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Collaborative study of expert laboratories for the definition of consensus values of RMs for ALP determination in milk

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Scope of the PT

In summer 2014, the Italian National Reference Laboratory for Milk and Milk Products (NRL-MMP) in collaboration with the Italian Reference Centre for Cow Milk Quality of IZSLER (RC-CMQ) organized the first national Proficiency Testing (PT) for the validation of a Reference Material (lyophilized milk) for the determination of the activity of alkaline phosphatase in milk with fluorimetric method ISO 11816:1).
That PT recruited 19 Italian laboratories (both public and private to reach the maximum possible number of participants), plus the EURL MMP.
In 2015, as first major improvement action, it was decided to organize a second round, using the same batch of samples used in 2014. This PT involved experienced laboratories of the net of the NRLs-MMP with the collaboration of EURL-MMP to verify the reliability of the results obtained during the first trial and to possibly improve the accuracy of the titles calculated for the Reference Materials and their relative uncertainties in that occasion.

Based on these considerations, in this report the z-score values calculated according to ISO 13528 are intentionally and arbitrarily intended as indirect indicators of the quality of the samples and not of the performances of the laboratories.
Finally, this trial was the occasion to test other dairy matrices different from milk (butter, cheese and cream of a small pilot production) with the preliminary scope to collect colleagues evaluations on their fitness for analysis in the perspective to improve a possible future production process. Data obtained were sufficient to get an idea on how realistic may be the possibility to produce Reference Materials for dairy products different from milk.

General informations

Participant laboratories

The PT involved 17 expert laboratories for the determination of alkaline phosphatase (ALP) activity in milk by reference method ISO 11816-1:2013 (tab. 1).

Table 1. Participant laboratories

Laboratory	Country
AGES- Austrian Agency for Health and Food Safety	Austria
Institute for Agricultural and Fisheries Research (ILVO)	Belgium
State Veterinary Istitute Prague	Czech Republic
Finnish Food Safety Authority Evira	Finland
ANSES - EURL MMP	France
Max Rubner-Institut Kiel	Germany
Veterinary Laboratory of Patras	Greece
National Food safety Office	Hungary
IZSLER	Italy
Università degli Studi di Milano – DeFENS STA	Italy
RIKILT Wageningen UR	Netherlands
National Veterinary Research Institute	Poland
INIAV IP – Polo do Lumiar	Portugal
Institute for Hygiene and Veterinary Public Health	Romania
UL Veterinary faculty NVI U Kranj	Slovenia
Laboratorio Agroalimentario de Santander	Spain
Agroscope, Biochemistry Lab	Switzerland

Organisation of the proficiency testing trial (PT)

Laboratories were invited by a circular mail (25th june 2015) to the PT and informed of: scope of the trial, method to apply, number of samples, kind of matrices, relevant dates for analysis and finally results transmission. Participant laboratories received the following documents:

- "receipt form " to fill upon samples reception
- "protocol " with instructions for samples reconstitution and analysis
- "results form" to fill with the analyses results

Samples were shipped by ordinary mail on the 9th July 2015.
No fixed date to run the analyses was required, thanks to the stability of the lyophilized samples.
The deadline for the forward of the results was stated at the 4th September 2015 but, due to instrumental problems of some participants and considering the scope of the PT, the deadline was postponed to 11th September.

Samples

Each laboratory received: 3 lyophilized milk samples to be analyzed in duplicate in repeatability conditions, plus 1 sample coded "calibrator" for the instrumental calibration:

- 1 sample "60" → lyophilized/ pasteurized /semi skimmed cow milk
- 1 sample "600" → lyophilized /thermized /semi skimmed cow milk
- 1 sample "6000" → lyophilized/no-heat-treated/semi skimmed cow milk (raw milk diluted 1: 100 with "ALP free" milk to obtain ready to use samples)
- 1 "calibrator" lyophilized/"ALP free"/semi skimmed cow milk

In addition, each laboratory received also 2 lyophilized samples randomly chosen among a limited pilot production of butter, cheese and cream. These samples were prepared from thermally treated matrices appropriately diluted to obtain instrumental measurements below 7000.
These samples were enclosed for a preliminary evaluation of their suitability for a possible use as reference materials. Also these samples had to be analyzed in duplicate, according to the instruction of the protocol (Annex 2).

All the samples were randomly coded as reported below:

Lab code	milk			butter	cheese	cream
	Sample "60"	Sample "600"	Sample "6000"			
1	M32	M24	M56		CH-01	CR-01
2	M37	M86	M62		CH-02	CR-02
3	M83	M64	M22		CH-03	CR-03
4	M19	M17	M65	B-04	CH-04	
5	M58	M48	M19	B-05	CH-05	
6	M40	M36	M88	B-06	CH-06	
7	M12	M09	M35	B-07	CH-07	
8	M27	M21	M89	B-08	CH-08	
9	M83	M44	M52	B-09	CH-09	
10	M26	M03	M81	B-10	CH-10	
11	M61	M63	M72	B-11		CR-11
12	M90	M46	M43			CR-12
13	M54	M53	M33	B-13		CR-13
14	M41	M47	M80	B-14		CR-14
15	M70	M79	M73	B-15		CR-15
16	M68	M28	M29	B-16		CR-16
18	M75	M04	M15		CH-26	CR-18

Milk samples

According to the operative protocol, all the samples had to be reconstituted with 2.0 g of demineralized water on a technical balance, throughly mixed (if necessary, vortexed) till complete dissolution and immediately analyzed.

Laboratories were also requested to report the following functionality controls:
- Daily A/D Test (302 ± 4; 602 ± 12)
- Reagent Control (< 1200)
- Calibration Ratio
- Phosphacheck Controls (facultative): Neg < 10; Normal < 40; Pos 500 ±100 mU/L).

Homogeneity and stability controls

The homogeneity of the samples was preliminaly tested according to ISO guide 35 (Reference materials — General and statistical principles for certification).

Before the 2014 interlaboratory test, a number of randomly chosen samples equivalent to the 5% the total samples prepared for each level of alkaline phosphatase was analysed in duplicate under repeatability conditions.

The between bottles standard deviation (Sbb) obtained by ANOVA analysis is summarized in the box below. Sbb was calculated as 2.20 mU/L for sample "60", 14.78 mU/L for sample "600", 173.37 mU/L for sample "6000". According to the criteria defined in ISO 13528: 2005 B.2, the level of homogeneity observed was deemed satisfactory, and taken in charge for the purposes of use in PT statistic evaluation.

between bottles Standard Deviation (Sbb):

Sbb sample "60" = 2.20 mU/L
Sbb sample "600" = 14.78 mU/L
Sbb sample "6000" = 173.37 mU/L
Sbb sample "6000" = 95 mU/L (evaluated during the 2014 PT on 2 samples for each participant)

Note: The homogeneity value calculated for each level of activity was successively used for the evaluation of the expanded uncertainty measurement of the samples.

Due to the lyophilized status of the samples, a long term stability evaluation of 7-10 years is ongoing, and at moment, a period of 1,5 year has just been tested and confirmed.
The last evaluation was repeated before shipping the samples, according to ISO 13528:2005. Samples for each level of alkaline phosphatase were randomly selected and analyzed in duplicate under repeatability conditions, starting from the sampling. Results obtained were fully satisfactory respect to ISO 13528:2005 limits for stability.

