Screening for Melamine in milk by Lactoscope FTIR

Application of NPN/Calculated Melamine (NPN/CM)

and a Melamine specific IR-model

Introduction

The sad consequences, which surfaced in the 2nd half of 2008, of adding melamine to milk in order to artificially increase the apparent protein content, have highlighted the need for screening methods for the early detection of such adulteration of milk. Several analytical methods (like ELISA & GCMS) for determining melamine in milk and milk products down to a few ppm or even ppb do exist, but these methods add significant cost and time if used on a routine basis. Achieving single digit ppm melamine detection limits with infrared analysis is impractical, but screening for melamine adulteration can be done effectively by using the Lactoscope FTIR at the same time as payment or incoming control tests are done, thus eliminating additional testing time and analytical costs.

The addition of melamine to milk increases the apparent protein content by approximately four times the amount of melamine added when the protein content is estimated by either N-Kjeldahl or standard methods of infrared analysis. Given the limited solubility of melamine in water (3 g/kg or 3000 ppm) at ambient temperature, the apparent protein content of milk can only be raised about 35%, or 12g/l.

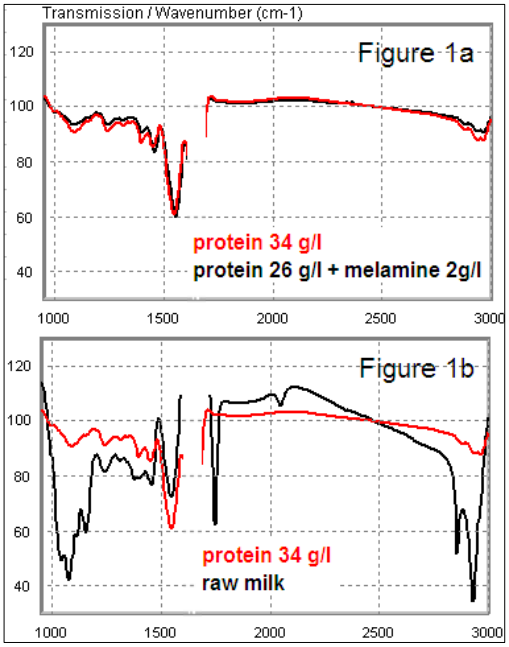
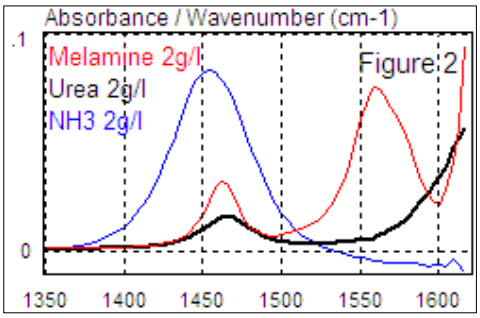


Figure 1a shows the close resemblance of infrared spectra of solutions of protein only (in red) and of protein plus 2g/l of added melamine (in black). The main infrared signal of protein, centered near 1545 cm^{-1} differs between the two spectra, but the overall absorption patterns appear similar. Spectra of adulterated milk are even more difficult to distinguish from those of regular milk because of the natural variability in milk composition of both major as well as minor components. (For reference purposes, a spectrum of raw milk is shown in figure 1b.)



Given these rather small spectral differences, a screening method based on pattern recognition would be rather insensitive unless specific knowledge of the contaminant's spectra is available. The spectrum of melamine dissolved in water (figure 2) displays two absorptions. The one centered at 1560 cm^{-1}, overlaps the main absorption of protein. The other centered near 1460 cm^{-1} coincides with the most prominent absorption of urea in milk. Based on a close resemblance in spectral response and the high correlation between

NPN and urea of regular cow milk, a method called NPN/calculated urea (NPN/CU) was developed by Delta Instruments BV for the Lactoscope FTIR (see the research note on NPN/CU). With this method, the urea content of regular milk is calculated from the NPN content as determined by the Lactoscope FTIR.
Using the same approach an estimate of the melamine content of adulterated milk can be calculated, which we will refer to as NPN/calculated melamine (NPN/CM). The specificity of this approach is rather restricted since urea and other NPN components like ammonia (see figure 2) contribute as well. But at the same time this approach may serve a wider purpose, since it can be used like wise as an early alert for the adulteration of milk with ammonia, which can be denoted with NPN/calculated ammonia (NPN/CA).
Since we recognized that greater specificity for melamine may be desirable, a full spectrum model was also developed as an alternative to the NPN/CM approach.
This paper reports on the applicability of both approaches and provides details on the performance achieved with the melamine specific model. Our studies show that the NPN/CM approach allows for the discrimination of melamine added to milk down to a level of 500 ppm. With the melamine specific model, the detection level is further reduced to a 100 ppm.

