The determination of residual lactose in lactose-reduced milk by Lactoscope FTIR advanced

Calibration, validation & application to milk & milk products

Abstract

This application note reports on the development of a calibration model, its validation and applicability for the determination of residual lactose in lactose-reduced milk and milk products using a Lactoscope FTIR Advanced (FTA) Instrument. The calibration model developed for milk was based on a set of 76 samples, including process samples, end-products of various brands of skim milk, semi skim and whole milk and a single brand of cat milk and mixes with regular milk, milk protein retentate and milk permeate. A similar set (39 samples) was prepared for the validation study, half a year later. Accuracies, as determined from the standard error of cross validation (SECV) for the calibration and the standard error of prediction (SEP) for the validation, were 0.07%m/m and 0.04%m/m respectively. Repeatability standard deviation (SDrep) was typically better than 0.025%m/m. An additional model for cream was established by inclusion of a sub set of 7 cream samples. Mean results of determinations in 5-fold for 5 samples of whole milk and 11 samples of half cream milk of different production dates but of a single brand were used to demonstrate the applicability of the method for end-criteria control of lactose levels in process monitoring. For both products mean results were close to "zero" %m/m lactose and revealed a minimal variation among samples, characterized by a standard deviation in the mean results (SD(mean)) of ≤0.02 %m/m. Tests on cream yielded similar positive results. In contrast, tests with lactose reduced chocolate milks and 2 brands of different cat milks had higher variability. Measured values based on the model differed from declared contents by several % in m/m, and chocolate milk results were highly variable (by several % m/m). Causes are discussed and possibilities of corrections explained.

Introduction

"Lactose-reduced" milk and milk products, also referred to as "low lactose" or "lactose-free" milk products, are of special commercial interest since the lactose present in regular milk products make it unsuitable for the majority of the world's adult population [1]. This is referred to as "lactose intolerance", i.e. the inability to cleave the disaccharide lactose into the monosaccharide's glucose and galactose, by lactase in the small intestine.

"Lactose-free" milk is mostly produced by treating milk with β-galactosidase, typically the lactase from Kluyveromyces Lactis [2]. The progress of the enzymatic reaction depends primarily on temperature, enzyme concentration and reaction inhibition by the galactose formed. At high lactose and galactose concentrations moreover, lactase shows significant transferase ability and produces b-1,6-linked galactosyl oligosaccharides. In industrial

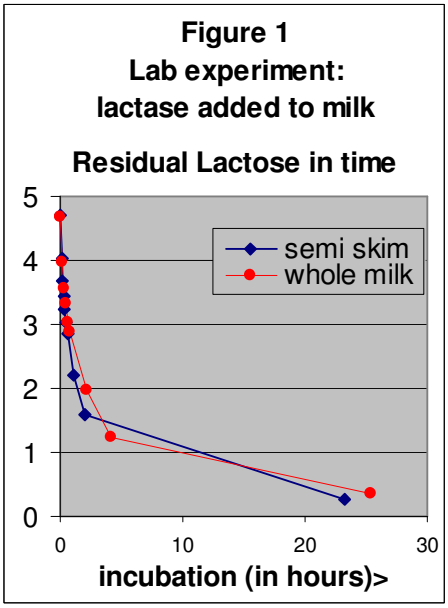
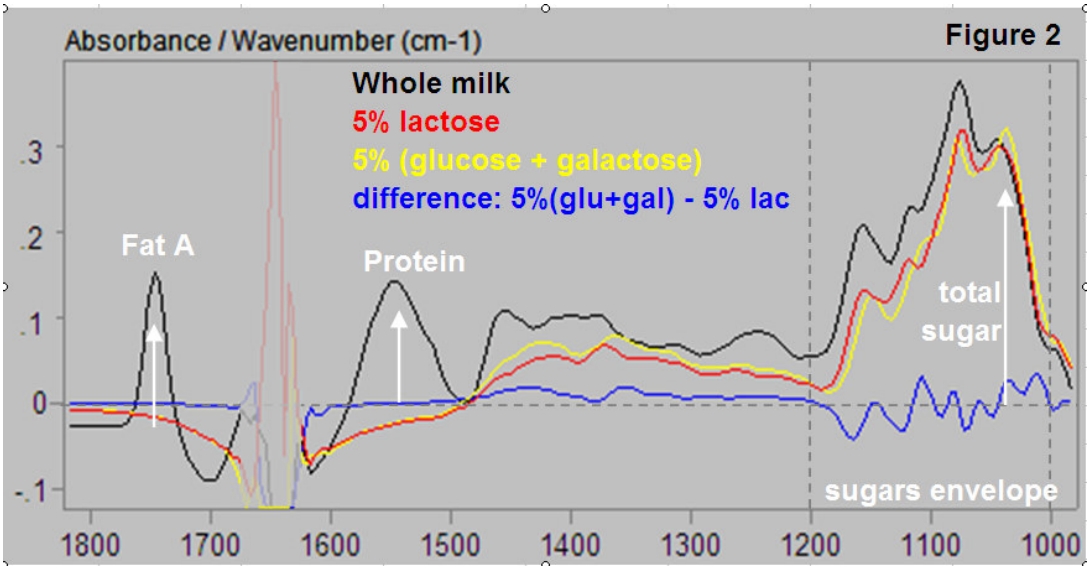