In addition, the thermal stability was arbitrarily checked on some samples kept at 44 °C for 1 week to evaluate the effect of deviations from usual temperature conditions of storage or shipment. No significant effect was evidenced.

Results and elaboration

References:
ISO 13528:2005,
ISO 11816-1:2013
ISO guide 35, 2006
ISO 5725:2003 Series (1-6)

(Excel®)spreadsheets and statistics procedures in code R (software MetRology, algA function) were used for the elaboration of results.

Data received

- No comments were received on transport or conditions at the samples reception.
- No problems were reported by any laboratory for milk samples analysis.
- All the 17 laboratories fully observed the indications supplied.

The test results are reported in Table 2.

Table 2. Results of the analyses (expressed in mU/L)

	Sample "60"		sample "600"		sample "6000"	
Lab code	Rep. A	Rep. B	Rep. A	Rep. B	Rep. A	Rep. B
1	90.6	85	708.4	697.4	< 10	< 10
2	152.2	149.4	954.3	928.1	9.029	8.812
3	96.3	98.2	805.0	801.4	6168.2	6219.5
4	70.8	69	587.5	583.4	4514	4487
5	104	102	788	771	6468	6316
6	98.4	100.2	783.3	781.5	6146	6137
7	94.2	96.5	768.6	794.8	6312	6468
8	106.0	106	784	786	5930	5958
9	80.9	97	773.2	730	6165	5949
10	98.8	97	759.4	766.8	4629	4597
11	102	90	795	746	6091	5926
12	98.8	98.8	744.7	748.4	5686	5724.6
13	94.2	96.1	780	775	5939	5999
14 *	95.2	99.3	838	834.4	6284	6381
15	89.6	92	840.3	819.3	6362	6482
16	90.1	90.1	746.6	730	5567	5700
18	97.9	97.9	781	782.9	5939	5958

*lab 14 partecipated with 2 operators, both working in duplicate. Only the single series of results casually chosen for the statistic elaboration is reported and coded as 14, whereas results of the second operator coded as 17, are not reported

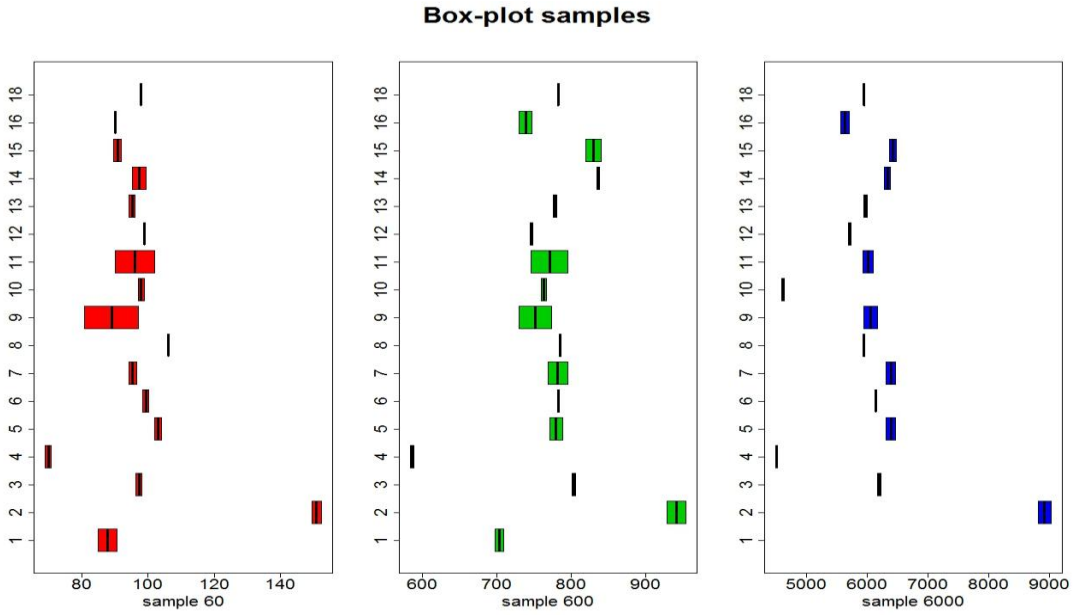
Instrumental functionality

- All the laboratories provided the requested results for Daily A/D test, Reagent Control (<1200) and Phosphacheck Controls.
- Calibration Ratio (no limit is given) showed good agreement with the only exception of 2 labs with values relatively different from the average (data no showed).

The calculation of the assigned values at the different levels of alkaline phosphatase was the consensus value from participants (robust estimation specified by ISO 13528:2005 5.6.1 and 5.6.2) without reiteration.
Only results for sample "6000" from lab 1 (<10) were considered "Not Valid" and eliminated.

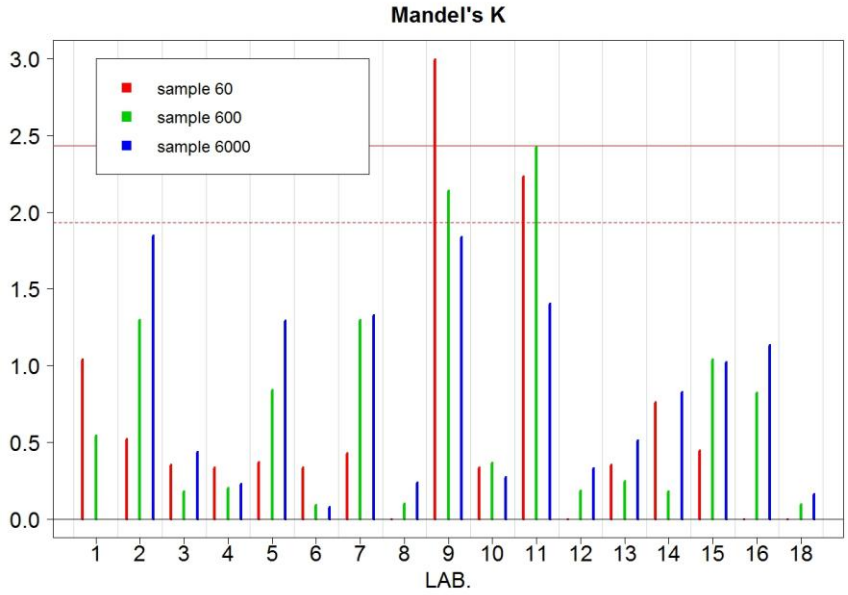
The global evaluation of the PT is illustrated in Fig 1 in which, for each participating laboratory and for each level of alkaline phosphatase activity, the distribution of the results and the differences between replicas are evidenced.

Figure 1. Box-plot- matrix milk



A further evaluation was made according to indication of ISO 13528:2005, 8.4. In particular, the repeatability of measurements was evaluated by Mandel's k statistics (Fig.2) (ISO 5725-2:1994). The horizontal lines represent the limits of the expected deviation, respectively, with P = 99% in whole line and P = 95% in dotted line for samples "6000" (in blue) , "600" (in green) and "60" (in red).
Very low k-ratio were estimated for sample "60" for labs 8, 12, 16 and 18 due to identical replicate results. The highest values were recorded for Lab 9 and 11 .

