A testing protocol for carry over in AMS using tracer-color dilution

Peter Løvendahl[1], Martin Bjerring[2], and Torben Larsen[2]

Department of Molecular Biology and Genetics[1];
Department of Animal Science[2], AU-Foulum, Aarhus University, DK-8830 Tjele, Denmark

Abstract

Carry-over (CO) is the phenomenon that a milk sample from cow B also contains some fraction of milk from cow A that was milked just before B.
This protocol describes a method for quantifying the fractional CO in a test-lab or on-farm situation, as suggested at ICAR Aarhus 2013. The estimate of CO covers the complete and complex system from the udder of cow to the sample ending in the milk testing laboratory. The protocol described here is based on our experiences from the application of previous versions of CO testing protocols. The protocol is designed to detect liquid volumes of milk using tracer-color dilution as principle. The protocol tests an AMS which is disconnected from the bulk tank pipeline and replace live cows with phantom-cows. All functional settings are for "recording-sampling". Two "phantom-cows" are milked twice each. One cow (named "Yellow") contains milk with a fluorescent (water and fat soluble; e.g. fluorescein) color tracer, and the other (named "White") contains pure milk without any color added. Fluorescence in samples is measured using spectroscopic fluorescence methods. Fractional CO is calculated as the percentage of "yellow" in the first "white" cow milk samples. The protocol takes advantage of standard laboratory procedures for determination of CO in as far as possible and gain accuracy by duplication. That means, using two color tracers, running colored and White two times before switching to new color, and repeating the whole series at least as duplicates, and better as triplicates, in order to obtain the most reliable results. Previously we have detected CO between 2.0 and 18.0 %, with the better results from well-adjusted AMS and samplers. In comparison, non-AMS CO values between 2.5 and 4.5% were detected using this method. Further insights are obtained by testing CO at various amounts of milk to provide a CO-profile. Implementing the protocol requires some investment in instruments but procedures are generally simple.

Key words: AMS, Carry over, Testing protocol, Detection, Guidelines.

Introduction

Milk samples obtained from AMS are more prone to carry-over than traditionally obtained samples because of the greater complexity of AMS including valves, pumps, containers and connecting tubes. Consequently, the CO observed in samples obtained from AMS is the sum or the product of

CO attributable to the inseparable parts of the milking and sampling process. Estimates of CO were shown by Løvendahl & Bjerring (2006), and Løvendahl, Bjerring & Larsen (2010), using either a statistically based method or a color tracer method. These methods gave reasonably similar results when applied to the same AMS, but are vastly different in their demand for data and labor and thereby also waiting time for obtaining a reliable result. Developments of new generations of AMS are expected over the coming years and better sampling of milk should be among the improvements. An agreed, practical and precise way of testing devices for CO would be advantageous during the development. A suggestion for a testing protocol with that aim is described here, based on our experiences from use of previous versions of CO testing protocols. As this is a suggested protocol, further enhancements or simplifications from other users (test centers) should be accumulated in order to obtain a final version which could be part of ICAR guidelines. The testing protocol described here use color tracer dilution.

Materials and Methods

PRINCIPLE.

The protocol is designed to detect liquid volumes of milk. However, if milk content (fat and protein) sticks to surfaces of milking equipment and are released again into the next milk sample this will also be counted as carry over. In case milk contains sticky components such as bacteria, they may have other characteristics for carry over than the liquid milk or its usual components, and may therefore require an extended testing procedure.

The protocol use an AMS strictly disconnected from the bulk tank pipeline, without live cows, but otherwise functional with settings for "recording-sampling". Instead of live cows, two "phantom-cows" are milked repeatedly. One cow (name "Yellow") contains milk with fluorescent water abd fat soluble color tracer, and the other (name "White") contains pure milk without any color added.

Following an initial wash cycle the test is started with a number of runs, where cow Yellow is milked twice, followed by twice milking of "White". Samples of milk are taken before each milking manually in the bucket, and after each milking in the collected samples. Color or fluorescence in samples is measured using appropriate spectro-photometric / fluorometric methods. Fractional carry over is then calculated as the percentage "yellow" in "white" cow milk samples (examples shown below).