Figure 1
Lab experiment:
lactase added to milk

practice the reaction is typically completed within a period of 4 to 24 hours. The reaction slows down towards the end (Figure 1, displays the decrease in lactose concentration as a function of time upon lactase incubation of whole milk and half cream milk in a laboratory experiment where the reaction took more than a day). A simple, rapid method for monitoring the residual lactose concentration would improve control of the process and evaluation of endpoint-criteria. With most “lactose-free” milk products the limit is typically set to 0.1g lactose/100g of product.

Several methods for the determination of residual lactose in lactose-reduced milk currently exist. These include: HPLC[3], colorimetry[4] and freezing point cryoscopy[5,6]. The former two methods are time-consuming. The latter is widely used in practice, but lacks the ability to discriminate between hydrolysis and transferase activity. Recent measurement of Lactose using FTIR methods by a number of authors have shown it to have promise as an alternative method. In 2004 a study was published by Cocciardi et al.[7] on the quantitative measurement of residual lactose in milk by single bounce ATR FTIR. In an earlier study, Hansen et al. (1999) [8] reported on the determination of residual lactose in milk using multivariate curve resolution, but quantitative results obtained through calibration were not given.



Monitoring lactose hydrolysis in milk to residual lactose levels of 0.1%m/m by FTIR spectroscopy requires the accurate determination of lactose in the presence of approximately 5%m/m of the hydrolysis products: glucose and galactose. Owing to the extensive overlap of the IR absorption bands of the 3 sugars, the difference IR absorption signals associated with lactose hydrolysis (Figure 2: blue line for 5%(glu+gal)-5%lac) are rather weak compared to the signals of the main components of milk. As a result, a calibration model for residual lactose requires multivariate calibration techniques such as partial least squares (PLS). This is in contrast to the calibration models for fat, protein or total sugars in milk, where the main signals are readily delineated from the spectra (Figure 2: white arrows) and quantification can be accomplished with a few analytical wavelengths in combination with multiple linear regression (MLR).

This note reports on the development and validation of PLS calibration models for the determination of residual lactose in milk and cream. Results of application tests demonstrate the utility and limitations of the method for monitoring hydrolysis processes and use in lactose-reduced milk product measurements.

Materials & methods

Calibration & validation set samples

Samples of the milk calibration set (N=77) composed at LUFA Nord West, were from 3 different producers in Germany, except for a single sample obtained from Valio in Finland. Among the samples were a series of skim milk and a series of half cream milk both collected at a dairy plant during their hydrolysis process at fixed time intervals of approx. 4 hours over a period of 24 hours. Additional calibration set and validation set samples (N=39) included lactose-reduced end products of skim, half cream milk, whole milk and a single brand of cat milk and mixes of these samples with regular milk, milk protein retentate and milk permeate. Further, a cream calibration subset of 7 samples was prepared from 2 low lactose cream samples mixed with regular cream of 30%.

