Journal of Thermal Analysis and Calorimetry, Vol. 71 (2003) 155–161

DETECTION OF BACTERIAL CONTAMINATED MILK BY MEANS OF A QUARTZ CRYSTAL MICROBALANCE BASED ELECTRONIC NOSE

Z. Ali, W. T. O'Hare and B. J. Theaker*

School of Science and Technology, University of Teesside, Middlesbrough, TS1 3BA, UK

Abstract

Headspace analysis by means of sensor arrays has been successfully applied to a wide range of qualitative applications. In this study, a six element array of coated Quartz Crystal Microbalance (QCM) sensors was used for the headspace analysis of milk volatiles. The sensors were exposed to uncontaminated samples of milk and samples contaminated with *Pseudomonas fragi* (*Ps. fragi*) or *Escherichia coli* (*E. coli*). Principal component analysis (PCA) was used to analyse the sensor array responses. No discrimination between uncontaminated milk samples and those contaminated with *Ps. fragi* was observed. This can be explained by *Ps. fragi* being a poor fermenter of milk. However, encouraging results were found for the discrimination between the milk samples and those contaminated with *E. coli*.

Keywords: bacterial contamination, milk, Quartz Crystal Microbalance, sensor array

Introduction

Food may become contaminated by micro-organisms during production, preparation and/or storage. This contamination may give the food undesirable characteristics such as off-flavours and odours or be potentially harmful to the consumer. This may be due to the micro-organism itself or their metabolites. Current quality control methods for determining the quality of milk samples can be performed using many methods. Enumeration of microflora in milk can be performed by means of serial dilutions and plate counting, reductase test [1], methylene blue test [1, 2] or resazurin dye reduction test [3]. Plate counting requires time for the bacteria to grow to sufficient numbers to be enumerated, usually a minimum of 24 h. The dye colouration methods require the milk to be stored prior to testing, in the case of the reductase test 5 h at 37°C, methylene blue 16.5–24 h at 17–21°C [2] and resazurin 3 days at 30°C [3].

Volatile compounds in milk have been extensively studied by instrumental methods, predominantly gas-chromatography (GC) and GC coupled with Mass-Spectrometry (GC-MS) [4–10]. Analyses of volatiles above aged milk samples have been per-

* Author for correspondence: E-mail: z.ali@tees.ac.uk

1418–2874/2003/ $ 20.00

Akadémiai Kiadó, Budapest
Kluwer Academic Publishers, Dordrecht

formed [4–6]. Poor quality milk produced increased concentrations of aldehydes, ketones and alcohols as well as additional volatiles such as esters when compared to good quality milk samples. This offers the possibility for the use of headspace analysis as a predictive tool for the determination of milk storage and off flavours.

Recent developments in electronic nose technology potentially offer alternative instrumental techniques to rapidly detect the presence of microflora in milk samples. Electronic noses based on doped metal oxide sensors and quartz crystal microbalances have previously been utilised to discriminate between samples of milk samples, which have been subjected to different heat treatments [11, 12].

A commercial electronic nose based on conducting polymer sensors was utilised to discriminate between uncontaminated milk samples and milk samples contaminated with various bacteria (*Ps. aureofaciens, Ps. fluorescens* and *B. cereus*) or yeasts (*Candida pseudotropicalis and Kluyveromyces lactis*) [13]. Milk samples were inoculated with initial populations of 10^3–10^4 CFU mL^{-1} prior to incubation at 30°C for 5 h and the sensor responses analysed cluster analysis, PCA and neural networks. Cluster analysis of the sensor responses showed a high similarity between milk samples and milk contaminated with *Ps. aureofaciens* (10^6 CFU mL^{-1}) however sample discrimination was clearer for larger inoculation populations ($3.5 \cdot 10^8$ and $8 \cdot 10^8$ CFU mL^{-1}). The authors postulate that for sample discrimination at the lower inoculation populations, longer incubation times may be required. PCA analysis of the sensor responses to milk samples contaminated with the various microflora showed relatively large within-class variation and minor between-class variation.

E. coli contaminated milk, both pasteurised and unpasteurised, has previously been implicated as the source of food poisoning outbreaks, [14–16] whilst *Ps. fragi* imparts an unpleasant fruity aroma into the milk.

