drugs are sulphonamides, β-lactams (e.g. penicillin), tetracyclines, aminoglycosides (e.g. streptomycin), macrolids (e.g. erythromycin), peptide antibiotics (e.g. virginiamycin) and ionophores (e.g. monensin). The betalactams and tetracycline are the most preferred drugs used in animal treatment.

Antibiotic residues determination in dairy products is significant to protect the quality and safety of milk and milk products. There are a number of different techniques for detection of antibiotic residues in milk (Molina *et al*., 2003). Generally, antibiotic residue analysis contains not only screening but also confirmatory methods. For screening, several commercially available test kits are available. The screening methods are inhibitory tests, receptor assays, immunoassays and confirmatory methods such as chromatography with UV, fluorescence or mass spectrometry detection (Setford *et al*., 1999; Reid *et al*., 2006; Rinken & Riik, 2006; Toldra & Reig, 2006; Le Breton *et al*., 2007). Rapid screening tests are widely preferred however, more accurate chromatographic methods are needed by goverment regulatory authorities to identify and confirm identity and quantity of antibiotic residue existence. In spite of the advantages, there are some drawbacks of screening tests. They can not determine the types of antibiotics are present. These tests may result in false-positive or false-negative. For instance, the presence of

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high somatic cell counts causes false positive results. They may detect antibiotic residues at levels far below the safe levels (Schenk & Callery, 1998). False-positive test kit results may cause unjustified waste of milk and economic losses. Inadequate screening can also affect the dairy industry by creating negative image among consumers, producers, veterinarians and regulatory personnel (Coffman *et al*., 1999).

The thermal analysis has been preferred for many years to characterise synthetic polymers. However, there is a growing interest to use of thermal analysis techniques for detection of water loss, uptake or migration, the denaturation of proteins and the crystallisation of starch, designing of new processes like freeze-drying, spray-drying, extrusion and hydro-thermal treatment and also to determination and improvement of food quality, safety and storage stability in food science. Differential scanning calorimetry (DSC) is a popular alternative thermal analysis technique. In this technique, the difference in the amount of heat required to increase the temperature of a sample and reference are determined as a function of temperature (De Meuter *et al*., 1999; Aktas & Kaya, 2001; Cordella *et al*., 2002).

Since the usage of antibiotic containing raw milk in ultra high temperature (UHT) milk processing is a common and illegal practice in Turkey, the origin of idea of this work is based on an interest to find a basic and rapid method for detection of antibiotic residues in UHT whole milk and milk products. For this purpose, an investigation was conducted to screen some of the thermo-physical properties of UHT milk by using DSC. To the best of our knowledge, there is no available literature on the detection of antibiotics in milk by using DSC. Melting and evaporation data including the heat of fusion, the evaporation temperature, the heat of evaporation and the melting temperature obtained from DSC measurements were used to characterise thermal behaviour of antibiotic free milk samples and milk samples fortified with Penicillin G and Ampicillin at a concentration level of 0, 2, 4, 8 ppb and Tetracycline at 0, 100, 250, 500 ppb. The differences between the peak temperatures and areas in DSC curves were compared to detect antibiotic residues in UHT whole milk.

Materials and methods

Materials and reagents

Ultra high temperature whole milk of the same brand was purchased from the local market in Izmir, Turkey, through the interval period from 2006 to 2008. The composition of the each samples were analysed by using Funke Gerber 3510 Lactostar milk content analyser (Funke Gerber, Berlin, Germany). The average fat %, protein %, lactose % and ash % were measured as 3.22 ± 0.12, 3.250 ± 0.015, 4.710 ± 0.023 and 0.50 ± 0.03, respectively. Prior to DSC analysis, UHT milk samples were subjected to Copan milk test (commercial rapid antibiotics test kit) and yogurt culture test to screen antibiotic residues. Negative samples were chosen as control and further fortified with different levels of antibiotics.

Penicillin G potassium salt (99.4%), Ampicillin trihydrate (98.1%) and Tetracycline hydrochloride (97.3%) were obtained from Riedel-de-Haën (Sigma Aldrich GmbH Quality Assurance, Munich, Germany). Copan Milk Test kits were supplied by Copan (Copan Italia Spa, Brescia, Italy).