Lactose mixes were prepared on a mass/mass basis and lactose levels calculated from the reference analyses carried out on the product and the process samples. There was good agreement between results for mixes obtained by calculation and from reference analysis. Tables 1a &1b give descriptive statistics and correlations among residual lactose and the main milk components of both the milk calibration and validation sets. The validation set was prepared at LUFA Nord West 6 months after the calibration set and analyzed twice on July-09 and July-16. With the 2nd measurement, the validation set was reduced to 27 samples, because some samples were no longer available and part of the set had turned sour (changes in pH of 0.1 to 1 pH unit) as determined from pH estimates by Lactoscope FTA.

Table 1a

Descriptive statistics - Calibration set (N=77)

	ref lactose %m/m	*FAT-IR %m/m*	*Protein-IR %m/m*	*Tot.sugar-IR %m/m*
Mean	0.8	2.7	3.4	4.6
SD	1.0	1.4	0.3	0.2
Range	4.8	5.2	2.0	1.5
Maximum	4.8	5.3	4.5	4.8
Minimum	0.05	0.1	2.5	3.3

Correlation (R) Calibration set (N=77)

	ref lactose %m/m	*FAT-IR %m/m*	*Protein-IR %m/m*	*Tot.sugar-IR %m/m*
ref lactose	1			
Fat	-0.39	1		
Protein	0.05	-0.13	1	
Total sugar	0.26	-0.29	-0.06	1

Table 1b

Descriptive statistics - Validation set (N=39)

	ref lactose %m/m	*FAT-IR %m/m*	*Protein-IR %m/m*	*Tot.sugar-IR %m/m*
Mean	0.4	3.7	3.3	4.6
SD	0.4	1.4	0.1	0.1
Range	1.0	3.7	0.7	0.3
Maximum	1.1	5.4	3.7	4.7
Minimum	0.05	1.7	3.0	4.4

Correlation (R) Validation set (N=39)

	ref lactose %m/m	*FAT-IR %m/m*	*Protein-IR %m/m*	*Tot.sugar-IR %m/m*
ref lactose	1			
Fat	-0.05	1		
Protein	0.21	-0.27	1	
Total sugar	0.18	-0.90	0.29	1

Reference analysis

The reference method used for Lactose analyses followed the standard protocol of LUFA Nord West (Lufa method nr: “AA 3/5C-513”) for the determination of sugars in dairy products by HPLC. This method involves the analysis of Carrez filtrates on an HPLC Amino column utilizing RI detection and an acetonitril/water gradient as the mobile phase. The working range for the method is from 0.03 to 6 %m/m lactose and the determination limit is 0.05%m/m.

Application test set of samples

For testing the applicability of the model(s) developed, samples of different expiry dates/production dates of whole milk (5), half cream milk (11), cream30% (4) and chocolate

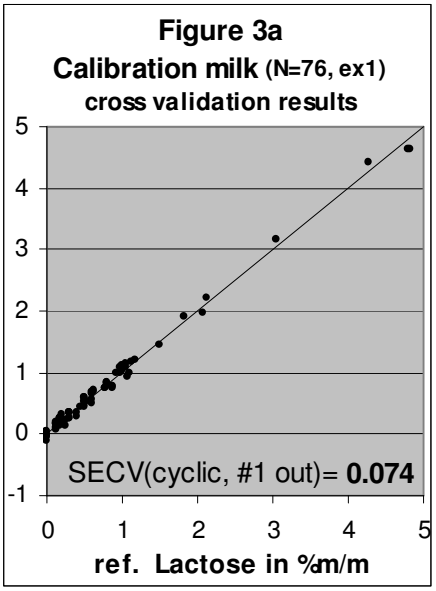
milk(5) of a single (Dutch) brand of lactose-reduced products, “Minus L”, were collected from local stores in the Netherlands and analyzed on a Lactoscope FTIR at Delta Instruments, in February and November. Samples of regular skim (2) and whole cream (2) UHT milk (“Lang Lekker” brand) and 2 samples of different brands of cat milk, “C1000 Kattenmelk” and “Whiskas CatMilk” were also evaluated.