Materials & methods

Milk samples spiked with melamine

Two crates (A & B) of 48 milk samples per crate, all sample remains of milk payment testing at the main milk control station in the Netherlands (“Qlip”) were selected (early Oct-2008) so as to span a maximum natural variation in fat, protein and lactose content.
Analytical grade melamine (Fluka) was dissolved to a concentration of 20 - 30 g/l in demineralized water at approx. 80°C and added on a mass per mass basis to the milk samples (preheated to 40°C) shortly before analysis. Melamine was added at 8 levels to samples of crate A; 6 samples per level; levels of approx. 0, 300, 400, 500, 750, 1500, 3000 & 6000 ppm melamine. Melamine was added at 7 levels to samples of crate B + one extra (N=49), levels of approx. 0, 200, 400, 600, 1000, 1400 & 1900 ppm melamine. Extra urea was added at 7 levels to these samples of crate B in an orthogonal fashion to the melamine added; urea levels of approx. 0, 150, 300, 500, 800, 1100 & 1500 ppm urea.

FPL milk calibration sets

Two standard milk calibration sets (FPL sets) obtained from Qlip, composed each of a concentration series of fat (F0..F8, 0 to 8%m/m), of protein (P1..P5, 1.5 to 5%m/m) and of lactose (L1..L5, 3 to 5.5%m/m) of 2 productions (May and July) were analyzed on the Lactoscope FTIR without melamine added. The 2nd set was analyzed twice, using a normal and a sub normal performing homogenizer (L0547 & L0791 resp.).

Milk dataset for evaluation

For evaluation purposes, a large dataset (N>17000) of (mainly) milk spectra collected during production at a major cheese plant in the Netherlands, over a continuous period of more than a year (from jan-2006 till may-2007) was used. Omitted from the set were samples (approx. 1%) for which either 1) duplicates were found to poorly reproduce or 2) the measurement had been interrupted or 3) protein (<0.5%m/m), fat (cream & double cream) or lactose (<3%, >6%) contents differed significantly from milk.

Calculation of NPN/CU, NPN/CM and NPN/CA

Results for NPN/calculated urea (NPN/CU) in ppm (mg/kg), were calculated automatically by the Lactoscope FTIR from predictions for the Non Protein Nitrogen contents (NPN) of the samples. In formula:

Calculated urea = 1/a * (NPN - b) **[1]**

Where a, and b are constants, derived from the study in 2004 after prediction of urea on the basis of the NPN contents of milk. For details, please refer to the research note on NPN/CU.

Results for NPN/calculated melamine (NPN/CM) in ppm, were calculated from the NPN/CU results, using:

Calculated melamine = 1/*fcm* * (NPN/CU - 200) **[2]**

Where the value of 200 ppm subtracted from the NPN/CU result, represents an estimate of the median urea content of the milk samples. *fcm* is the response factor of melamine, with the given model for NPN/CU on the Lactoscope FTIR, as determined from standard addition of melamine to milk (see results & discussion, figure 3 a,b).

Results for NPN/calculated ammonia (NPN/CA) in ppm ammonia (mg NH_3/kg) were calculated in a similar way using:

Calculated ammonia = 1/*fca* * (NPN/CU - 200) **[3]**

Where *fca,* the response factor of ammonia, with the given model for NPN/CU on the Lactoscope, as determined from standard addition of ammonium chloride to milk (in mg NH_3/kg milk, see results & discussion, figure 7 a,b). And the value of 200 as above represents the median urea content of the milk samples of crate A.