MATERIALS

Phantom cows, are constructed from 4 plastic teats bored to tightly fit plastic tubing, (NALGENE, od. 7 mm, id 5 mm, L 2 meter). Tubes are collected 2 + 2 into thick plastic tubing pieces, L 100 mm, i.d. 12 mm. Each of the two phantom cows should have numbered cow-ID transponders, identified in the system, with numbers being easy to separate from normal cows. These should have full access to milking at any time, and should be entered as "healthy" so that milk will follow the normal collection and pumping routes.

Raw milk: a bulk volume (e.g. 200 L) of milk from the herd is separated into a vat (small tank holding at least 200 L). This should be a mixture of milk from a small number of cows.
Tracer coloring agents. (Fluorescein; 4MeU). The tracer was prepared by dissolving 600 mg of AY73 (Fluorescein Sodium salt, Sigma-Aldrich, Fluka 46960) and 600 mg of 4MeU (4-Methylumbelliferon Sodium salt, Sigma-Aldrich M1508), first in 40 mL of milk and then mixed up in 35 L milk.
Tracer milk is produced from the bulk milk by dissolving 600 mg of each color agent into 35 L of white milk. This is done by dissolving the powder form color into a stock solution of 40 mL milk in a 100 mL vial by shaking or inverting that vigorously until it appears homogenous, which usually takes some minutes. This stock solution is further diluted into the 35 L milk under constant stirring or other form of agitation until a stable coloring is established, again taking some minutes. Milk should be kept warm (38°C) using lids on buckets and immersion heaters in containers.
Large samples (300 mL) are taken from raw White and Yellow milk for later use in lab to calibrate instruments.

DETAILED SCHEDULE
Overall schedule

Step	Actions	Time per step
1	Prepare raw milk	
2	Prepare tracer milk	
3	Prepare phantom cows and ID-tags	
4	Stop milking	
5	Run cleaning – washing program	
6	Disconnect from pipeline	
7	EXPERIMENTAL PHANTOM COW MILKINGS	
8	Run total wash	
	Re-connect to pipelines	
	Resume milking	

The sampling schedule example indicate numbering of samples (30 mL) taken in each serial run. A complete run contains 4 milkings.

Series	Content	Milk in	Bucket	Sampler	Recorded,	Remark
		Kg	Sample_ID	Sample_ID	Kg	(time)
1.1	Yellow	8.0	11.1	11.2	?	
1.2	Yellow	8.0	12.1	12.2	?	
1.3	White	8.0	13.1	13.2	?	
1.4	White	8.0	14.1	14.2	?	
2.1	Yellow	8.0	21.1	21.2	?	
2.2	Yellow	8.0	22.1	22.2		
2.3	White	8.0	23.1	23.2		

2.4	White	8.0	24.1	24.2		
3.1	Yellow	12.0	31.1	31.2		
.	Yellow	12.0	32.1	32.2		
.	White	12.0	33.1	.		
.	White	12.0	.	.		
.	.	.	.	.		

It is further suggested to use a range of different volumes to obtain a clearer picture of the performance profile going down to 3 and up above 25 to 30 Kg.

ASSAYING and CALCULATIONS

The milk samples are assayed using fluorometric measurements, calibrated by diluting the full color tracer milk into white milk. Samples can be arranged on microtitre plates if a suitable reader is available. Alternative readers using single cuvettes (methacrylate for UV) are also available, and less expensive, but still with good precision.