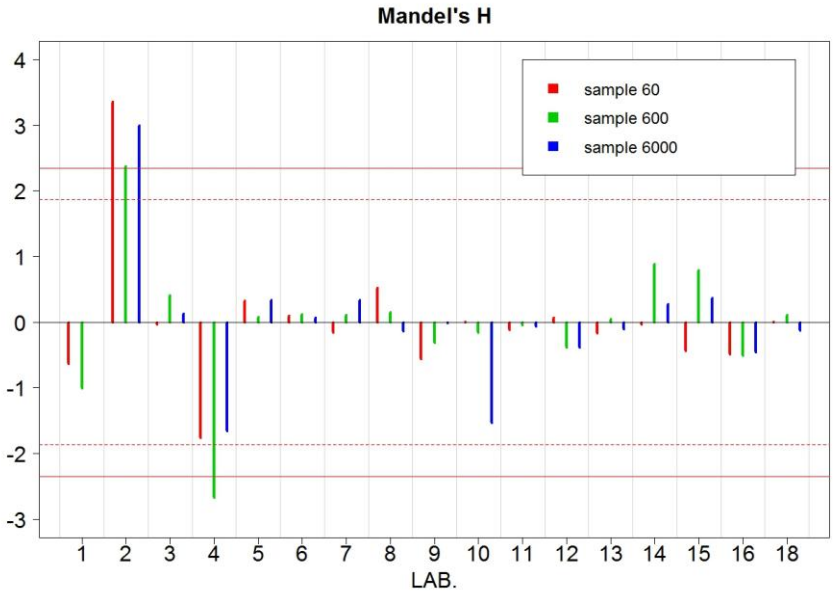
Figure 2. k-ratios of the laboratories



The dispersion between laboratories was analyzed through Mandel's h statistics (ISO 5725-2:1994), that represent in Fig 3, for each laboratory, the means of the three samples analyzed, also in this case, according to the limits of significance, respectively: P= 99% in whole line and P = 95% in dotted line.

To note: Laboratory 2 for the 3 samples (P=99%) and Laboratory 4 for sample "600" (P = 99%)

Figure 3. h-ratios of the laboratories



Note: it should be noted that these statistical analyses give an overall evaluation of the trial confirming the level of repeatability (Mandel's k) and the low dispersion of the averaged results given by participants (Mandel's h), but do not individuate real outliers.

The statistics computed and used below for the performance evaluation (robust mean and robust standard deviation) do not require the selection of "valid" data.

Definition of the standard deviation assigned for the test: $\hat{\sigma}$

In the absence of criteria available for all the 3 levels of alkaline phosphatase activity of the samples for the reference method (ISO 11816-1:2013), we choose to estimate the standard deviation $\hat{\sigma}$ for the test, by the "robust standard deviation" of the averaged mean values of each laboratory, as shown in ISO 13528: 2005 point 6.6.1 to reduce the effect of more dispersed averaged mean values.

Assigned standard deviation for the PT:
$\hat{\sigma}$ sample "60" = 6.70 mU/L
$\hat{\sigma}$ sample "600" = 43.00 mU/L
$\hat{\sigma}$ sample "6000"= 406.07 mU/L

Definition of the assigned value X and of its uncertainty for each sample

Expected values of each level of alkaline phosphatase were calculated as the robust mean "X" according to ISO 13528:2005 C.1 and resulted:

Assigned value X:
X sample "60" = 96.10 mU/L
X sample "600" = 775.91 mU/L
X sample "6000"= 6040.65 mU/L

Consequently, the uncertainty u_x of the assigned values was estimated:as:

$$u_X = \frac{1.25 \times s^*}{\sqrt{p}}$$

where:
s * = robust standard deviation of the averages of each laboratory (6.70 for the sample "60", 43.00 for the sample "600", 406.07 for sample "6000").
p = 17, number of laboratories.

Standard measurement uncertainty u_x:
sample "60" = 2.03 mU/L
sample "600" = 13.03 mU/L
sample "6000"= 126.89 mU/L

The estimated uncertainties are slightly higher than the criterium stated in ISO 13528:2005 4.2 ($u_X \leq 0.3\ \widehat{\sigma}\ \sigma$) but acceptable for the scope of the trial .

Statistic calculation of results

Results were evaluated in terms of z-score, intended as indicator of the quality of the samples:

$$z = \frac{(x - X)}{\hat{\sigma}}$$

Table 3: Sample "60"

Laboratory	media	n° determinations	z – score
1	87,80	2	-1,24
2	150,80	2	8,16
3	97,25	2	0,17
4	69,90	2	-3,91
5	103,00	2	1,03
6	99,30	2	0,48
7	95,35	2	-0,11
8	106,00	2	1,48
9	88,95	2	-1,07
10	97,90	2	0,27
11	96,00	2	-0,02
12	98,80	2	0,40
13	95,15	2	-0,14
14	97,25	2	0,17
15	90,80	2	-0,79
16	90,10	2	-0,90
18	97,90	2	0,27

Table 4: Sample "600"

Laboratory	media	n° data	z – score
1	702,90	2	-1,70
2	941,20	2	3,84
3	803,20	2	0,63
4	585,45	2	-4,43
5	779,50	2	0,08
6	782,40	2	0,15
7	781,70	2	0,13
8	785,00	2	0,21
9	751,60	2	-0,57
10	763,10	2	-0,30
11	770,50	2	-0,13
12	746,55	2	-0,68
13	777,50	2	0,04
14	836,20	2	1,40
15	829,80	2	1,25
16	738,30	2	-0,87
18	781,95	2	0,14

Table 5. Sample "6000"

Laboratory	media	n° data	z – score
2	8920,50	2	7,09
3	6193,85	2	0,38
4	4500,50	2	-3,79
5	6392,00	2	0,87
6	6141,50	2	0,25
7	6390,00	2	0,86
8	5944,00	2	-0,24
9	6057,00	2	0,04
10	4613,00	2	-3,52
11	6008,50	2	-0,08
12	5705,30	2	-0,83
13	5969,00	2	-0,18
14	6332,50	2	0,72
15	6422,00	2	0,94
16	5633,50	2	-1,00
18	5948,50	2	-0,23

Figure 4. Graphical representation of z-scores for sample "60"

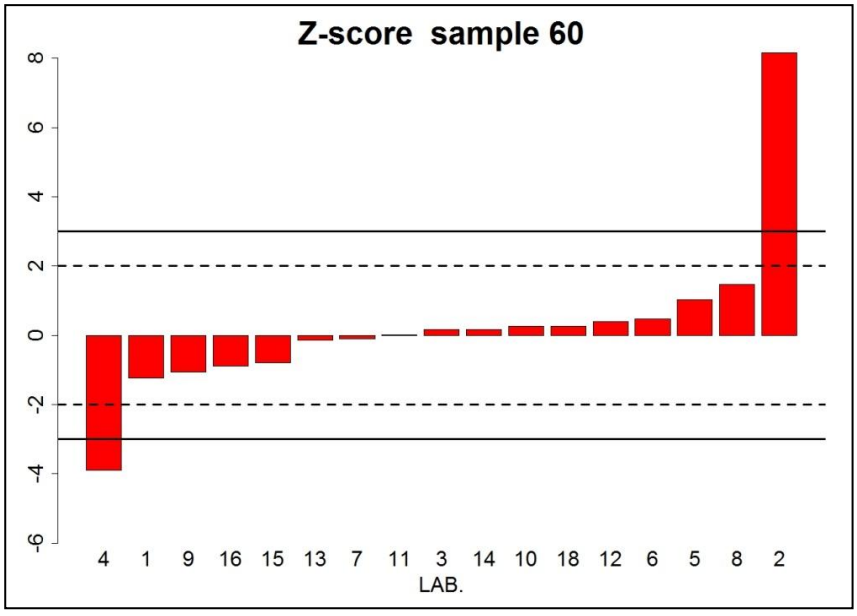


Figure 5. Graphical representation of z-scores for sample "600"