Preparation of spiked milk samples

Penicillin G potassium salt ($C_{16}H_{17}KN_2O_4S$, 99.4%, 372.48 g mol^{-1}), Ampicillin trihydrate ($C_{16}H_{19}N_3O_4S.3H_2O$, 98.1%, 403.45 g mol^{-1}) and Tetracycline hydrochloride ($C_{22}H_{25}ClN_2O_8$, 97.3%, 480.90 g mol^{-1}) were used to prepare standard solution. Working standard solutions of Penicillin G, Ampicillin and Tetracycline were prepared by diluting the stock standard solution with ultra pure deionised water. Target concentrations (2, 4, 8 ppb for Penicillin G and Ampicillin; 100, 250 and 500 ppb for Tetracycline) were selected in accordance with EU and Turkish Food Codex MRLs in UHT whole milk.

Copan milk test kits procedure

This assay is based on the rapid growth and acid production of test organism, *Bacillus stearothermophilus* var. *calidolactis* C 953 spores. In principle, if there is no antibiotic residue in the milk sample or the concentration is lower than the limits of detection, the *Bacillus* spores germinate, grow and metabolise the sugar. The acid produced from the fermentation of glucose changes the colour of the indicator Bromocresol Purple in the medium to a yellow colour. But, if antimicrobial substances are present in the milk sample then germination and growth of the *Bacillus* spores are inhibited. This means there is no fermentation of glucose and acid production, and therefore the Bromocresol Purple indicator in the medium remains purple. Yellow/Purple (Partially Positive) means that no inhibitors are present or that the presence is lower than the limit detectable by the test. Detection limits and MRL's for target antibiotics are presented in Table 1.

Table 1 Detection limits and MRL's for Copan milk test (Source: Copan 2008)

Antibiotics	Copan test detection limit (ppb)	MRL (ppb)
Penicillin G	1–2	4
Ampicillin	<2	4
Tetracycline	250–500	100

100 μL of milk to be tested was added directly onto the surface of the agar and then incubated at 64 ± 0.5 °C in an incubator for 3 h ± 15 min. The results were evaluated according to manufacturers instructions described previously.

Yogurt culture test

The milk samples were inoculated with a yogurt culture. Yogurt cultures consisted of equal mixtures (2%) of *Streptococcus thermophilus* (St 95/1) and *Lactobacillus delbrueckii* sub-sp. *bulgaricus* (Lb 54). They were isolated from traditional yogurt samples of Toros mountain region of Turkey. Phenotypic and genotypic characterisations of the cultures were performed by Molecular Food Biotechnology research group at Izmir Institute of Technology (Erkuş *et al.*, 2006). Milk samples were incubated at 43 °C for 6 h. Initially, pHs of all milk samples were measured. Then, antibiotic free milk samples and milk containing Penicillin G, Ampicillin and Tetracycline at prescribed concentrations were incubated for 6 h. Duplicate measurements of pH were done immediately at the second, fourth, sixth hours of incubation by using Mettler Toledo Seven Easy pH meter (Mettler Toledo, USA). The decrease of pH in milk and change in consistency were observed and compared (Yamani *et al.*, 1999).

Differential scanning calorimetry analysis

Q10 DSC (TA Instruments, New Castle, DE, USA) was used to obtain data concerning the net heat changes produced by milk samples (control and fortified) during their heating. DSC was previously calibrated according to manufacturer instructions. Runs were conducted from –30 °C to 200 °C to obtain the complete thermal behaviour of milk samples. The heating and cooling were carried out under a constant nitrogen flow. The experimental conditions (temperature range, type of crucible, temperature programming, heating rate (10 °C min^{-1}), cooling rate (10 °C min^{-1}), and weighed mass of sample) was described using DSC software program (Cordella *et al.*, 2003). Approximately 5–10 mg of the samples were weighed and hermetically sealed into an aluminium pan by using a sealer. Then, DSC measurements were performed to determine thermal parameters including the heat of fusion, the evaporation temperature, the heat of evaporation and the melting temperature of each sample at a prescribed temperature range (De Meuter *et al.*, 1999).

Statistical analysis

The statistical analyses were performed using the statistical software Minitab Statistical Software 14 Trial version (Minitab Inc., State College, PA, USA). DSC results of milk samples were expressed as the mean and standard deviation. All DSC measurements were replicated three times. One-way analysis of variance (ANOVA) with Fisher's, individual error rate test was carried out in order to evaluate the effect of the concentration of an antibiotic in milk samples at the level of $P < 0.05$.

Results and discussions

Copan milk test and yogurt culture test results

Before all the measurements, eight different UHT whole milk samples of the different brands were screened by using Copan Milk Test for antibiotic residues. In our previous work (unpublished), among three rapid antibiotic test kits, Copan milk test was found simple to interpret, easy to use and having long shelf life at differentiating the antibiotic residues in UHT milk. Therefore it was chosen for the further studies. The visual interpretation was in accordance with the colour card for Copan Milk Test. Only three brands were found to be free of antibiotic residues. One negative milk sample was selected and further subjected to yogurt culture test in order to confirm and eliminate any false-positive outcomes originated from the rapid test.

