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Automatic determination of fat in milk by use of a flow injection system with a piezoelectric detector

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Abstract

A new method for the direct determination of total fat in milk by use of a quartz crystal microbalance was developed. The method is based on a (micro) extraction procedure that is carried out in a flow injection system. Samples are diluted with an ethanol–water mixture and injected into the flow system, where they are mixed with *n*-hexane in an extraction coil. The analyte is determined in the organic phase. The calibration thus achieved is linear in the range 0.20–0.45% w/v, and the relative standard deviation is ±3.2% ($n = 11$; $P = 0.05$). The throughput is 12 samples per hour. The proposed method was used to determine fat matter in milk samples; the results were found to be competitive with those of official methods for the same purpose.

Keywords: Flow system; Piezoelectric detection; Fat; Milk

1. Introduction

The use of the piezoelectric quartz crystal as microbalance detector was originally conceived by Bruckenstein and Shay [1], who developed a circuit that allowed the simultaneous measurement of the in situ frequency change accompanying electrolysis at the working electrode as a function of either electrode potential or current. Also, they proposed using an oscillating quartz crystal as an electrochemical quartz microbalance. The in situ mass sensitivity of the crystal as determined by electrodeposition of silver, is quite consistent with the value predicted from the Sauerbrey [2] equation. This author also investigated the effects of the solution temperature and viscosity, and the thickness of the liquid film on top of the crystal, and correlated his results with theory. Subsequently Alder and McCallum [3] reviewed the theory and applications of piezoelectric crystals for mass measuring. They concluded that many of the shortcomings and advantages of piezoelectric crystal detectors had been identified and that equipment and data processing had developed to an extent allowing one to identify appropriate situations for application of the piezoelectric crystal detector under real-life conditions. Thus, applications where only a modest level of selectivity is required (e.g. to monitor fam-

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ilies of compounds), seem quite feasible. Uses for short-term (e.g. working day) rather than long-term monitoring with disposable crystals also seems attractive. The performance of the piezoelectric quartz crystal was studied in basic terms by Sauerbrey [2]. He derived the following equation relating the change in oscillating frequency, ΔF, to the mass change, ΔM, at the crystal surface:

$$\Delta F = \left(\frac{-F^2 \Delta M}{N A \rho} \right) \quad (1)$$

where N is the frequency constant, ρ the density of quartz, A the piezoelectric active area and F the basic oscillation frequency of the crystal. Sauerbrey [4] also derived a linear relation between the mass added to the crystal surface and the frequency change:

$$\Delta F = -2.3 \times 10^6 \left(\frac{F^2 \Delta M}{A} \right) \quad (2)$$

A number of analytical applications of the piezoelectric quartz crystal have recently been reported. Thus, Muratsugu et al. [5] used a quartz crystal microbalance to detect microgram amounts of human serum albumin. Chang and Shih [6] employed a piezoelectric quartz crystal as a gas-chromatografic detector for various organic molecules. Li and Thompson [7] studied the mass sensitivity of the acoustic tube wave sensor. Previously, Yang and Thompson [8] studied the effects of different experimental variables of liquids in the response of a thickness shear mode (TSM) bulk acoustic wave sensor (AT–cut quartz piezoelectric crystal coated with gold electrode). Dickert et al. [9] developed chemical sensors based on a highly mass sensitive quartz crystal microbalance (QCM), coated with sensitive layers; the layers are specially suitable for the trace analysis of organic solvent vapours such as halogenated or aromatic hydrocarbons. Zhihong et al. [10] proposed a method based on flow injection analysis and a piezoelectric detector for the determination of norepinephrine bitartrate by ion association with an anionic surfactant; this system introduced a substantial change in the conventional approach to the technique. Magna et al. [11] reported the determination of brix in both sugar cane and alcoholic fermentation broth by use of a flow injector and piezoelectric detection; they placed special emphasis on the fact that the quartz crystal used was uncoated. Because small changes in mass at the crystal surface reduce or increase the frequency, a piezoelectric quartz crystal can serve as an effective mass sensor enabling faster, more reproducible assays in flow injection analysis (FIA) [12–13]. Hepher and Reilly [14] presented an exhaustive review of applications of the piezoelectric quartz crystal as a sensor system for environmental monitoring, in the fields of gas analysis, aerosols particles and liquid sensors.