Table 1 Descriptive statistics - Crate B

	Fat	*Protein*	*Lactose*	*Melamine added*	*Urea added*
	%m/m	%m/m	%m/m	in ppm	in ppm
Mean	4.46	3.54	4.50	774	611
SD	0.34	0.22	0.20	642	494
Maximum	5.4	4.0	4.8	1976	1570
Minimum	3.8	3.1	3.9	0	0
Range	1.6	0.9	0.9	1976	1570
Count	49	49	49	49	49

Correlations (R) - Crate B only

Crate B only	*Fat*	*Protein*	*Lactose*	*Melamine added*
Fat	1			-0.56
Protein	0.61	1		-0.53
Lactose	0.44	0.38	1	-0.81
Urea added	-0.23	-0.1	-0.02	0.02

Correlations (R) - Calibration set

Crate B & 3 sets FPL	*Fat*	*Protein*	*Lactose*	*Melamine added*
Fat	1			-0.05
Protein	-0.05	1		-0.09
Lactose	-0.16	0.0	1	-0.35
Urea added	-0.01	0.0	-0.17	0.46

Melamine model development & statistics

A full spectrum IR-model for the detection of melamine with high specificity was developed using Grams/PLSIQ software. The calibration set was composed of the samples of crate B and the FPL sets. Included was a single replicate per analysis (carried out in duplicate or more). Melamine added to crate B was varied orthogonal to the extra urea added. See table 1 for descriptive statistics of crate B and correlations among the constituents of both the calibration set and crate B alone. The optimum number of factors was determined from cross

validation (cyclic, leaving out one sample at a time), yielding a standard error of cross validation (SECV). The model was validated first, on the basis of results for melamine added to crate A, for which a standard error of prediction (SEP) was calculated, corrected for a slight deviation (±1%) in slope (denoted with SEP*), attributed to some analytical error in the preparation of the samples. Further validation took the reanalysis of production datasets of milk. Among these was a dataset including sour milk samples that revealed a modest error in predicted values for melamine as a function of pH (not detailed here). Based on those data, a pH correction was calculated that was applied to all melamine results reported here and is referred to as "Melamine (pH corrected)". Final validation of the pH corrected model was on the basis of the production milk dataset of more than 17000 samples collected at a major cheese plant (on another Lactoscope FTIR). The mean and standard deviation (SD) in the melamine responses were calculated over this dataset as a whole.

Results and discussion

The NPN/CM Approach

In the Lactoscope FTIR (both FTA and Combi versions), the urea content of milk is calculated from the IR determination of the Non Protein Nitrogen content (NPN) by a slope/intercept calculation according to formula [1] above and dubbed " NPN/CU". This model has been shown to predict the urea content of regular cow milk with good accuracy. The NPN/CU model is considered rugged and robust thanks to the wide variety of milk samples used in the calibration, consisting of both herd and individual cow milk samples collected from all over the Netherlands. The good accuracy achieved with this approach largely derives from the close correlation between urea and NPN in milk (For further information see the research note on NPN/CU).