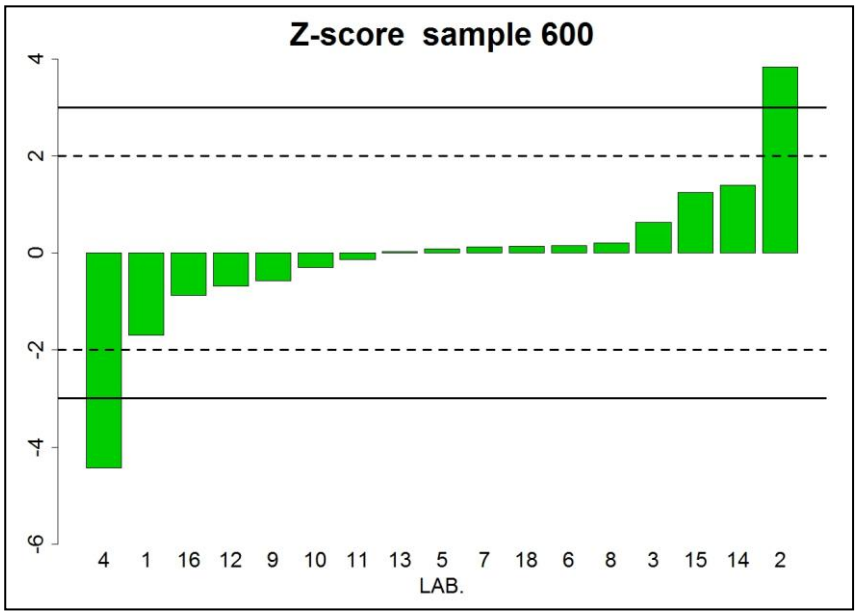
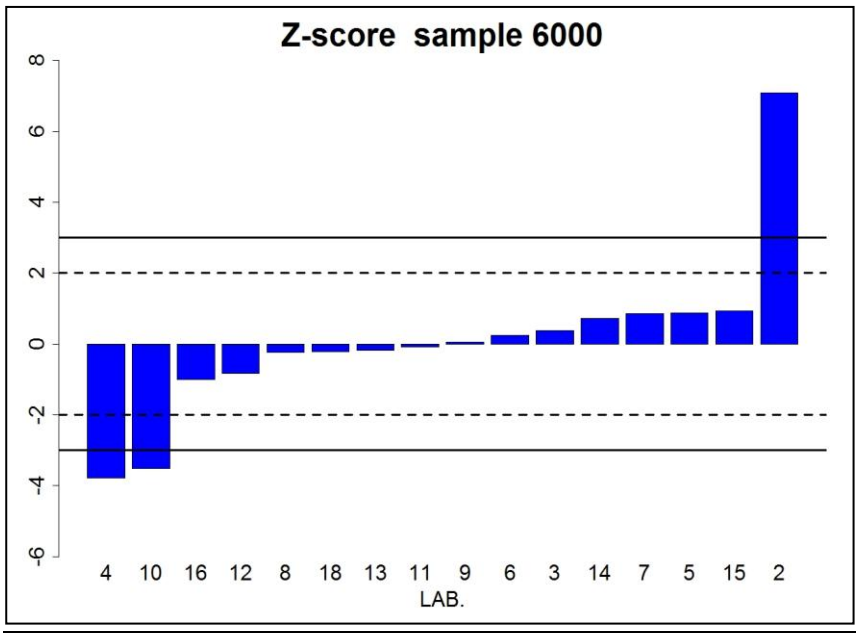


Figure 6. Graphical representation of z-scores for sample "6000"



Evaluation of results and conclusions

Overall, the outcome was considered satisfactory since the major part of the z-scores, intended as indicators of the quality of the samples resulted, for all the 3 samples, in the range ± 1, whereas, on the opposite, only few results (from 2 laboratories on a total of 17) for sample 60, from 2 laboratories for sample 600 and from 3 laboratories for sample 6000 fall outside z-score 2 confirming the stability and homogeneity of samples proved in 2014 PT (Annex 1).
Results for the definition of titles and uncertainties of the Reference Materials are reported in Annex I.
Results for matrices different from milk are reported in Annex II.

Conclusions

The experience of these two years, provided us an objective confirmation of fulfilling the basic requirements of a Reference Material for lyophilized milk samples: good level of homogeneity , fitness for the purpose uncertainty, stability over the time (at moment verified on samples kept in refrigerated conditions for 18 months), and last but not least, smart sample management.
For this last aspect, it is worth remembering that we decided to ship samples by ordinary postal service in no refrigerated conditions thanks to the positive results of a short term stability test performed at 44° C for one week. Among the other advantages, this allowed to limit the cost expenses of the shipping even in a period, the beginning of july, when in the current year, the temperature in Rome ranged 31-36 °C.

ANNEX I

Comparison between the 2 PT using the same batch of Reference Material

As reported in the scope, the same batch of sample was used in 2014 and in 2015 PTs.
The comparison between the results from the two PTs is summarized in Table 1.

Table 1. Comparison of results from 2014 PT and 2015 PT.

Sample	n° observ.	2014 PT		n° observ.	2015 PT	
		Assigned value (mU/L)	u_x (mU/L)		Assigned value (mU/L)	u_x (mU/L)
"60"	48	90.77	7.46	34	96.10	2.03
"600"	48	792.65	36.76	34	775.91	13.03
"6000"	96	6297.20	330.88	32	6040.65	126.89

It is evident the significant improvement of the uncertainty in the 2015 trial, in spite of a slight change in the assigned value. This result confirm the expected better perfomances provided by expert laboratories in the second experience.

Statistical elaboration of the comparison

Due to some operative differences between 2014 and 2015 trials (triple analysis of all the samples and two aliquots for the sample "6000" in 2014) we thought not appropriate a global evaluation of the pooled data. Anyhow, due to the fact that a unique batch of samples was used, a graphical comparison between the two experiences, was simulated.

In the figure below, it is possible to evaluate the usual indicators of homogeneity of the replicas and the dispersions between the laboratories for the 2014 trial (N: National) and respectively for the 2015 one (I: International). It appears clear the lower dispersion of the 2015 results .