In this paper, a simple, reliable, responsive piezoelectric sensor was developed for the determination of total fat in milk in response to the need for analytical tools for expeditious monitoring quality control parameters in manufacturing processes. One case in point is cow milk, the total fat content in which is routinely determined to classify different types of milk. Particularly those aimed at infants and dieting individuals (low-fat milk).

2. Experimental

2.1. Reagents

A stock standard solution containing 1% palmitic acid was prepared by dissolving 1 g of product (Sigma ultra) in 100 ml of *n*-hexane (Scharlau, high purity). Standard working solutions were prepared by appropriate dilution of the stock. Dilute solutions were obtained by mixing an appropriate volume of palmitic acid solution with three parts of ethanol (analytical grade), two parts of ultrapure water (milli-Q) and 0.3 ml of NH_3 (30%, Panreac).

2.2. Standard and sample preparation

The synthetic samples used were standard solutions of fat prepared from the palmitic acid stock solution. The palmitic acid was used because it represented the majority of the lipid fraction in the milk composition [15]. The real samples employed were milks of different brands that were diluted five or ten times with the ethanol solution. This was the sole pretreatment required for the milk samples prior to their introduction in the flow system. The ethanol solution in ammonia was demonstrated to be efficient to release bound

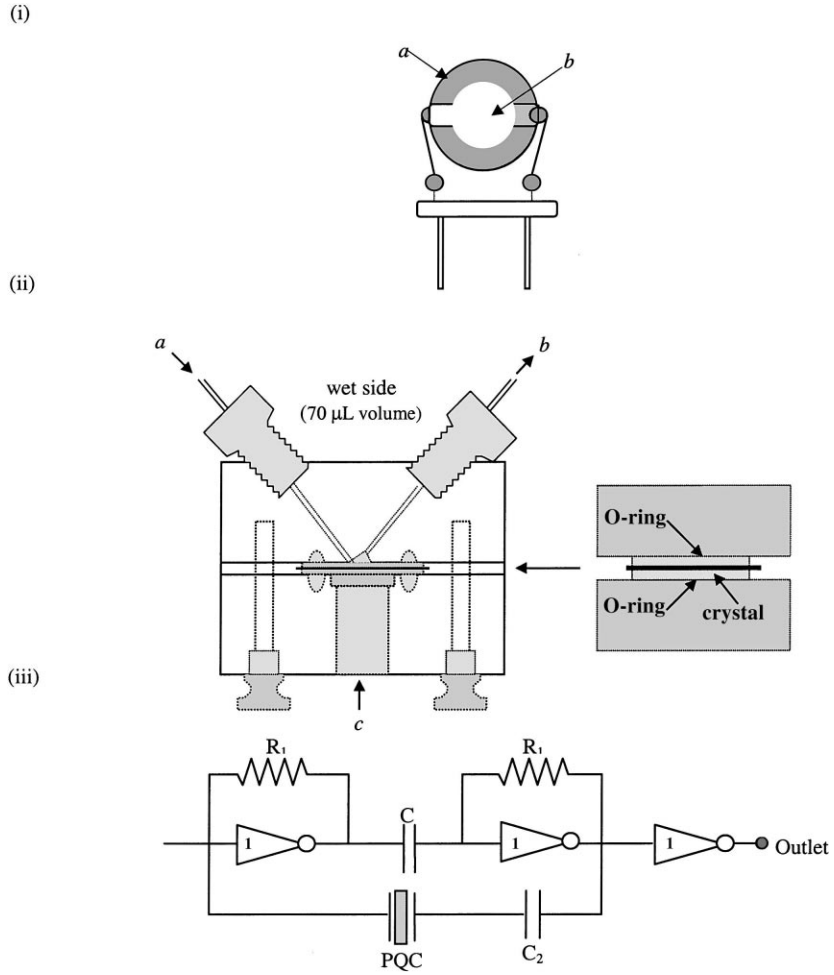