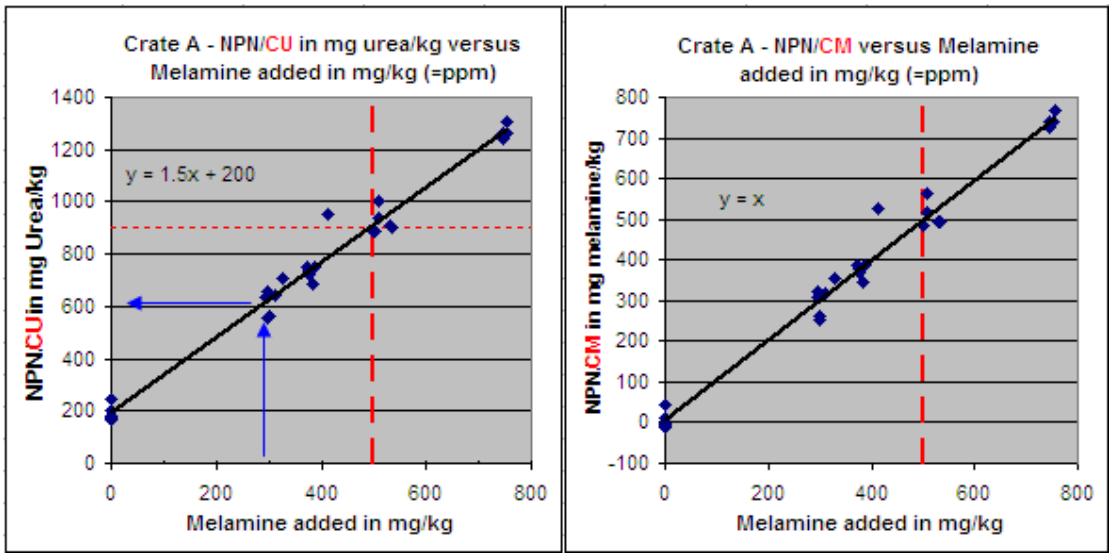


Figure **3a** - **NPN/CU & NPN/CM** - Figure **3b**

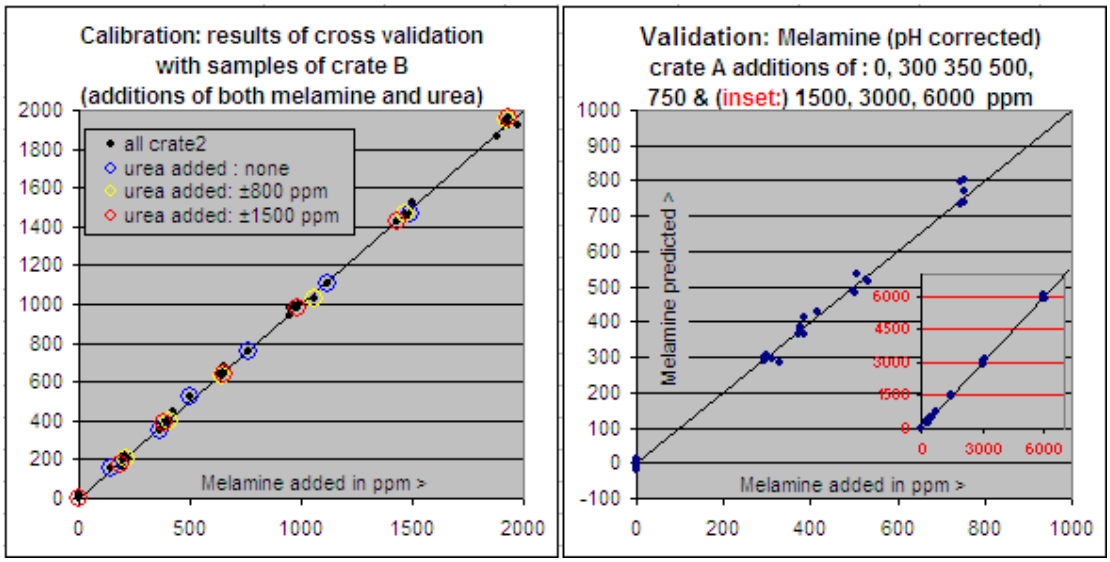
Given the strong overlap of the most prominent absorption of urea in water and one of the absorptions of melamine, centered at 1460cm-1 (figure 2), any model for NPN or urea in milk developed on the basis of regular cow milk is expected to show a strong response to added melamine. This is also what was observed, for melamine added to samples of crate A, for

which NPN/CU results are displayed in figure 3a as a function of melamine added in ppm (= mg/kg). Without melamine added, NPN/CU averages approx. 200 ppm (or 200 mg urea/kg milk), which is a regular level of urea in milk and, in this case, about the median urea content of samples of crate A. Upon addition of 300 ppm melamine, NPN/CU readings rise to on average 650 ppm. With 500 ppm added the NPN/CU respons increases to 900 ppm. Values of 800 ppm urea and higher are rarely observed with regular herd milk, thus a discrimination limit for samples considered "suspicious or not" can be set to a level of 500 ppm or 0.05%m/m melamine added to milk when using the NPN/CM appraoch.
Based on the slope ("fcm") of 1.5 in the response for melamine on the NPN/CU scale and the median content of 200 ppm urea, results have been rescaled according to formula [2] into results for what we want to call NPN/calculated melamine (NPN/CM) in figure 3b. Deviations from the line y=x in this plot largely reflect the natural variation in urea content of the samples.