Figure 1. Box plot : 2014 trial (N) and 2015 (I)

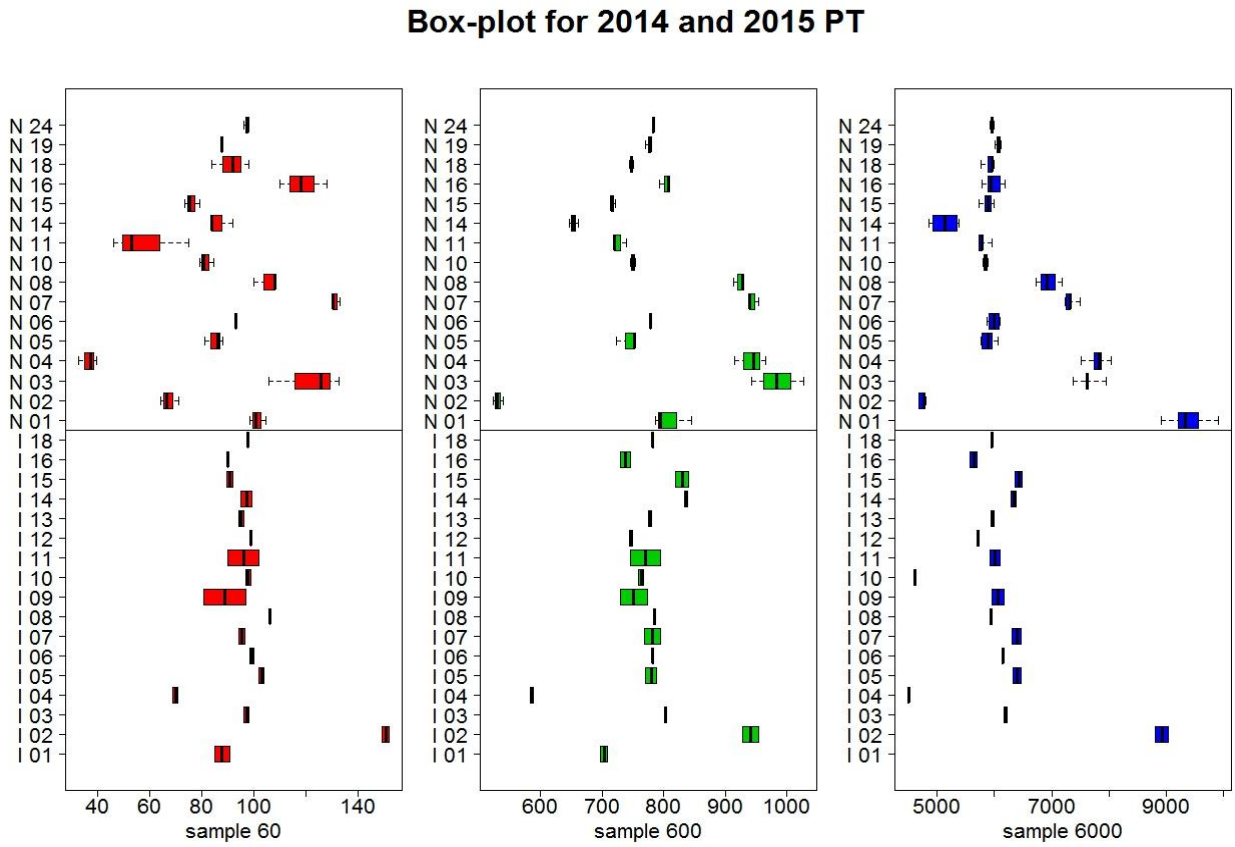


Fig 2, 3 and 4 show, for each of the three samples, the comparison among the assigned value (solid lines) and the respective uncertainties (dashed lines), individually for 2014 and 2015 trials and for the hypothetical two trials pooled (green lines 2014+2015). It is evident that a combined elaboration of 2014 and 2015 data, could lead to a theoretical important improvement of titles and uncertainties for the first trial.

Figure 2. Comparison for sample "60"

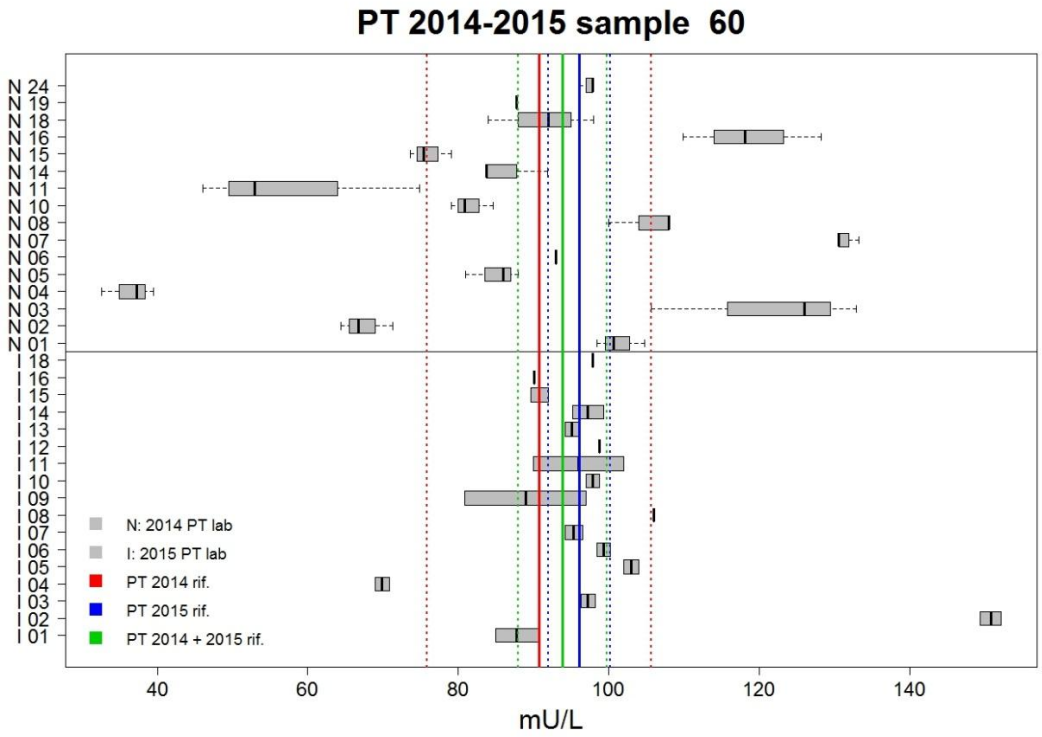


Figure 3. Comparison for sample "600"