Fig. 1. Parts of the detection system: (i) 10 MHz piezoelectric crystal, including 14 mm diameter, 0.17 mm thick quartz disc (a) and 7.8 mm diameter gold electrode (b). (ii) flow-cell: (a) is inlet, (b) Outlet, (c) dry side. (iii) oscillator circuit: $C_1 = 0.01\,\mu F$, $C_2 = 20\,\rho F$, $R_1 = 390\,\Omega$, IC → 7404, PQC: piezoelectric quartz crystal, 1: oscillator.

lipids from the milk matrix. Also, the use of ethanol avoids emulsification [16], which is very important to get reproducible results.

2.3. Apparatus

An AT-cut 10 MHz piezoelectric quartz crystal (14 mm diameter, 0.17 mm thick) coated with gold plated electrodes on both sides (Fig. 1a) was used. The quartz crystal was housed in flow-through PEEK™ cell and clamped between two O-rings recessed into the housing (Fig. 1b); one crystal face was exposed to the sample in a cell of 70 μl. The piezoelectric end PEEK™ was supplied by Universal Sensors. A laboratory-made oscillator circuit (Fig. 1c) was connected to the electrode via platinum foil; the resonant frequency was monitored with a Hewlett Packard® HP-53181A/225 MHz frequency counter that was connected to a PC-pentium® microcomputer via an HP-IB interface (Hewlett Packard®). HP-34812A Bench Link software (HP BenchLink/Meter) was used to acquire and store data. A Gilson Minipuls-3 peristaltic pump and a Rheodyne 5020 injection valve were also used.

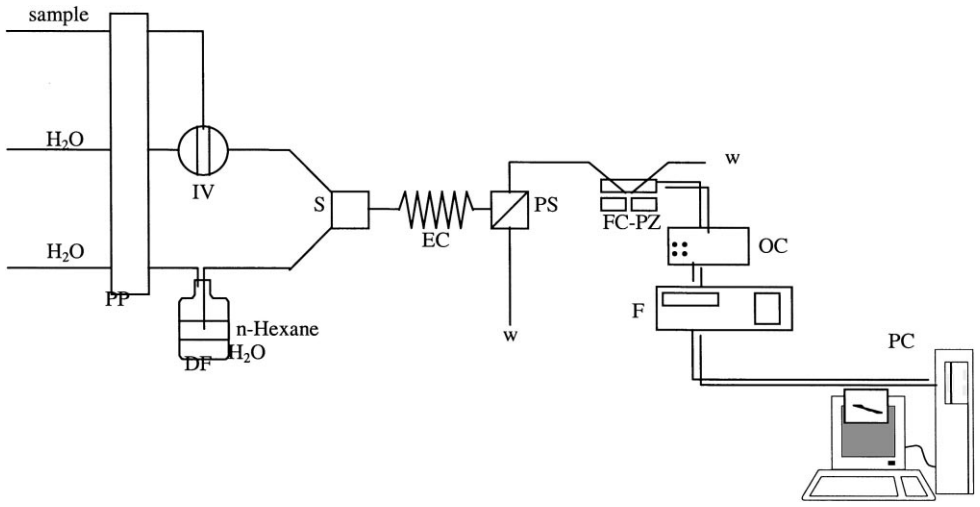

Fig. 2. Manifold for the determination of fat in milk. PP: peristaltic pump, IV: injection valve, DF: displacement flask, S: segmenter, EC: extraction coil, PS: phase separator, w: waste, FC-PZ: flow-cell and piezoelectric crystal, OC: oscillator circuitry, F: frequency counter and PC: interfaced personal computer.