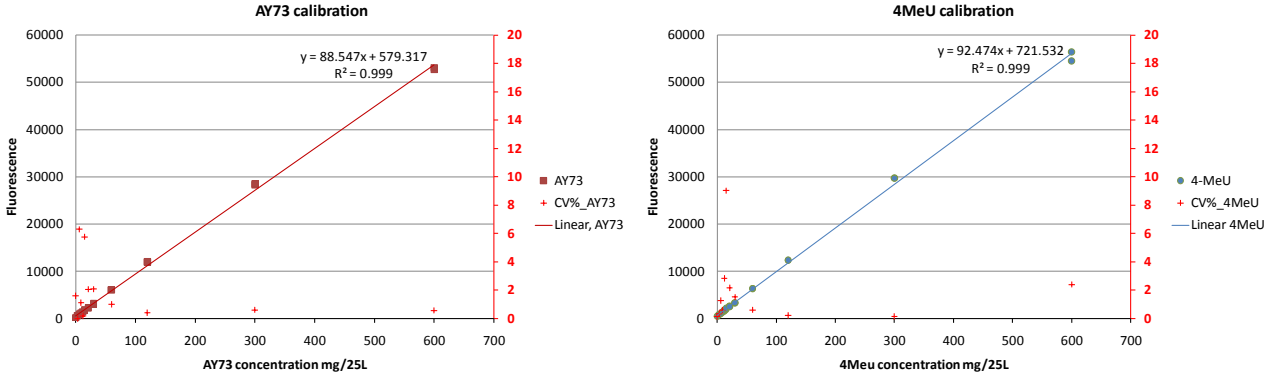
Before samples are measured they are diluted as follows, using two buffers (A, TRIS pH 8.5 [36.3 g TRIS/L, adjust to pH 8.5]; B Stop-buffer, pH 10.8, [6.68 g Titriplex = Na-EDTA/ L + 50.0 g glycin/L, adjust to pH 10.8]) and water to obtain pH values giving optimal readings. Milk samples are diluted stepwise as follows:

a. 50 µL milk + 200 µL TRIS, (5x)
b. 125 µL a + 125 µL TRIS, (2x)
c. 50 µL + 200 µL H_2O (5x, total 50x).
MEASURE c at Blue light (485 nm / emission 520 nm)
d. Add 50 µL Stop-buffer B, (1.2x, total 60x)
MEASURE d at UV light (355 nm / emission 460 nm).

A linear relationship between concentrations and fluorescence readings are expected as shown in the figure below.

Fluorescence intensity was measured using excitation filter at 485 nm and emission filter at 520 nm for the AY73 and at 355 / 460 nm for the 4MeU. “White” milk readings were always low, but were included as the 0 (zero) calibrator. The colored sample from each batch was defined as 600 arbitrary units, being equal to 100 % color saturation. Ten more (10) calibrators were initially made for all intermediate values by mixing white and colored milk. Calibrators and samples were dispensed into duplicate wells of microtitre plates, so that all readings were in duplicate. All calibrators and samples were heated to 39°C before measurement on a fluorometer. Calibration curves were checked for linearity by back-calculating values derived from a simple 2-point calibration. For routine use a 5 point curve was used (0, 10, 20, 30, 100 %). In this setup, carry-over is expressed in units of maximum colored milk which was proportional to percentage by division with 6.00.

Calibration curves for the two fluorochromes are shown below in figures 1 and 2 (from a previous experiment using 25 instead of 35 L tracer milk). The fluorescence readings were directly proportional to the concentrations of each tracer over the range used.



Figures 1 (left) and **2** (right). Calibration curves and precision profiles for two color tracers. Precision is shown as CV% of duplicates (right red axis, + markers).

Results and Discussion

ASSAYING OF SAMPLES

The linearity of both sets of standards was almost perfect over the range of concentrations and fluorescence readings. The precision profile showed that duplicate replication was always better than a CV% of 10% and typically around 2 to 4 % in the range of interest for use, namely between 12 and 120 mg/25L, equal to 2 to 20 % carry-over. "White" milk had a baseline reading of 600 to 700 fluorescence counts, which was subtracted from calculated concentrations using the trend-line as calibration formula. Similar new curves were produced for every batch of tracer and white milk, and used for actual concentrations. In effect, a sample containing 60 units, would have a precision of 2 color units or less, equal to 0.3 % units at a carry-over at 10%.

CALCULATIONS

If calibrations are performed with "100.00" as the full color, and "0.00" as the raw white, carry over is simple to calculate. The example below shows findings from a recent test using the suggested protocol.