Yogurt formation was observed at the end of 6 hours incubation time. Yogurt culture test results for the selected milk sample are given in Table 2. The change in the consistency and pH during yogurt formation was observed for milk samples containing Penicillin G, Ampicillin, and Tetracycline at selected concentrations. The control sample (0 ppb) reached the maximum curd firmless at about pH 4.6 due to sufficient acidity generated at the end of 6 h to form a coagulum. Milk samples fortified with Penicillin G, Ampicillin and Tetracycline resulted in no curd formation. The

Table 2 Consistency and pH of the selected milk sample containing selected concentrations of penicillin G, ampicillin, and tetracycline

	Concentration (ppb)	pH (at the end of incubation time)	Consistency (visually)
Penicillin G	0	4.487 ± 0.1224	Custard-like curd
	2	5.707 ± 0.1490	No yogurt formation
	4	5.766 ± 0.1188	No yogurt formation
	8	5.974 ± 0.1203	No yogurt formation
Ampicillin	0	4.578 ± 0.2079	Custard-like curd
	2	6.428 ± 0.1303	No yogurt formation
	4	6.444 ± 0.1165	No yogurt formation
	8	6.465 ± 0.1036	No yogurt formation
Tetracycline	0	4.470 ± 0.2026	Custard-like curd
	100	5.583 ± 0.2042	No yogurt formation
	250	5.854 ± 0.1805	No yogurt formation
	500	6.037 ± 0.1203	No yogurt formation

antibiotic positive milk samples had higher pH level compared to negative milk samples. Yogurt formation was not observed at and above 2 ppb Penicillin G and Ampicillin concentration, and at and above 100 ppb Tetracycline concentration. After the Copan Milk Test and yogurt culture test, one negative milk sample was chosen for the DSC measurements and fortified with Penicillin G, Ampicillin and Tetracycline at the selected concentrations.

Differential scanning calorimetry results

Melting and evaporation data were used to characterise thermal behaviour of control (0 ppb) and milk samples fortified with Penicillin G (2, 4, 8 ppb), Ampicillin (2, 4, 8 ppb) and Tetracycline (100, 250, 500 ppb). Melting and evaporation points were recorded at the maximum of endothermic peaks.

Figure 1 shows effects of antibiotic concentration on melting temperature, heat of fusion, evaporation temperature and heat of evaporation of control and fortified samples. The heat of fusion, evaporation temperature, heat of evaporation parameters of fortified milk samples exhibited increasing trend depending on an increase in the concentration of Penicillin G, Ampicillin and Tetracycline. On the other hand, melting temperature of these samples showed a decreasing trend while antibiotic concentrations were increasing.

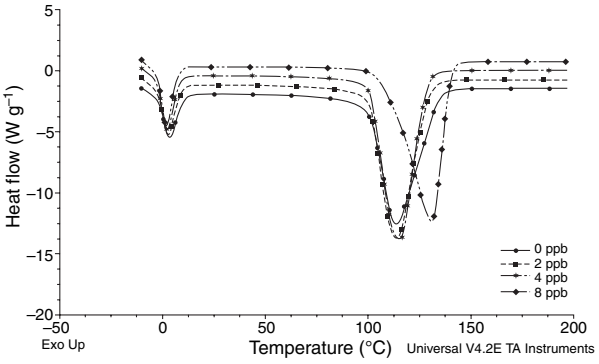

Figure 2 DSC curves of milk samples containing 0, 2, 4, 8 ppb penicillin G.

However, the shape of the curves were different for each antibiotic residues.

Heat flow curves (DSC curves) depicted in Figs 2–4 for control and fortified milk samples at selected concentrations showed big endothermic peaks. Recognisable differences in melting temperatures, evaporation temperatures, and onset temperatures of transitions were obtained. For Penicillin G residues, the DSC thermogram of milk samples showed two endothermic signals changing from 3.600 °C to 1.985 °C, and 112.625 °C to 129.970 °C, respectively (Fig. 2). It was concluded that first thermal event was a melting