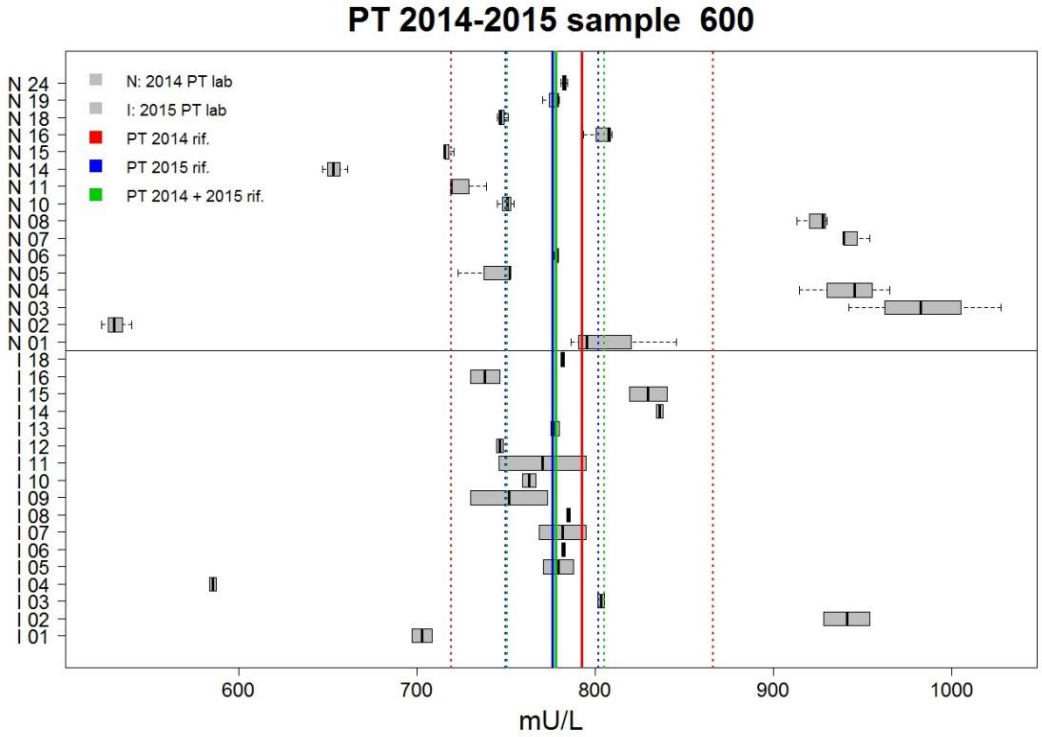
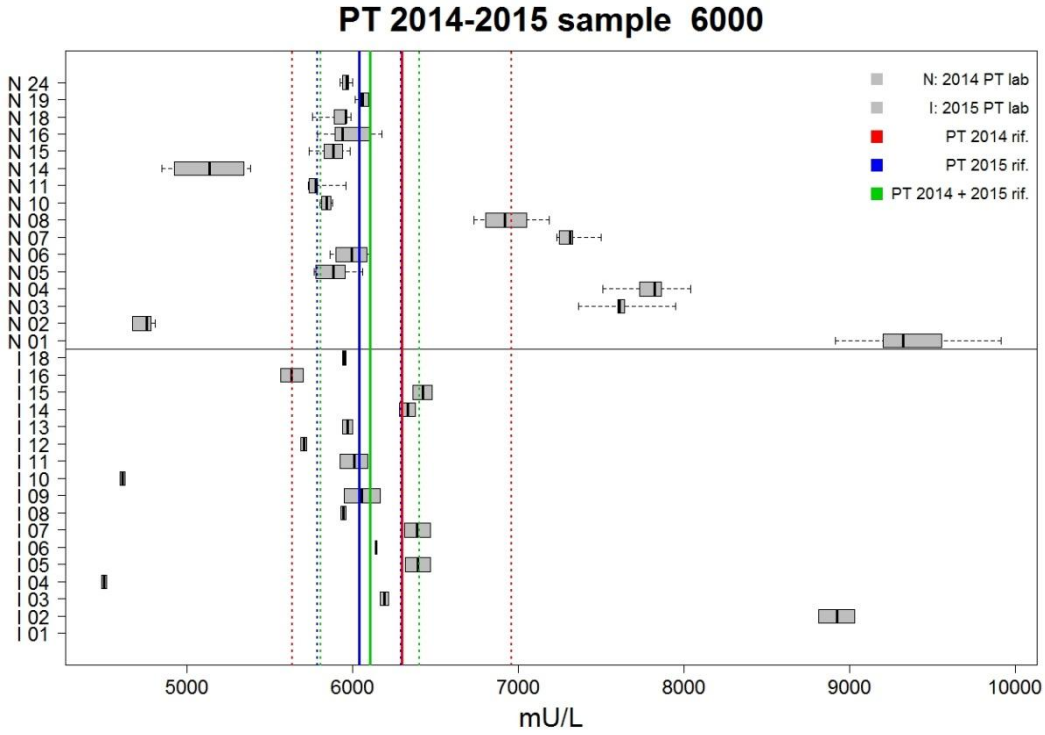


Figure 4. Comparison for sample "6000"



Comparison between titles of the Reference Materials derived from 2014 PT and 2015 PT

Table 2 shows the title values attributed to the Reference Material as result of the 2014 PT and, for comparison, those calculated computing data of the 2015 PT.

Table 2. Comparison of Reference Material Definition 2014-2015

Sample	Outliers/n° Observ.	2014 Ref. Material		Outliers/n° Observ.	2015 Ref.Material Hypotetic	
		value (mU/L)	U (p95, K=2)		value (mU/L)	U (p95, K=2)
"60"	12/48	90,9	9,0	8/34	96,7	5,2
"600"	9/48	759,87	65,1	4/34	775,3	34,3
"6000"	12/96	6267,1	567,7	6/32	6087,5	374,6

IMPORTANT NOTE : According to ISO Guide 35:2005, the assigned values for Reference Materials are calculated eliminating "outlier" values so as the extended uncertainties (U) of the RMs are calculated computing also the homogeneity of the products (calculated in 2014 trial). It is interesting to note how for 2014 data, the elimination of outliers in RM elaboration, significantly improves the U values of the PT (see Table 1). On the contrary, the U values of the hypothetical attribution in 2015 result greater than the U of 2015 PT for the higher effect of the homogeneity uncertainty respect to the characterization uncertainty (Tab 3).

Table 3. ANOVA elaboration

	Lab n.	Obs n.	Ref. Val.	charact. u	homog. u	u charact. + u homog.	Expanded U (P95, K=2)	U%
2014								
60	12	36	90.94	3.951	2.196	4.52	9.04	9.9%
600	13	39	759.77	29	14.78	32.55	65.09	8.6%
6000	14	84	6267.09	224.8	173.4	283.9	567.74	9.1%
2015								
60	13	26	96.66	1.39	2.20	2.60	5.20	5.4%
600	15	30	775.35	8.70	14.78	17.15	34.29	4.4%
6000	13	26	6087.51	70.88	173.37	187.30	374.60	6.2%

Due to some operative differences between 2014 and 2015 trials (triple analysis of all the samples and two aliquots for the sample "6000" in 2014) we thought not appropriate a global evaluation of the

pooled data. Anyhow, due to the fact the samples were of the same batch, a graphical comparison between the two experiences, was simulated.
Althought this elaboration was not exploited for updating the titles of the batches, it was useful to estimate the level of uncertainty achievable for future similar productions. Besides specific statistical considerations, Table 2 shows magnitudes of uncertainties fit for the use of this material in PT and in internal quality control of instrumental functionality,

ANNEX II

Butter, cheese and cream

The trial was the occasion to test other dairy matrices different from milk (heat treated butter, cheese and cream) with the preliminary scope to collect colleagues evaluations on the fitness for analysis of these samples of new production.