Series	Cont.	*Bucket*			*Samp.*			**Ratios**	
			Fluresc	4MeU		Fluresc.	4MeU	**Fluresc.**	**4MeU**
1.1	Yellow	*11.1*	610,6	597,4	*11.2*	542,5	539,8	542,5/610,6 = 0,89	539,8/597,4 = 0,90
1.2	Yellow	*12.1*	620,4	605,0	*12.2*	606,1	600,5	606,1/620,4 = 0,98	600,5/605,0 = 0,99
1.3	White	*13.1*	-3,9	-0,2	*13.2*	61,7	56,9	**61,7/606,1 = 0,102**	**56,9/600,5 = 0,095**
1.4	White	*14.1*	-0,4	0,6	*14.2*	0,5	4,6		

2.1	Yellow	*21.1*	602,7	587,6	*21.2*	593,7	583,3		
2.2	Yellow	*22.1*	595,6	585,6	*22.2*	616,2	618,8		
2.3	White	*23.1*	-1,3	1,5	*23.2*	61,6	59,9	**61,6/616,2 = 0,100**	**59,9/618,8 = 0,097**
2.4	White	*24.1*	-1,1	4,7	*24.2*	3,7	7,0		

The **primary carry over** is here calculated as the ratio between the SAMPLE color intensity in the first White following immediately after the last colored, and here showing on average 0.10, or 10% CO.

The example also shows that the first colored milking is not enough to saturate the system, as the ratio is only 0.90, but in the second milking reaches 0.99. However, this phenomenon was not seen in the second series. Anyway, if secondary carry-over exist, the second milking in each series is effective in saturating the system.

Secondary carry over could be estimated also, but will be prone to much error because the measured units come close to the white milk background. The white milk background was also seen to fluctuate between -3.9 and 4.7, (STD = 2.5).

The example was based on tests all using 8 Kg of milk. In case different volumes are used, and each one is repeated, the CO estimates should be plotted against milk volume so that a CO profile could be estimated.

EVALUATION OF RESULTS AND UNCERTAINTIES

The suggested protocol takes advantage of standard laboratory replication practice in as far as possible. That means, using two color tracers, running colored and White two times before switching to new color, and repeating the whole series at least as duplicates, and better as triplicates.

As shown above, the baseline color reading has a noise around it (STD = 2.5 units) which sets the accuracy at that level, thus giving the detection limit. At the high end values in the bucket samples also varied from 620.4 to 585.6 giving a STD of 11,6 units, or around 2 % units. At the focus point the STD was 2.24 units which gives the estimated CO (mean value 10%), a STD of only 0.3 percent units, thus giving highly accurate estimates. The above calculations of accuracy are rather simple and only provided here as examples. However, they are very similar to our findings during previous testing sessions (not shown here). Anyway, the calculated CO fractions should be shown along with their accuracy estimates in order to evaluate if changes to the construction details have impacted on the CO performance.

DISCLAIMER

We take absolutely no responsibility of any sort for violations of any national or local regulations on food safety or similar from using this testing protocol.

However, we are not aware of any safety risk involved in using the two suggested color tracers. We are not aware of regulations and restrictions for food safety prohibiting the use of these colors for

this purpose as outlined in this protocol, including the full wash cycle and replacement of filters that have been in contact with colored milk. However, to the best of our knowledge these reagents are largely harmless and in fact used i.v. for human medical diagnostics (fluorescein – eye diagnostics).

List of References

ICAR, 2007. ICAR Recording Guidelines: International Agreement of Recording Practices. ICAR Guidelines approved by the General Assembly held in Kuopio, Finland on 9th June 2006. Available as PDF from www.icar.org 474 pp.

Løvendahl, P. and Bjerring M, 2006. Detection of Carry-Over in Automated Milk Sampling Equipment. Journal of Dairy Science, 89:3645–3652

Løvendahl, P., Bjerring, M. and Larsen, T. 2010. Determination of carry-over in automated milking, recording and sampling systems using fluorescent tracers. Proc. ICAR 37th Annual Meeting – Riga, Latvia (31 May - 4 June, 2010).

Løvendahl, P., Bjerring, M., Larsen, T. van den Bijgaart H., and Baumgartner, C. 2013. Protocol for assessment of fractional carry over in samples from automatic milking systems. ICAR Technical Meeting Aarhus (Denmark, 29 - 31 May 2013).