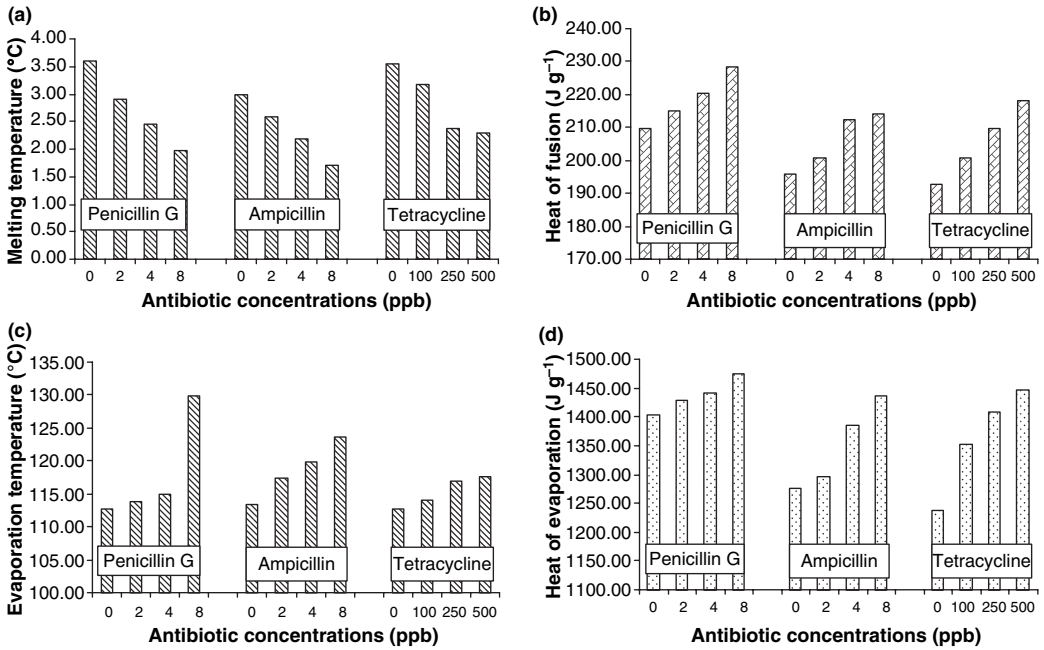

Figure 1 Effects of penicillin G, ampicillin and tetracycline concentrations on the thermal parameters (a) melting temperature, (b) heat of fusion, (c) evaporation temperature, (d) heat of evaporation of the milk samples.

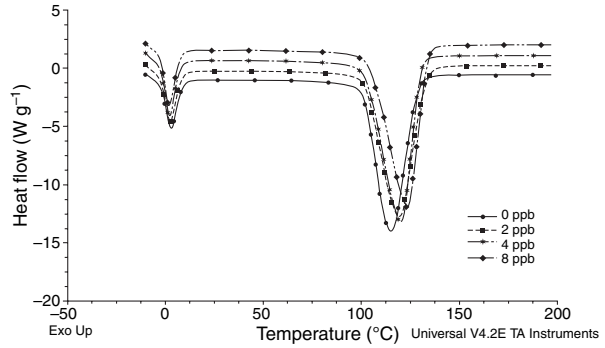

Figure 3 DSC curves of milk samples containing 0, 2, 4, 8 ppb ampicillin.

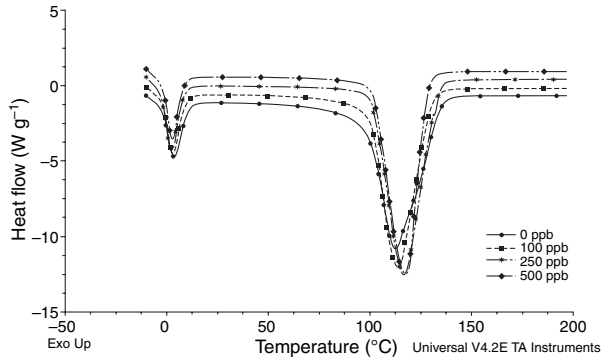

Figure 4 DSC curves of milk samples containing 0, 100, 250, 500 ppb tetracycline.

endotherm and second one was an evaporation endotherm. The maximum temperature difference was 44.86% in the melting endotherm. The maximum area difference was 8.89% (Table 3).

For Ampicillin residues, first endothermic peak was in the temperature range of 2.9850–1.7050 °C (Fig. 3). Second endothermic peak was wide and intense endothermic peak in the temperature range of 113.380–123.565 °C. The maximum temperature difference was 42.88%. The maximum area difference was calculated as 12.58% (Table 3).

For Tetracycline residues, the average values of first endothermic peak temperature of milk samples at selected concentrations was changed from 3.5600 °C to 2.3050 °C (Fig. 4). Second endothermic peak temperature was in between 192.60 °C and 218.30 °C. The maximum temperature and area differences were 35.25% and 16.83%, respectively (Table 3). It was observed that the peak temperatures and areas increased with increasing antibiotic concentrations. Peaks appeared sharper and clearer with the increase of endothermic enthalpy.