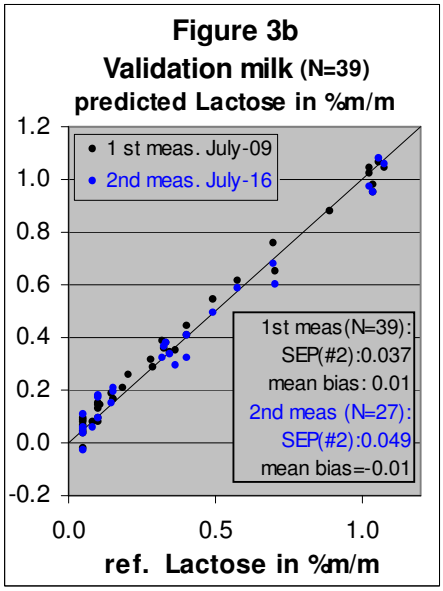
Measurements by Lactoscope FTIR advanced (Lactoscope FTA)

Lactose measurements by Lactoscope FTA were carried out at both LUFA (calibration & validation sets) and Delta Instruments (application test set) following a standard protocol. Standard models for determining fat, protein, total sugars (= lactose) in regular raw milk and cream were employed. The milk added sugar model (“MAS IR”) was used for determining sucrose in the chocolate milk samples. Results at LUFA were collected in duplicate (2 replicate measurements per product sample drawn). 5 replicates per product sample were done for the application tests conducted at Delta Instruments.

Model development & statistics

PLS models for predicting residual lactose in milk and cream were developed using both Bruker OPUS (LUFA Nord West) and Grams PLSIQ (Delta Instruments) software. Final models, reported here and available for use on the Lactoscope FTA, were generated using Grams (based on a single replicate (#2nd) per sample) and predict the lactose content of a sample from selected spectral segments, including the main part of the sugars envelope (see figure 2). Except for 1 sample (N=76), the milk calibration set was used in generating the milk model, referred to as LLmp3601.cal (LSM-parmxx0x). The same set, minus the mixes with permeate and retentate, but including the subset of 7 mixes of cream, was used in generating the cream model, referred to as LLFR3601.cal (or LSC-parmyy0y). The optimum number of factors was determined by cross validation (cyclic, leaving out one sample at a time), yielding a standard error of cross validation (SECV in %m/m). The repeatability of measurements, defined as the repeatability standard deviation, SDrep in %m/m, was evaluated from replicate measurements of the validation set and from 5-fold replicate measurements of the application test set.





Results and discussion

Calibration & Validation

The PLS model for predicting residual lactose in milk was established on the basis of a milk calibration set (N=77) including end products of three different producers, manufacturing process samples collected during hydrolysis and mixes of these samples with regular milk, milk protein retentate and permeate. This set mimicked the variation met in practice and achieved sufficient orthogonality in the variation among the main components of milk (see for details M&M and the correlations given in table 1) for reliable model development. To enhance model stability/performance for measurements near the end point of hydrolysis (i.e. for lactose <0.1%m/m), lactose contents of most of the samples was chosen to be less than 1%m/m and less than 0.6%m/m for half of the samples. Figure 3a, displays cross validation results for samples of the milk calibration set minus one sample, which was identified as a "concentration" outlier. A nice linear relationship is observed, characterized by a standard error of 0.074 %m/m (SECV, computed over the set N=76). Evaluation of spectral residuals and mahalanobis distances for samples of the calibration set, indicated that the distribution among the samples of the set was homogeneous, except for samples of cat milk which were found to add a separate group. Similar results were seen for the single sample of milk from Valio, which differed significantly in total sugars content (3.3%m/m, compared to the rest of samples: between 4.2 and 4.8%m/m). Results (Figure 3b) obtained half a year later on the validation set prepared in much the same way, but restricted to the lower range in residual lactose ≤1%m/m, revealed good model stability: mean bias ±0.01%m/m lactose upon 1st and 2nd analysis and the accuracy in the results characterized by a standard error of prediction (SEP) of 0.037%m/m on 1st analysis and a slightly higher SEP=0.049%m/m on 2nd analysis of part of the same set a week later. Upon validation, the cat milk samples were found to readily fit the milk calibration. On the other hand readings of samples omitted from the 2nd series of measurements of the validation set did not. These readings deviated significantly from readings on the 1st series of measurements with differences ranging from 0.07 up to 0.35 %m/m in lactose. These samples had turned sour while in storage over a period of one week.