2.4. Manifold design and procedure

The configuration of the manifold used is shown in Fig. 2. The carrier solution (H_2O) was pumped through tygon tubes. A displacement flask (DF) was used to avoid passage of the organic solvent through the pump tubes. Samples and standard solutions were injected by means of an injection valve (IV). The solution held in the sample loop (500 μl) was transported by the water carrier to a segmenter (S), where it was mixed with *n*-hexane. Then, it was transferred to the extraction coil (EC) and finally to the phase separator (PS), containing a Fluoropore (PTFE)® membrane. The organic phase was passed through the piezoelectric flow-cell (FC-PZ), where the signal was detected, transmitted to the frequency counter (F) and recorded (PC). Samples and calibration standard solutions were injected into the flow once the base resonant frequency (F_b) levelled off and measurements were found to be $\Delta F = F_p - F_b$, where F_p is the maximum frequency during each run.

3. Results and discussion

3.1. Mass-sensitive detection

The behaviour of the 10 MHz AT-cut quartz crystal was consistent with the reported information [17]. Thus, the frequency decreases on bringing one face of the crystal into contact with the organic phase containing the analyte. Fig. 3 shows the frequency–time profile for an injection of a 0.35% w/v palmitic acid solution; the fast response of the crystal allows the analyte concentration to be determined within a few seconds. The interval of readings (1–38 in *x*-axis Fig. 3) shows the baseline frequency (F_b); the interval (38–75) shows the noise that is produced in the instant of introducing the sample through the injection valve; the interval (112–223) shows the frequency when the palmitic acid solution is in contact with the detector (F_p). The measurement is given by difference between the baseline frequency and the frequency during the run measurement. Viscosity and density remained unchanged as the temperature was kept constant ($22^\circ C \pm 1$). This suggests that the frequency change is caused by the small mass change in the quartz electrode surface [2] and hence that it depends exclusively on the analyte concentration. In fact, the frequency change (detector response) was found to be linearly related to the concentration of the injected palmitic acid solution.

3.2. Optimization of variables

Preliminary experiments showed the flow-rate, length of the extraction coil and nature of the extrac-