Quartz Crystal Microbalances are robust and sensitive sensors. Added to this is their versatility due to the wide range of coating materials. Arrays of Quartz Crystal Microbalances are suitable for a wide variety of qualitative problems. However, they appear to relatively under utilised within this field of headspace analysis of milk volatiles.

Experimental

Bacterial preparation

Cultures of *Pseudomonas fragi* (*Ps. fragi*) and *Escherichia coli* (*E. coli*) were obtained from NCIMB Ltd. Aberdeen. These were stored under refrigerated conditions at approximately 4°C. Working aseptically with the aid of a Bunsen flame, a loop (10 μL) of culture was added to general purpose nutrient broth (100 mL). The bacterial broth was placed in the orbital shaker 20°C at 100 RPM. After 12 h, the optical density of the bacterial broth was measured against a nutrient broth reference using a UV/VIS spectrophotometer (PYE-UNICAM PU 8600) at 600 nm (OD_{600}). The OD_{600} was standardised to 0.6 by dilution with sterile 50 mM phosphate buffer (pH 7.6) to enable comparative additions of bacteria (approx. 10^6 cells mL^{-1} milk). Standardised bacterial broth (10 mL) was added to a sterilised plastic centrifuge tube by means of a sterilised pipette.

The broth was then centrifuged for 3 min and the supernatant liquid decanted off. The bacterial pellet was washed with 10 mL sterile phosphate buffer (50 mM, pH 7.6) using a vortex mixer. The bacterial suspension was centrifuged again, rinsed with phosphate buffer and vortexed for 3×1 s. This procedure was then repeated to further rinse the bacteria.

Serial dilution plate counts were performed in triplicate to enumerate and standardise the the number of bacteria inoculated into the milk samples. Bacterial inoculant (1 mL) was added to sterile pH 7.6 phosphate buffer solution (9 mL). The mixture was then vortexed, 1 mL removed and diluted with sterile buffer solution (9 mL). From each dilution sample, 100 μL was aseptically transferred onto a sterile general purpose agar plate. The agar plates incubated overnight at 37°C, prior to quantification.

Apparatus

The apparatus used for the study of headspace above milk samples is shown in Fig. 1. Buffered bacterial solution or buffer (1 mL) was added to UHT milk (20 mL) in a Dreschel bottle (volume=125 mL) with the polypropylene syringe added to one of the arms (Whatman International Ltd., Maidstone, Kent). Ambient air (100 mL) was introduced through the withdrawing of the syringe. A PTFE filter (0.45 μm pore size, Whatman International) ensured no particulate matter or bacteria were introduced to the sample at this stage. The milk samples were then incubated in an orbital shaker (6 h, 25°C at 100 RPM).

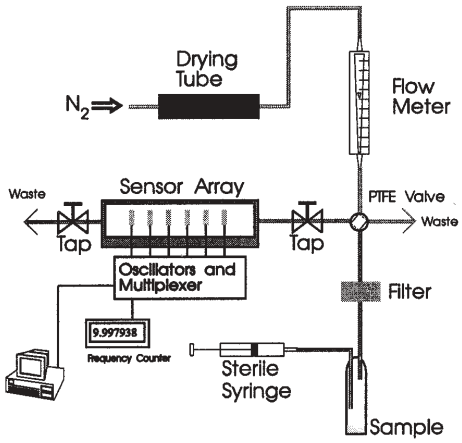

Fig. 1 Schematic representation of the static headspace analysis apparatus

The sensor array chamber contained six coated QCM sensors (8 mm diameter with 5 mm unpolished gold electrodes) supplied by SES Piezo Ltd. Portsmouth. The coating methodology has been previously described [17]. The sensor coatings used are shown in Table 1.

Table 1 The coating materials used for the QCM sensor array and associated frequency shifts due to the applied coatings

Sensor	Coating	Total frequency shift/KHz
1	Silar 10C	2.780
2	DEGS	3.095
3	Carbowax 20M	3.175
4	OV-17	3.429
5	OV-210	3.009
6	OV-1	2.875

Headspace sampling

For each measurement cycle, the sensor chamber was flushed with nitrogen (5 min at 50 mL min^{-1}) to refresh the sensors after the previous sample. The nitrogen flow rate was controlled by the flow meter. A drying tube consisting of a combined molecular sieve and silica gel, supplied by Phase Separations Ltd., Deeside, was added to remove possible trace levels of oil and moisture from the nitrogen supply. The nitrogen flow was then stopped and the sensors were allowed to stabilise to their resonant frequencies and these frequencies were monitored every 10 s for 1 min as a reference value. After such time, the headspace of a sample was introduced into the sensor chamber by plunging the syringe. After sample introduction, the sensor chamber was isolated by means of the taps at either end of the chamber, (1/16 inch Swagelok Teesside Valve & Fitting, Newton Aycliffe, Co. Durham). The frequencies of each sensor were then monitored for a further 5 min. After this time, the taps were opened and the sensor chamber was again flushed with nitrogen prior to the introduction of the subsequent sample.