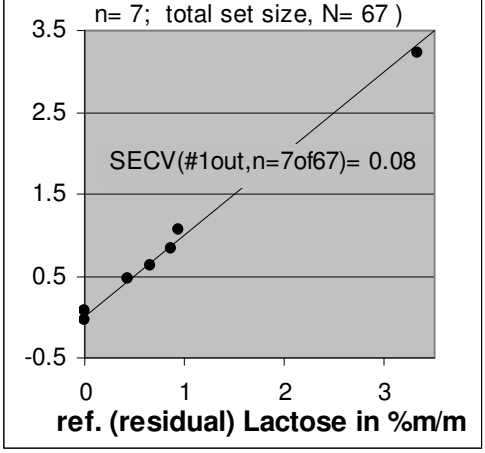
The repeatability of the measurements was evaluated from the repeatability standard deviation, SDrep and was 0.026%m/m as calculated from duplicate measurements of the validation set. Whereas somewhat less, between 0.015 and 0.020%m/m with the determinations in 5-fold with the measurements of lactose-reduced and regular milks (see below, table 2) studied in the application test.

A preliminary model for predicting residual lactose in lactose-reduced cream was developed on the basis of 7 mixes of cream 30% prepared from regular and lactose-reduced cream. These samples were included with the milk calibration set, minus the milk mixes prepared with permeate and retentate. For both the set as a whole and the subset of 7 cream mixes, the SECV was found to be approx. 0.08%m/m. Results of the prediction on cross validation for the sub set of 7 cream samples displayed in figure 4 again demonstrate a good linear relationship over the

Figure 4

Calibration cream

cross validation, (cream sub set, n= 7; total set size, N= 67)



full range from zero up to 3.3%m/m lactose, the regular lactose content of cream.

Application test

Given the specification of "<0.1%m/m lactose" with most lactose-reduced products and the fact that SECV of 0.07%m/m derived for the milk calibration and the SEP of 0.04%m/m with the milk validation are on the order of the lactose levels to be measured, it was felt that the calibration model should be evaluated on sample products to verify its applicability.

Since, minor differences in milk matrices associated with "non-milk" ingredients, variability in lactase transferase activity resulting from differences in process conditions and souring can influence results, it was decided to restrict the tests to a single brand of lactose-reduced milk products, named Minus L, which are readily available in the Netherlands.

Results obtained for lactose-reduced whole milk (5) , half cream milk (11) and cream30% (4) samples of different expiration dates, spanning production over several months are in Table 2a. The uncorrected results are displayed in Figures 5a & 5b. Measurements were carried out in February and November.