The Full Spectrum Melamine Approach

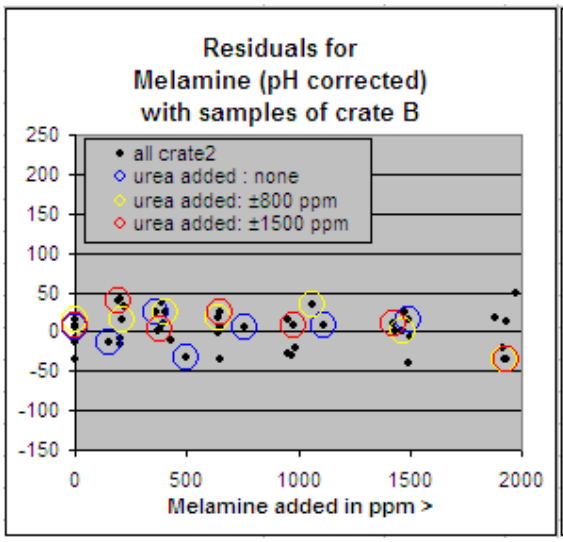
As an alternative to the NPN/CM approach, a full spectrum model has been developed for the detection of melamine in milk with better specificity. The calibration set applied consisted of milk samples of crate B with melamine and urea added in an orthogonal fashion and three milk calibration sets, referred to as FPL sets (see Materials & Methods). Figure 4a, displays the results of the cross validation, for samples of crate B. A nice linear relationship is observed, characterized by a standard error of 17 ppm (SECV, computed over the complete set). As illustrated by figure 4a, all points fall close on the line y=x, irrespective the amount of urea (0 to 1500 ppm) added to the samples.

Figure **4a** - **Melamine specific model** - Figure **4b**



Thus with this full spectrum model, the melamine response is free of interference from urea. The same was found to hold with regard to (not detailed here:) interference from ammonia added to milk upto 500 ppm NH3 in the form of NH4Cl. In contrast, the souring of milk was found to adversely affect the melamine readings, but easily corrected for by incorporation of a pH correction (not detailed either). All data on the melamine specific model reported were corrected and results are refered to as "melamine (pH corrected)".

Residuals for the calibration set samples displayed in figure 5, reinforce the independence in melamine response as a function of urea content as well as fat, protein and lactose contents over the ranges 0 to 8% fat (F0..F8), 1.5 to 5 %protein (P1..P5) and 3 to 6% lactose (L1..L5). The validation on the basis of samples of crate A, of which a graph is given in figure 4b, demonstrated the accuracy and good linearity in response over the range up to 6000 ppm melamine.



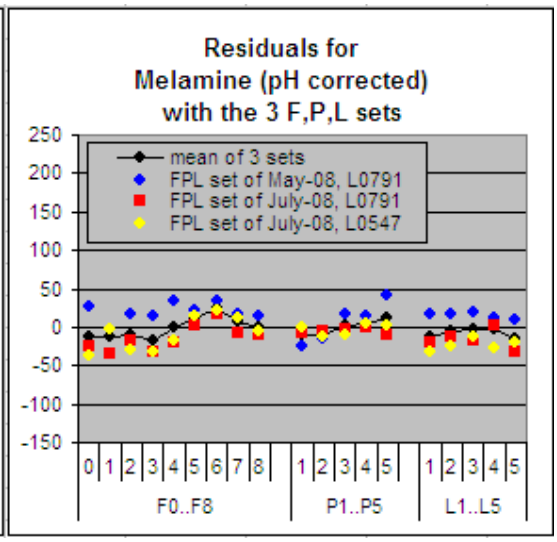


Figure **5a** - **Melamine calibration residuals** - Figure **5b**

Statistics for the melamine specific model have been summarized in table2. Calculated were standard errors of 21 ppm both for the standard error of calibration (SEC) and for the standard error of prediction (SEP) for samples of crate A (N=36, up to 1500 ppm, i.e. exclusive the 2*6 samples with additions as high as 3000 and 6000ppm). The repeatability standard deviation (SDrep(i,i+1)) as deduced from consecutive measurements of samples analyzed in duplicate or triplicate was typically 10 ppm.