Results

A total of 58 results were obtained over a two-week period, 16 from uncontaminated UHT milk samples, 25 from milk samples contaminated with *E. coli* and 17 from milk samples contaminated with *Ps. fragi*. The inoculation populations for *Ps. fragi* were in the range $9.5 \cdot 10^5$–$2.65 \cdot 10^6$ CFU mL^{-1} (n=3) and for *E. coli* 1.1–$2.2 \cdot 10^6$ CFU mL^{-1} (n=6).

Discrimination between uncontaminated milk samples and samples contaminated with *Ps. fragi* was not possible. It has previously been demonstrated by GC analysis that *Ps. fragi* is a poor fermenter of milk. Trace levels of ethanol and methyl sulfide were found in milk (Initial population $1.2 \cdot 10^2$ CFU mL^{-1}, incubation conditions 25°C for 32 h resulting in a final population of $2 \cdot 10^8$ CFU mL^{-1}) [7]. Large increases of up to 5 fold in the respective concentrations of ethyl butyrate and hexanoate in milk with the presence of *Ps. fragi* have also been shown. (incubation conditions 21°C for 72 h final population 10^8 CFU mL^{-1}) [8, 9]. However, the concentration of the esters did not exceed 2.0 ppm through the incubation period. The pres-

ence of such esters, even at such low concentrations, may explain the fruity odour observed from the samples in this current study and previously reported [8, 9]. It is therefore suggested that the volatiles produced by the *Ps. fragi* were below the sensitivity limits of the non-selective coatings. In addition, reproducible array responses for *Ps. fragi* contaminated milk samples could not obtained.

In comparison, *E. coli* has previously been shown to be an efficient fermenter of milk producing volatile compounds. (Initial population 10^1–10^2 CFU mL^{-1}, incubation conditions 25°C for 33 h resulting in a final population of 10^8–10^9 CFU mL^{-1}) [7, 10].

Principal component analysis

Figure 2 shows the first two principal components in a PCA scores plot for the milk samples. Discrimination between the sample classes was encouraging. Although distinct clustering for the two sample classes is not demonstrated, a majority of the uncontaminated milk samples have negative PC_1 scores whilst a majority of *E. coli* contaminated milk samples have positive PC_1 scores. Differences in the respective PC_1 scores of the sample clusters were observed to be statistically significant ($p<0.05$).

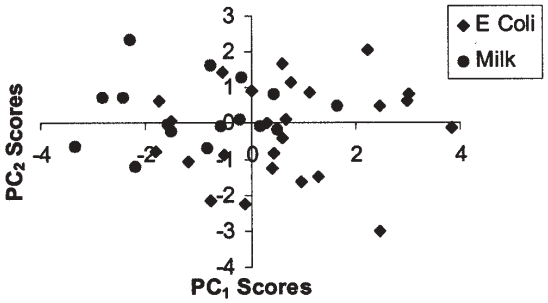

Fig. 2 Principal component scores plot of the 5 QCM sensor responses to *E. Coli* contaminated and uncontaminated milk samples

Discussion

The coated Quartz Crystal Microbalance sensor array was unable to clearly discriminate between the uncontaminated milk and the contaminated milk samples. A possible hypothesis for the lack of sample discrimination may be due to the high water content of milk. Coated QCM sensors have previously been shown to be susceptible to adsorption of water vapour [18]. The compounds in milk associated with bacterial degradation are present around ppm concentrations, while the water content of milk is approx. 90% thus subtle changes in the composition of the milk volatiles is effectively swamped by water content of milk.

A possible solution could be the use of a pre-concentration unit such as Tenax or Porapak. Tenax has previously been used as to pre-concentrate milk volatiles prior to GC analysis [4–6]. Alternatively, an array of high temperature sensors based on metal oxide sensors, which would not adsorb the water molecules allowing the volatile milk

compounds to be adsorbed, may allow potential greater sample discrimination to be performed. Recently, an array of 5 electrochemical electrodes has been utilised to discriminate between fresh and bacterially contaminated milk [19].