Table 2a Application test Milk & Cream - brand: Minus L

	"residual lactose" by IR			total	
in %m/m	SDrep(#5)	SD(mean)	**Mean**	sugars IR	N
semi skim	0.016	0.020	**-0.059**	4.61	11
whole milk	0.014	0.011	**-0.019**	4.45	5
Cream	0.030	0.029	**0.14**	3.28	8(4)

Table 2b Regular milks

	"residual lactose" by IR		total		
in %m/m	SDrep(#5)	Mean	sugars IR	**ratio**	N
skim milk	0.017	4.82	4.79	101%	5(2)
whole milk	0.018	4.64	4.57	102%	5(2)

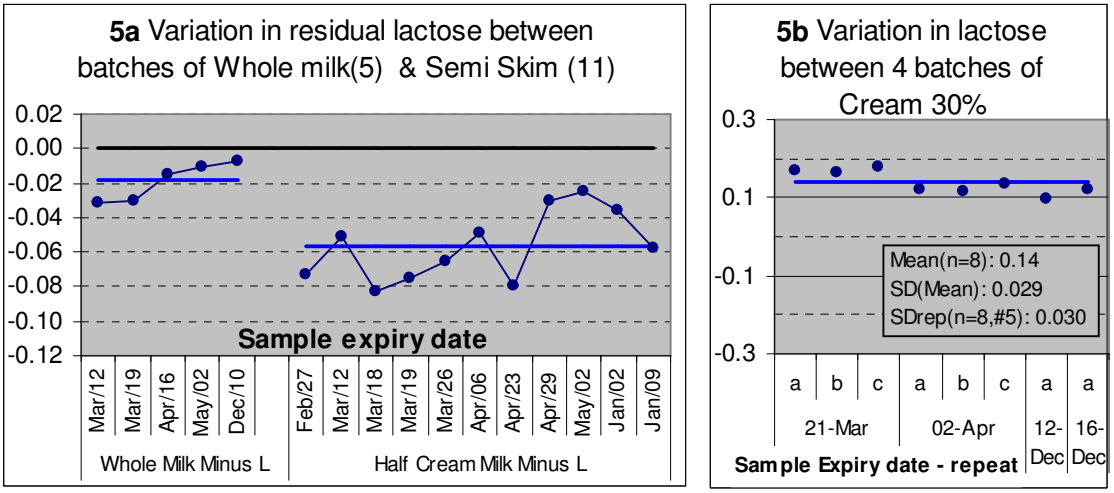
Where: Mean: the mean in the results over all samples, SD(mean): SD in the mean SDrep(#5): the repeatability stadard deviation computed from #5 replicates per sample; N: the number of samples of different expiry dates, either given as such or between brackets (with cream and the regular milks)

The mean result calculated over the mean responses (averages over 5 replicate measurements per sample) for 11 half cream milk samples is -0.06%m/m, while the standard deviation (SD(mean)) in the mean responses is 0.02%m/m only. A bias (or offset) correction of 0.06%m/m sets the mean result equal to zero and yields a maximum value (for the sample with the expiry date May/02) of less than 0.04%m/m residual lactose, which is still well below the product specification of <0.1%m/m. Based on the SD(mean) of 0.02%m/m, the end-point criterion for this calibration model can be readily set to 0.05%m/m, with the assurance that the product specification is met with a probability better than 95% (2xSD).

The same conclusions can be drawn from the results obtained for the 5 samples of whole milk, for which the mean in the result for residual lactose is even closer to zero, -0.02%m/m. In case of the 4 batches of cream, for which measurements for 2 of the batches were repeated on 3 days over a period of a week in February, the repeatability in the measurements as determined from the SDrep(#5) is 0.03%m/m, somewhat worse compared

to milk and the mean in the uncorrected results is larger, 0.14%m/m, but the standard deviation in the mean (SD mean) is still quite acceptable at 0.029%m/m.
As already mentioned above, we can only speculate on the origin of the differences in mean results from zero %m/m for the half cream milk and the cream30%. However, with cream it is likely that the degree of homogenization of the fat can have a significant influence on the results. This may have been the cause of differences (0.1 and 0.5%m/m, not presented here) in residual lactose response for 2 samples of cream which were beyond expiry date upon the day of measurement.