Table2
Statistics "Melamine (pH corrected)"

	in ppm
SEC(N=106)	21
SEP*(A, ex highest 12)	21
SDrep(i,i+1)	10
SD(milk set, N≈17000)	16

A further validation on the basis of a milk set of over 17000 analyses obtained from a large cheese plant in the Netherlands, collected over a period of more than a year, yielded a mean response for melamine of 10 ppm and a value of 16 ppm for the standard deviation in the results. A graph displaying all of the results for Melamine (pH corrected) is displayed in figure 6a. Although the vast majority of samples analyzed were just milk samples, the set was found to include some more samples and the variability in milk samples analyzed was quite large. This is illustrated by the graph of the fat results (figure 6c), showing that the fat content of a majority of samples was around 4%m/m or at another discrete level e.g. close to 2% or 0% fat, but the range in fat runs from 0 up to 8%. Eliminated were some samples more extreme in composition (see material & methods). Almost all results for Melamine (pH corrected) fall in a narrow range around 0 ppm of ±50 ppm. Observed in negative direction are a few outlying points, but in number these still are negligible. The highest result appears 91 ppm, shortly before (a day) preceded by a sample for which the response is 73 ppm. Both results however were found to concern samples of

whey of which some 20 more samples were present in the set and for which the average melamine response turned out to amount 46 ppm, with a standard deviation of 19 ppm in the results. We may conclude that adaptation to a matrix like whey can be made by a simple bias correction of about 50 ppm. This brings us to the conclusion that the discrimination limit regarding "suspected or not" for Melamine (pHcorr) can be safely set for milk at 100 ppm.

Why the NPN/CU limit cannot be as low as the full spectrum model is evident from the results for the uncontaminated milk set of figure 6b. Choosing a limit of 500 ppm is certainly safe, and might even be lowered somewhat if the variation in NPN/CU content (figure 6d) stays in a 100 to 400 ppm urea band with some seasonal dependence.