These findings were also supported with statistical analysis. Results of Fisher's individual error rate test were given in Table 3 for control and milk samples fortified with Penicillin G, Ampicillin and Tetracycline at selected concentrations. The statistical testing showed that antibiotic concentration had a significant effect on the thermal parameters (melting temperature, heat of fusion, evaporation temperature and heat of evaporation) at a 95% confidence level. Overall, it was concluded that the first peak temperature (melting temperature) and first peak area of Penicilin G, first peak area of Ampicillin and first peak area of Tetracycline observed in DSC curves were determined to be significant point to distinguish the presence of antibiotic residues in UHT whole milk. It is speculated that the change in the temperature causes some structural modifications (transformations) or decompositions in

Table 3 Influence of antibiotic concentration on the DSC curves of milk samples (based on the results of Fisher's, individual error rate test)

Type of antibiotic	Concentration (ppb)	Peak	Temperature (°C)	Peak	Area (J g^{-1})
		1. peak	2. peak	1. peak	2. peak
Penicillin G	0	3.6000 ± 0.0707^{a}	112.625 ± 0.035^{a}	209.80 ± 2.40^{a}	1404.00 ± 18.38^{a}
	2	2.8950 ± 0.0495^{b}	113.770 ± 0.042^{a}	215.10 ± 0.42^{b}	1427.50 ± 0.71^{ab}
	4	2.4500 ± 0.0849^{c}	114.940 ± 0.014^{a}	220.20 ± 0.42^{c}	1441.50 ± 6.36^{bc}
	8	1.9850 ± 0.1061^{d}	129.970 ± 1.513^{b}	228.45 ± 0.64^{d}	1473.50 ± 0.71^{c}
Ampicillin	0	2.9850 ± 0.0212^{a}	113.380 ± 0.792^{a}	195.75 ± 0.92^{a}	1276.0 ± 33.9^{a}
	2	2.5800 ± 0.2263^{ab}	117.415 ± 0.035^{b}	200.90 ± 1.41^{b}	1296.0 ± 52.3^{a}
	4	2.2000 ± 0.0990^{b}	119.775 ± 0.474^{c}	212.20 ± 1.27^{c}	1386.5 ± 2.1^{ab}
	8	1.7050 ± 0.0919^{c}	123.565 ± 0.389^{d}	214.30 ± 0.57^{c}	1436.5 ± 17.7^{b}
Tetracycline	0	3.5600 ± 0.0141^{a}	112.605 ± 0.064^{a}	192.60 ± 5.66^{a}	1238.5 ± 7.8^{a}
	100	3.1850 ± 0.0495^{b}	114.010 ± 0.127^{b}	200.65 ± 3.61^{ab}	1351.5 ± 14.8^{b}
	250	2.3700 ± 0.0283^{c}	116.835 ± 0.021^{c}	209.80 ± 2.40^{bc}	1407.5 ± 9.2^{bc}
	500	2.3050 ± 0.0071^{c}	117.645 ± 0.120^{d}	218.30 ± 0.00^{c}	1447.0 ± 35.4^{c}

±: Standard deviation for milk samples containing target antibiotics at selected concentrations. $^{a–d}$Values in a column with the same superscript are not significantly different by Fisher's test ($P < 0.05$).

milk samples. The common transformations observed by DSC are the denaturation of proteins, crystallisation of lipids and interactions of other chemical compounds. Additionally, multiple interactions can arise between milk components and lead to some modifications of the thermal behaviour. The study to be conducted to determine the mechanism of how an antibiotic modifies the thermal properties of milk is still underway.

Conclusion

The effects of Penicillin G, Ampicillin in the range of 2, 4, 8 ppb and Tetracycline in the range of 100, 250, 500 ppb on the thermal properties of UHT whole milk were determined by using DSC. It was demonstrated that an increase in antibiotic concentration levels of milk samples resulted in an increase in values of heat of fusion, evaporation temperature and heat of evaporation and a decrease in values of melting temperature of milk samples. Although the developed method can not determine the types of antibiotics that are present in milk samples, prelimenary results show that it is a sensitive and easy method, and the time of analysis is short (< 20 min). It can be alternative to screeing tests and can not be considered as a confirmatory method.

It was concluded that DSC can be a promising technique for detecting antibiotics in UHT whole milk but needs expertise to run the measurements. Still, more work is necessary to improve this method. On the other hand, future work dealing with the contribution of DSC to the detection of antibiotic residues in naturally contaminated milk samples drawn from treated cows is necessary for validation of this technique.

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