Figure 5 Residual lactose (in %m/m) by Lactoscope FTA for different batches of "Minus L"



Given the good results obtained with the preliminary model for cream and the inclusion of cat milk samples in the calibration set, an additional test was carried out on 2 samples of different brands of cat milk sold in the Netherlands. This evaluation yielded the "poor" results summarized in Table 3. This was unexpected since results for the 2 validation set samples of cat milk of the single brand of German origin of the validation set were in close agreement with the reference results obtained by HPLC (<0.05%m/m). Results for residual-lactose for the two cat milks bought in the Netherlands exceeded by far the contents declared on the packages (i.e. <0.4%m/m with "C1000" and <0.2%m/m with "Whiskas"). With Whiskas it was even 1.8 %m/m, almost 10-fold the declared upper limit. As a result, the samples were signaled as outliers by the PLSIQ prediction routine (on the basis of both mahalanobis

Table 3 Lactose reduced - cat milks

in % brand	Fat IR	Protein IR	Total sugars IR	Solids IR	**Residual lactose IR**	added sugars
Cat milk samples of the validation set						
Humana1	5.4	3.2	4.4	13.8	**-0.02**	?
Humana2	5.3	3.2	4.4	13.8	**0.04**	?
Additional brands of cat milk from the Netherlands:						
C1000	3.4	3.4	5.5	13.1	**1.0**	inuline, sucrose
Whiskas	3.4	3.5	5.8	13.5	**1.8**	malt
declared	3.3	3.4	-	15&13	**<0.4&<0.2**	

distance and spectral residuals). The likely cause is “non-milk” ingredients, probably the sugars added to these two cat milks. The actual composition of the German brand of cat milk remains unknown. Visual inspection and further chemometric analysis of the spectra revealed a closer homology over the sugars envelope among regular milks and the cat milk of German origin than compared to the two cat milks bought in the Netherlands.
Whether inclusion of a sub set of samples of a given brand of either C1000 or Whiskas cat milk may yield a reliable/stable model for predicting residual lactose in the specific brands of cat milk is uncertain. This is because of the extra sugars added and the natural variability in composition of these sugars, which need to be taken into consideration in establishing reliable models. For example, with Whiskas is specified that “malt” is added. This is a complex mixture of the disaccharide maltose, the monosaccharide glucose and further oligosaccharides which may vary in composition dependent on origin and processing of the malt. Specified on the C1000 cat milk label are the additions of sucrose and inuline (>0.4%m/m), the latter essentially is a polysaccharide of fructose.
With the aim of investigating the possibility of further compensation for additional sugar(s), a few chocolate milk samples of brand Minus L were analyzed. According to the product label, the single sugar, sucrose (= saccharose = regular table sugar) is added to the product at a concentration of 2.6%m/m. Rather unexpectedly, the test results obtained were found to “scatter in all directions”. See the results summarized in Table 4. Results for sucrose, determined using a model developed earlier, referred to as “milk added sugar” (or sucrose by “IR MAS”), revealed excessive (and therefore unexpected) variation in sucrose among samples of different expiry dates. Of the three samples analyzed in February, the (3rd) sample only (, for which the expiry date was still more than a 100 days away,) the estimated sucrose content (2.8%m/m) was considered in reasonable accordance with the declared content of 2.6%m/m. Reanalysis of another sample of the same production run (the 4th in the table), on November the 3rd, i.e. more than 100 days past the expiry date, yielded the lowest predicted result in sucrose, 0.4%m/m only. Concurrently, the predicted residual lactose for the sample went down from 4%m/m to 1% m/m. The cause of the changes was found to be due to hydrolysis of the sucrose over the storage period.