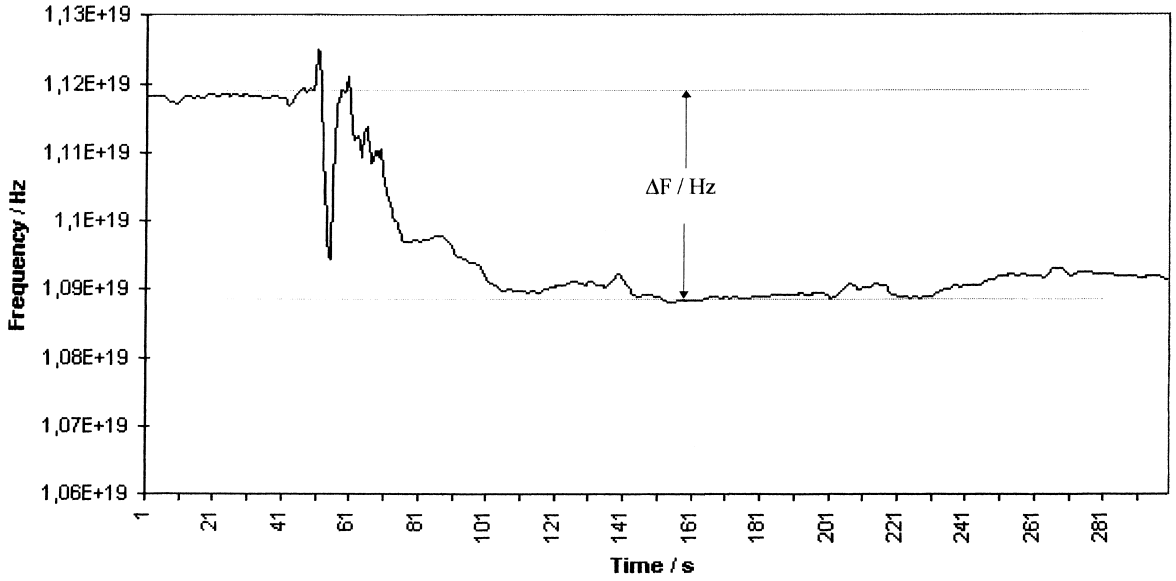

Fig. 3. Frequency vs. reading (time response) for a 0.35% w/v palmitic acid solution, flow-rate 0.25 ml/min, injected volume 500 μl, coil length 50 cm (0.8 mm i.d.).

tion solvent used to influence the frequency change in the detector. The palmitic acid concentration used in these experiments (fat fraction) was 0.35% w/v.

3.2.1. Flow-rate

The flow-rate affected the frequency. Also, it had a strong effect on the phase separator efficiency. Thus, increasing flow-rates in the range 0.10–0.30 ml/min resulted in increasing frequency changes. In order to boost throughput, a flow-rate of 0.25 ml/min was used as a compromise.

3.2.2. Extraction coil

The length of the extraction coil (0.8 mm i.d.) also affected the frequency of the detector. The optimum length was found to be 50 cm, which provided a stable baseline. Longer lengths resulted in increased dispersion in the detector response whereas shorter lengths caused a significant loss of sensitivity.

3.2.3. Influence of the extraction solvent

Two different solvents among those used in conventional methods for the extraction of fat [18] were studied, namely *n*-hexane and diethyl ether. Both were used to run calibration graphs, the regression coefficients of which were 0.9982 for *n*-hexane and 0.9881 for diethyl ether. *n*-Hexane was therefore, selected as solvent for fat extraction as it was more compatible with the chemical composition of the Fluoropore® membrane.

3.3. Figures of merit of the proposed method

Standard solutions containing palmitic acid (fat fraction) at concentrations between 0.20 and 0.45% w/v were used to construct the calibration graph to determine fat. The figures of merit of the proposed method are listed in Table 1. The calibration graph (run from triplicate measurements at each point) was linear over the range 0.20–0.45% w/v. The sensitivity of the method is 1061.9 ΔF (Hz)/% for palmitic acid. The precision of the method, expressed as relative standard deviation, for 0.35% w/v palmitic acid was $\pm 3.2\%$ ($n = 11$; $P = 0.05$). The estimated throughput was 12 samples per hour. The proposed method is thus an expeditious alternative to the official method, which takes about 1.5 h per analysis.

3.4. Validation of the method

Table 2 shows the results obtained in the validation of the proposed method against the official one [17] by

Table 1
Figures of merit of the proposed method for the determination of fat in milk

Equation[a] ($n=15$)	$S=1061.895C-77.429$
Regression coefficient	0.9982
R^2	99.6%
Standard deviation of residuals, $S_{y/x}$	2.04
Determination range (% w/v)	0.20–0.45
Throughput (samples^{-1})	12
Detection limit (% w/v)[b]	0.06

[a] Dependent variable: S, Signal measured [frequency difference ΔF (Hz)], Independent variable: C, % w/v fat.
[b] Defined as the blank signal plus three times its standard deviation.