Each laboratory received two of the three matrices available at moment. Samples had to be analyzed in duplicate. Due to the finality, no protocol with indications of specific channel and calibration procedure was required. Data obtained were sufficient for a preliminary statistical elaboration of the performances of these samples .
Only to give a "reference" for participant labs , the instrumental values of these prototypic samples may be evaluated around :
Butter 550
Cheese 370
Cream 220

Remarks from participants

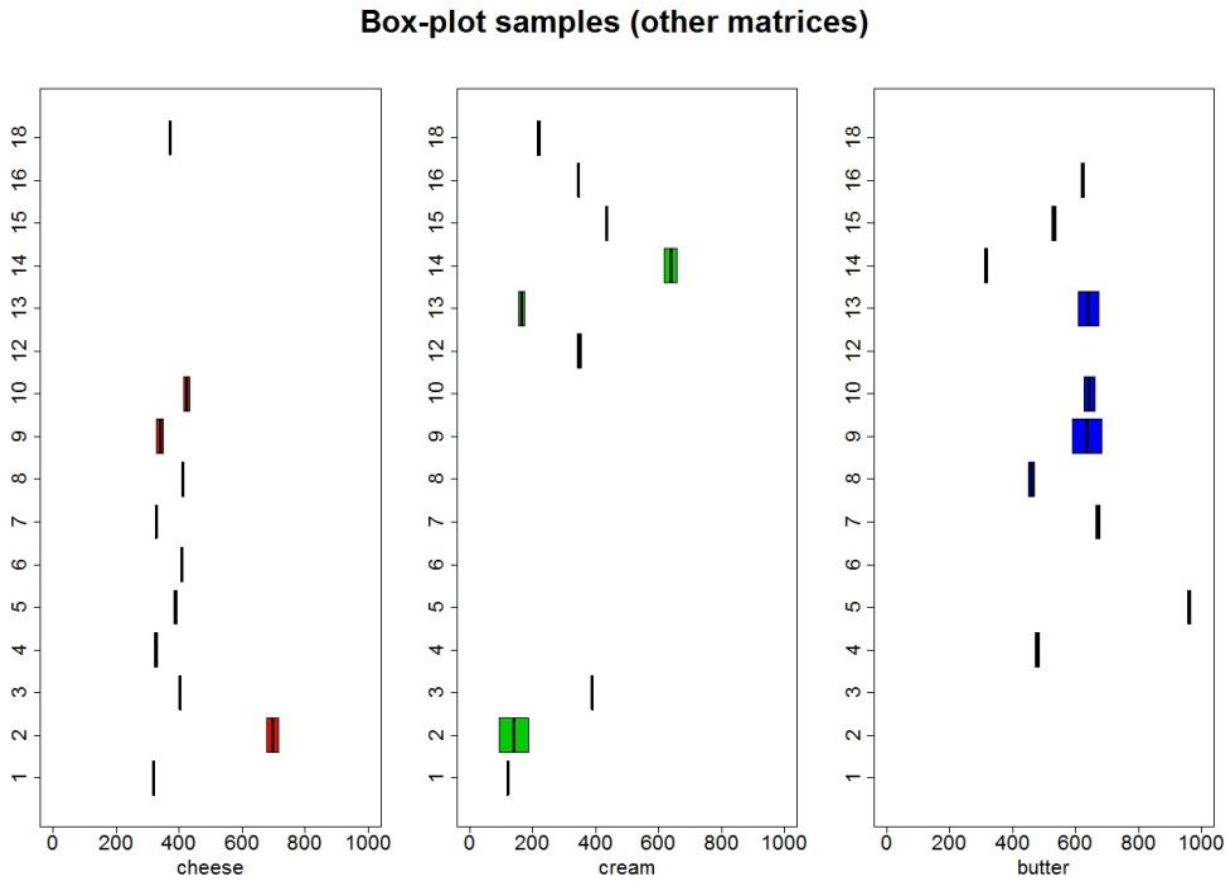
- Solution difficulties for cream samples (4 labs) and butter samples (3 labs)
- Instrumental instability in the reading step for butter sample (1 lab)

The no totally homogeneous consistency of the samples after reconstitution seemed to be a problem rather "aesthetic" than substantial. Actually, this problem did not affect the results of the laboratories who noticed the anomaly. A light heating of the samples (<38 ° C) after reconstitution and a repeated and vigorous mixing can partially reduce the no-homogeneity of the sample.

Elaboration of results

Figure1 shows a box-plot elaboration to appreciate dispersion and repeatability of the dairy samples tested in the PT. Considering the small number of laboratories for each matrix, the results provide a picture of good uniformity for the cheese matrix. On the contrary, it is evident the need of further production improvement for butter and cream samples.

Figure 1. Box-plot of the results for samples matrices cheese, cream and butter



k -ratio (ISO 5725-2:2003) for the statistical evaluation of the intralaboratory repeatability is reported in Fig 2. The horizontal lines represent the limits of the expected deviations, respectively, with P = 99% solid line and dotted line P = 95% for the 3 different matrices.
h-ratio (ISO 5725-2:2003) for the evaluation of the dispersion between laboratories averages of the three samples analyzed, is shown in Fig 3 . The horizontal lines represent the limits of the expected deviations respectively: P = 99% whole line, P = 95% dashed line.

Figure 2. k-ratio

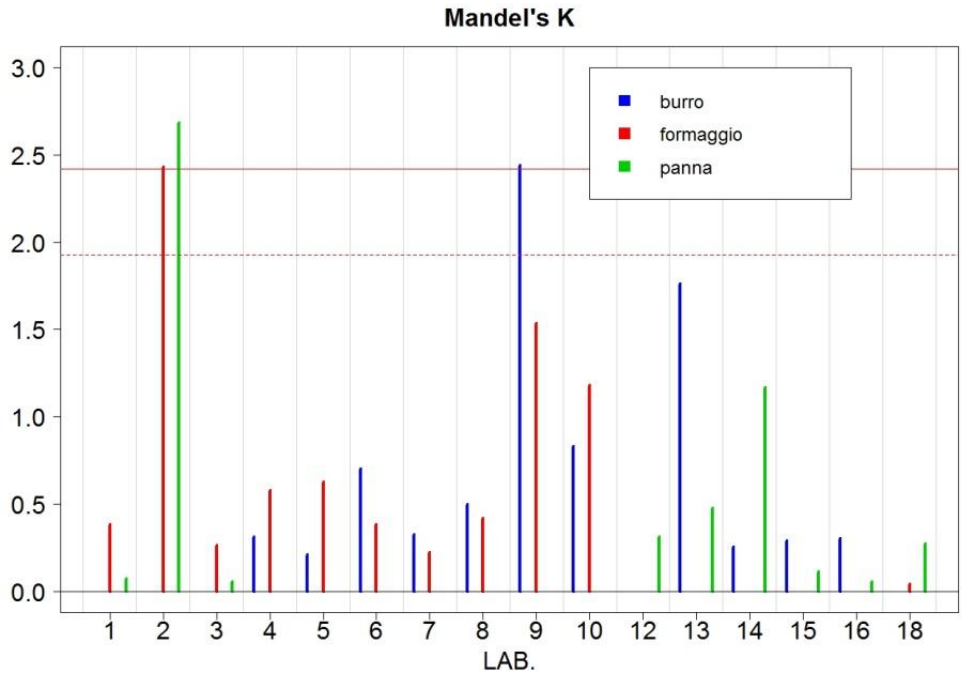
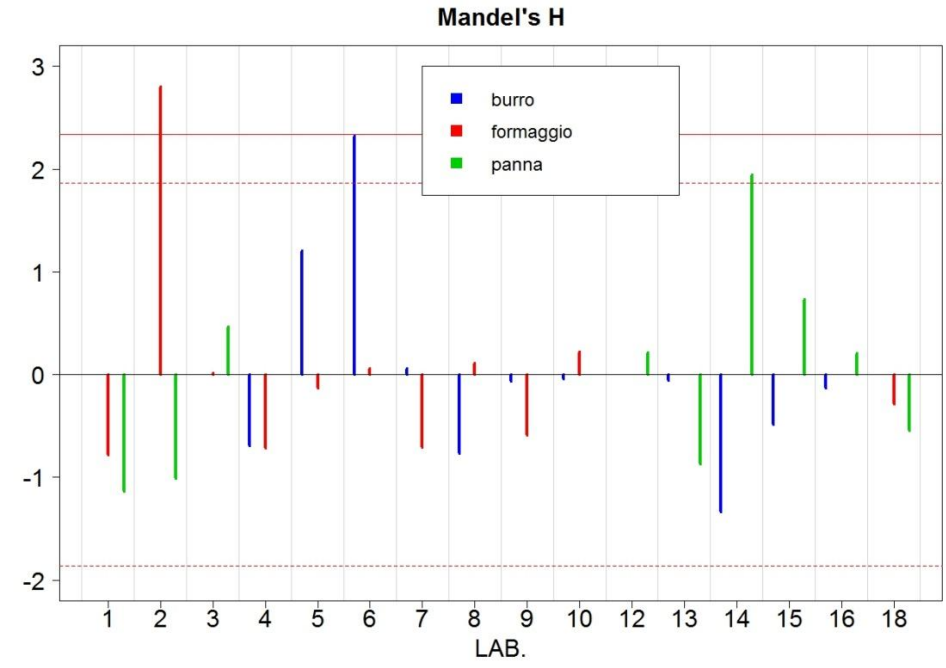