The array was immersed in milk samples and measurements taken every 36 min with the milk being incubated at 21, 24 and 25.5°C. The milk samples were compared to a blank sample containing sodium azide as a bacteriastat. The authors made no attempt to identify the bacterial strain present. The electrode responses were analysed by means of PCA and neural networks. Milk samples containing bacteriastat were clearly discriminated from the other samples. The samples stored at 21 and 25.5°C could also be separated from the samples stored at 24°C. The samples stored at 24°C were clustered closely together showing good reproducibility of these samples. The bacterial counts were generally in the area 10^2 to 10^8 CFU mL^{-1}. The sample data were then analysed by means of PLS and a NN, in order to try to predict the true values of bacteria contained in each sample, with varying degrees of success.

* * *

The authors initially wish to acknowledge HEFCE for funding the project, acknowledge the assistance of Bill Ling and Doug McClelland in the preparation and culturing of the bacteria, Kevin Middleton for constructing the sensor array chamber and Jill Legg for compiling the data acquisition program.

References

1 C. H. Collins, Microbiological Methods (2nd Edition), Butterworths, London 1967, p. 317.
2 D. D. Muir in R. K. Robinson (Ed.), Dairy Microbiology, Vol. 1, The Microbiology of Milk (2nd Edition), Elsevier, London 1990, p. 210.
3 A. H. Varnam and J. P. Sutherland, Milk and Milk Products, Chapman & Hall, London 1994, p. 98.
4 B. Vallejo-Cordoba and S. Nakai, J. Agric. Food Chem., 41 (1993) 2378.
5 B. Vallejo-Cordoba and S. Nakai, J. Agric. Food Chem., 42 (1994) 989.
6 B. Vallejo-Cordoba and S. Nakai, J. Agric. Food Chem., 42 (1994) 994.
7 R. Bassette and T. J. Claydon, J. Dairy Sci., 48 (1965) 775.
8 M. C. Reddy, D. D. Bills, R. C. Lindsay, L. M. Libbey, A. Miller and M. E. Morgan, J. Dairy Sci., 51 (1968) 656.
9 M. C. Reddy, D. D. Bills and R. C. Lindsay, Appl. Microbiol., 17 (1969) 78.
10 R. E. Bawdon and R. Bassette, J. Dairy Sci., 49 (1966) 624.
11 G. Sberveglieri, E. Comini, G. Faglia, G. Niederjaufner, G. Benussi, G. Contarini and M. Povolo, W. J. Hurst (Eds) Seminars in Food Analysis Chapman & Hall, London Vol. 3, No. 1, 1998, p. 67.
12 C. Di Natale, A. Macagnano, R. Paolesse, A. Mantini, E. Tarizzo, A. D'Amico, F. Sinesio, F. M. Bucarelli, E. Moneta and G. B. Quaglia, Sens. Acts B, 50 (1998) 246.
13 N. Magan, A. Pavlou and I. Chrysanthakis, Sens. Acts B, 72 (2001) 28.
14 A. Ammon, Eurosurveillance, 2 (1997) 91.
15 W. E. Keene, K. Hedberg, D. E. Herriott, D. D. Hancock, R. W. McKay, T. Barett and D. W. Fleming, J. Infect. Dis., 176 (1997) 815.

16 R. P. Johnson, R. C. Clarke, J. B.Wilson, S. C. Read, K. Rahn, S. A. Renwick, K. A. Sandhu, D. Alves, M. A. Karmali, H. Lior, S. A. McEwen, J. S. Spika and C. L. Gyles, J. Food Prot., 59 (1996) 1112.
17 Z. Ali, D. James, W. T. O'Hare, F. J. Rowell, T. Sarkodie-Gyan, S. M. Scott and B. J. Theaker, J. W. Gardner and K. C. Persuad (Eds) Electronic Noses and Olfaction 2000 IOP Publishing, Bristol 2000, p. 229.
18 F. Welle, A. Mauer, E.-M. Kiel and M. Slama, Sens. Acts B, 69 (2000) 372.
19 F. Winquist, C. Krantz-Rülcker, P. Wide and I. Lundström, Meas. Sci. Technol., 9 (1998) 1937.