Table 4 Lactase treated chocolate milk - brand: Minus L

Sucrose MAS: Saccharose by FTIR model "MAS"
A - B: days until (-) and past (+) expiry date

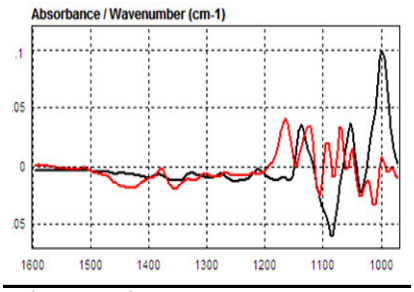
Date of analysis **A**	Date of expiry **B**	total sugars IR (in %m/m)	Sucrose MAS IR (in %m/m)	Residual lactose IR (in %m/m)	A - B days
27-Feb	26-Dec	8.6	1.6	2.4	63
27-Feb	25-Feb	8.8	1.0	1.9	2
27-Feb	**19-Jun**	8.8	**2.8**	4.4	**-112**
03-Nov	**19-Jun**	8.5	**0.4**	1.1	137
03-Nov	20-Nov	8.8	1.8	3.0	**-17**
03-Nov	11-Dec	8.9	2.2	3.7	**-38**

Declared composition (in g/100 ml) chocolate milk Minus L

Fat	Protein	tot.sugars	Sucrose	Lactose
1.7	3.4	7.8	2.6	<0.1

Fig 6. Sugar difference spectra

red: 5% lac - 5% 1:1 glu/gal
black: 4% sac - 4% 1:1 glu/fru



lactose: lac sucrose: sac
glucose: glu fructose: fru
galactose: gal

Where glucose and galactose result from hydrolysis of the disaccharide lactose, hydrolysis of the disaccharide sucrose yields glucose and fructose. Over the storage period of more than 200 days, the hydrolysis of the specific sample (expiry date June 19th) was virtually complete, whereas in February the hydrolysis was most likely in its early stages. The

hydrolysis of sucrose was readily recognized from difference spectra computed from spectra of the single sample analyzed both in February and November. It revealed a difference spectrum virtually identical to the difference spectrum obtained when subtracting summed spectra of 1:1 glucose/fructose from the spectrum of sucrose of equal concentration on a m/m basis. As an illustration, difference spectra representative of the hydrolysis of lactose and saccharose are displayed in figure 6. The difference spectra are clearly different from one another. Difference signals for hydrolysis of sucrose (4%m/m) are relatively large compared to those for lactose (5%m/m). Development of a model for hydrolysis of sucrose, in close analogy with the model for hydrolyzed lactose, thus seems feasible.
The question of whether an additional single extra sugar can be readily discriminated remains unanswered and requires further investigation. The addition of extra sugars increases the complexity of discriminating them. Disregarding transferase side reactions, the detection of residual lactose in lactose-reduced milk only requires the differentiation in a variable milk matrix of a binary sugar mixture: lactose and glucose+galactose. With added sugar, we move to differentiation in a ternary mixture. With sucrose hydrolysis added on top of that: a quaternary mixture.

Calibration Control
For reasons discussed above the method developed is expected to work optimally in monitoring end-point criteria with specific/single lines of products. Calibration control, under those circumstances can be kept simple and inexpensive. The response slope can be expected to be equal to 1 or at least constant over time. An offset (or bias) correction will take one or more samples of end products collected from production runs (As for the half cream milk samples of Minus L considered above). With milk products at least, the response can be checked from the ratio in the response for residual lactose relative to the response for total sugars (= lactose) for normal untreated milk, which in this case should be close to 100%. This was the case with the application tests, for which two lots of both regular skim milk and regular whole milk were analyzed and results are displayed in Table 2b.
Reference data for calibration control should preferably be obtained by a direct method of analysis like HPLC as used with this study, instead of by indirect methods, like the enzymatic determination of lactose based on the determination of the galactose formed.

Conclusions

The method developed for determining residual-lactose by Lactoscope FTA provides a good alternative to the existing cryoscopic method. Models derived are readily applicable with lactose-reduced milk and cream. Results obtained for the single sample of “carbohydrate reduced low lactose” milk from Valio (3.3%m/m total sugars, lactose <0.05%m/m) and the single brand of cat milk included with the calibration set, suggest a wider range of application beyond “regular” lactose-reduced milk and cream. With soured products for example, verification of this may require the inclusion of another/extra sub set of sour samples in the milk calibration set. The weakness of this model, in general, is its variable response to extra ingredients (sugars /carbohydrates) added to the product.

Acknowledgement

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