Figure 3. h-ratio



As said before, in this PT the z-score was arbitrarily intended as indirect indicator of "the fitness of the samples to the analysis" and not an indicator of laboratories performance. Fig 4 5 and 6 report z-scores for butter, cheese and cream samples. With this key reading we consider the results for these matrices very satisfactory. In fact, besides a single exception of one laboratory for the cheese sample, all the other results are in the range of clear acceptability.

Figure 4. . Graphical representation of z-scores for butter

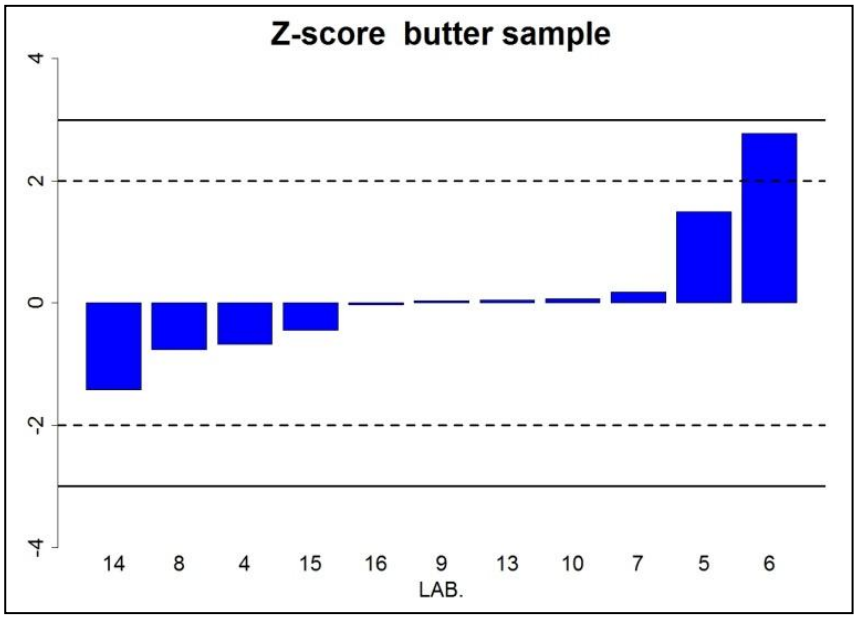


Figure 5: . Graphical representation of z-scores for cheese

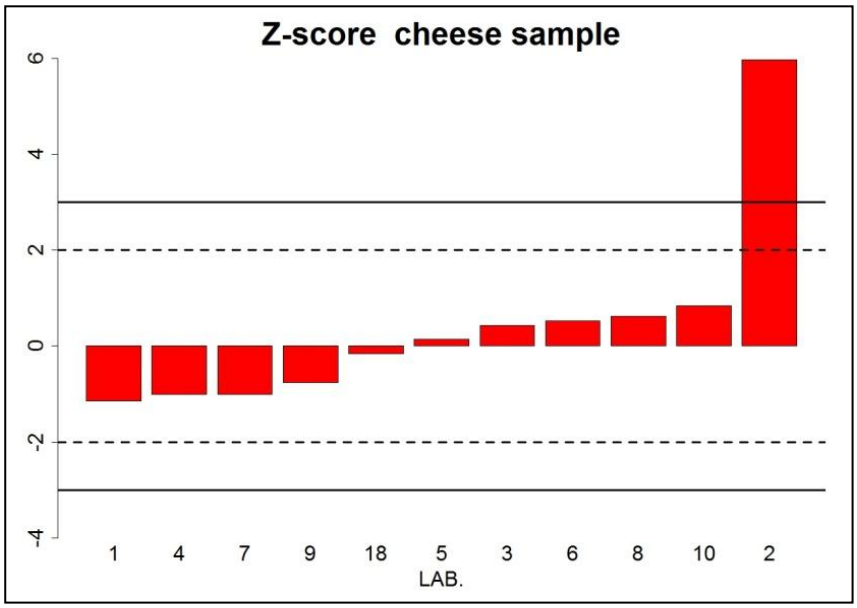
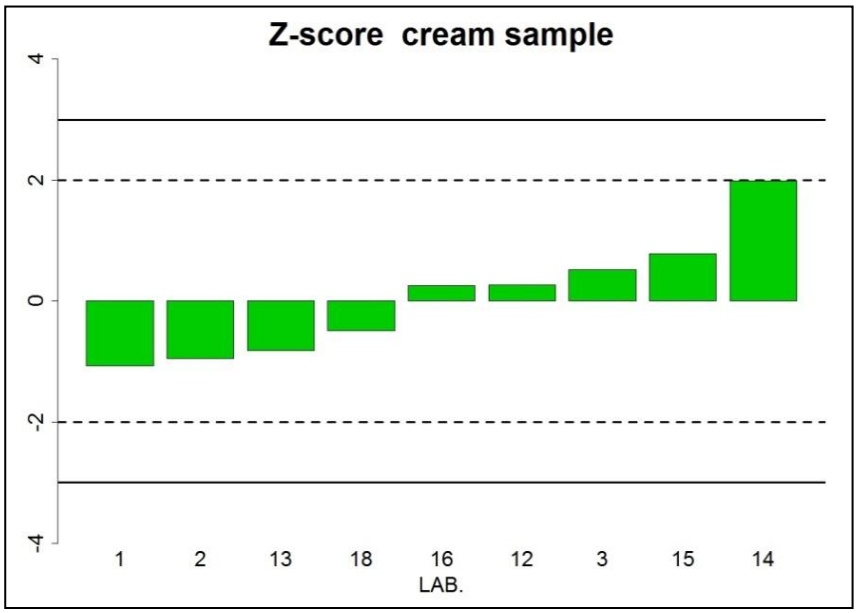


Figure 6: . Graphical representation of z-scores for cream



Conclusions

The test performed on butter, cheese and cream provided interesting considerations on the experimental products and will be very useful in the near future to make the necessary adjustments and improve the production process in the possible perspective of preparation of future reference materials in different matrices.

Just from now, the global result is satisfactory and encouraging to demonstrate the feasibility of the process of production of a reference material for the determination of alkaline phosphatase also in dairy matrices different from milk with levels of homogeneity, stability, and handiness comparable to those of milk samples.

We thank all the colleagues who shared with us and made possible this study.