Table 2
Validation of the proposed method by regression analysis against the official one

Dependent variable Y:	Independent variable X:	
Parameters	Estimate	Standard error
Intercept a:	0.104	0.07
Slope b:	0.994	0.03
Correlation coefficient, r	0.996	
R^2 (%)	99.1	
Standard deviation of residuals, $S_{y/x}$	0.131	

regression analysis. The results provided by the official method were used as independent variable (x-axis), and those obtained with the proposed method as dependent variable (y-axis). The calculations showed standard errors to be insignificant. With 13 degrees of freedom ($n-2$), the following confidence limits ($P=0.05$) were obtained for the slope and the intercept: $a=0.1042\pm0.1499$ and $b=0.9937\pm0.0575$. Based on these results, the slope and intercept were not significantly different from the 'ideal' values of 1 and 0, respectively, so no systematic differences between both series of results existed. Therefore, the proposed method is traceable to the reference one.

Table 3
Analysis of synthetic samples containing palmitic acid as fat

Concentration added (% w/v)	Concentration found (% w/v)	Error (%)
0.20	0.19 ± 0.02	−5.0
0.25	0.25 ± 0.02	0.0
0.31	0.31 ± 0.04	0.0
0.35	0.35 ± 0.02	−5.7
0.40	0.41 ± 0.04	+2.5
0.45	0.44 ± 0.03	−2.2
0.23	0.22 ± 0.02	−4.3
0.32	0.32 ± 0.04	0.0
0.30	0.29 ± 0.01	−3.3

3.5. Analysis of synthetic and real samples

The applicability of the proposed method was initially checked by analysing synthetic samples of fat. The results obtained are shown in Table 3. As can be seen, differences between the concentrations found and those added were in general small (less than 4%). Subsequently, the method was applied to real samples (milks of different brands). The matrix effect was cancelled by effect of the samples being extracted within the flow system. As it is demonstrated in Table 4, and especially in Table 5, any significant interference can be supposed because of the other components besides fatty acids in milk. The results obtained were compared with those provided by an officially recommended method [17]. As can be seen from Table 4, both were quite consistent, as further confirmed by Table 5, which shows the results of the t-paired

Table 4
Analysis of milk samples by using the proposed and official methods

Sample	Concentration found (% w/v)		
	Proposed method	Official method	Difference (d)[a]
Whole milk (RAM)	3.65 ± 0.04	3.70 ± 0.20	-0.05 ± 0.24
Low fat milk (Covap)	1.58 ± 0.01	1.38 ± 0.02	0.20 ± 0.03
Whole milk (Pascual)	3.93 ± 0.03	3.94 ± 0.04	-0.01 ± 0.07
Low fat milk (RAM)	1.48 ± 0.01	1.35 ± 0.04	0.13 ± 0.05
Low fat milk (Eroski)	1.19 ± 0.09	1.01 ± 0.04	0.18 ± 0.13

[a] Difference (d) = Proposed method − official method.

Table 5
Results obtained by applying the paired *t*-test to compare the proposed and official methods for the determination of fat in milk[a]

Sample statistics:	Number of observations	5
	Average	0.09
	Variance	0.0129
	Standard deviation	0.1136
	Median	0.13
Confidence interval for mean:		95%
Sample		$-0.050802 + 0.0230802$ 4 degrees of freedom
Hypothesis test: H_0: Mean = 0		Critical value for $t = 2.776$
H_a: Mean $\neq$ 0		Experimental t value = 1.775
at $P = 0.05$		So H_0 should be accepted

[a] Difference (d) = Proposed method − official method.

comparison test. The null hypothesis was H_0: mean (d) = 0, versus the alternative one H_1: mean (d) $\neq$ 0. The computed value of t in Table 5 was less than the critical value (with $\nu = 4$ degrees of freedom, at the 95% confidence level, t_{crit} is 2.776), so H_0 is not rejected and no differences exist between both methods. Consequently, the proposed method is applicable to real samples.

4. Conclusions

The proposed method provides an automatic, simple miniaturized tool for the determination of fat in milk. Its simplicity greatly reduces analysis times and potential sources of error. Its principal is that is uses small amounts of organic solvent, which lowers toxicity risk and decreases reagent costs relative to the official method. The proposed method is thus a useful screening tool.

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