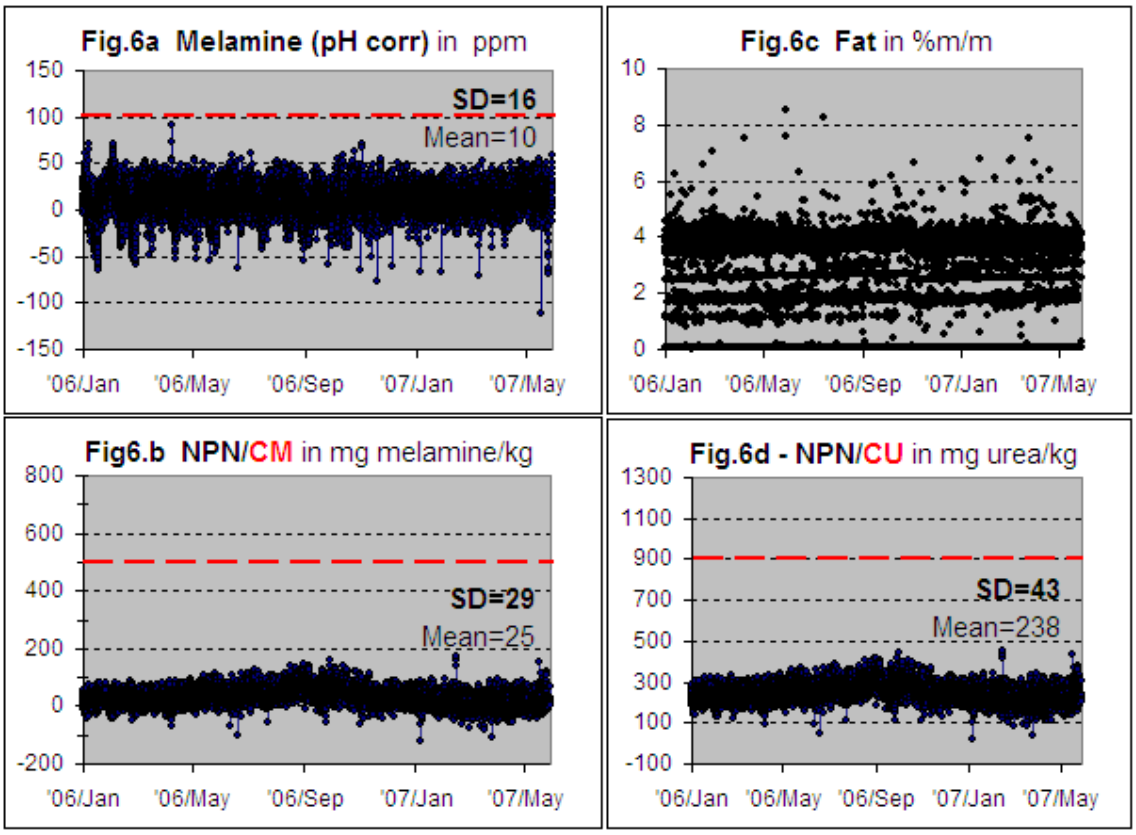


Figure **6** **Milk set from practice (N≈17000)**

The NPN/CA Method

Finally, what has been baptized NPN/calculated ammonia (NPN/CA) deserves some attention. Figure 7a, below, displays the increase in NPN/CU response in milk as a function of ammonia (NH3) added in the form of ammonium chloride (NH4Cl, i.e. in such a form that the pH of the milk sample stays neutral). Ammonium chloride was added in various amounts to a single sample of fresh milk, so the results fall on a straight line. Expressed in ppm urea versus ppm ammonia, the slope of the line (fca) amounts 2.1. Rescaling according to equation [3] of Material & Methods yields the values expressed in NPN/CA displayed in figure 7b. Following the same line of reasoning used in the case of NPN/CM, the

discrimination limit for “adulterated or not” by the addition of ammonia can be set in this case to 350 ppm.

Whereas N-Kjeldahl would show elevated protein results when ammonia has been added to milk, neither added ammonia nor added urea would result in higher protein readings when using standard infrared analysis methods. However, if ammonia (in the form of NH4OH) is added to restore the pH of sour milk to mask its poor quality, the NPN/CA approach could be of value in detecting this form of adulteration.

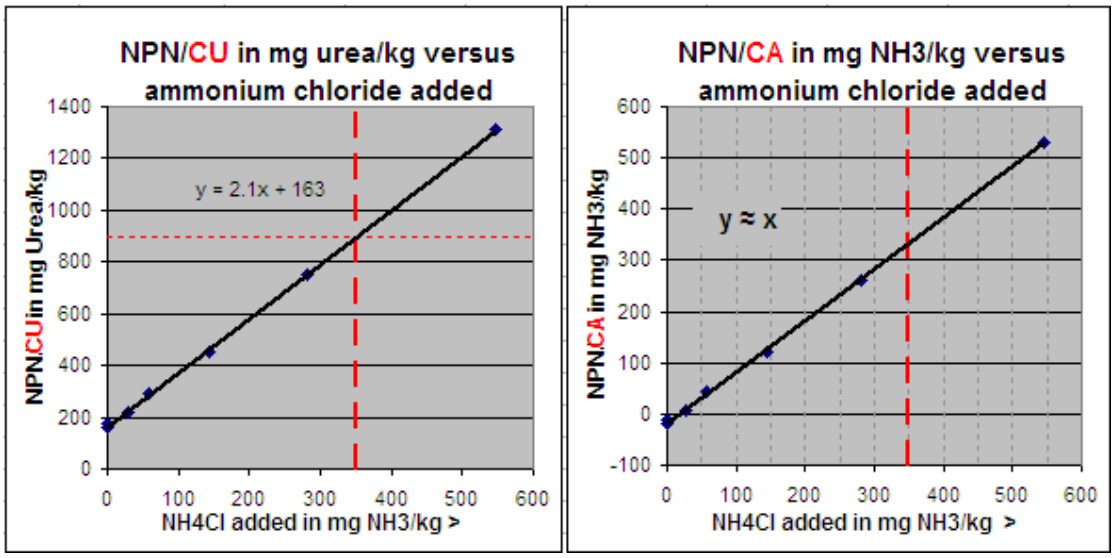


Figure **7a** - **NPN/CU & NPN/CA** - Figure **7b**

Conclusion

The present work demonstrates the practicality of screening raw milk for the presence of melamine down to a level of 500 ppm or 0.05%m/m using the NPN/CM approach on a Lactoscope FTIR. In addition, the discrimination limit can be further reduced to 100 ppm (0.01%m/m) using the melamine specific detection model.

Given the infrared multiplying factor of between 3 and 4 for melamine (in %m/m) on IR based protein (in %m/m) results, added melamine will only become detectable when added in sufficient quantities to raise the apparent protein content (normally about 3%) by 0.2%m/m or 0.04%m/m respectively.