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Interlaboratory Proficiency Test

Determination of alkaline phosphatase activity in milk with fluorimetric method in semi-skimmed lyophilized cow milk

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Determination of alkaline phosphatase activity in milk with fluorimetric method

in semi skimmed lyophilized cow milk

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Introduction

The activity of phosphatase alkaline is considered to be the main pasteurization tracer of milk.

COMMISSION REGULATION (EC) No 1664/2006 amending Regulation (EC) No 2074/2005, establishes that ISO 11816:1 "Milk and milk products – determination of alkaline phosphatase activity – Part 1: Fluorimetric method for milk and milk-based drinks" is the method to apply as reference method for the determination of alkaline phosphatase activity in milk. The same Regulation establishes also that:

2. *The alkaline phosphatase activity is expressed as milliunits of enzyme activity per litre (mU/l). A unit of alkaline phosphatase activity is the amount of alkaline phosphatase enzyme that catalyses the transformation of 1 micromole of substrate per minute.*

3. An alkaline phosphatase test is considered to give a negative result if the measured activity in cow's milk is not higher than 350 mU/l.

4. The use of alternative analytical methods is acceptable when the methods are validated against the reference method ISO 11816:1 in accordance with internationally accepted protocols.

The Italian National Reference Laboratory for Milk and Milk Products (NRL-MMP) in concert with the Italian Reference Centre for Cow Milk Quality (RC-CMQ) organized in summer 2014 an interlaboratory comparison (PVI) for the laboratories in charge of the official control in Italy (IIZZSS) to evaluate their performance with regard to the determination of the activity of alkaline phosphatase in cow milk with the method prescribed for the official control ISO 11816:1. As consequence, the number of participant IIZZSS was strongly limited by the availability of the instrument Fluorophos Test System (Advanced Instruments, Norwood, Us.) and by the possibility to run the analyses with ISO 11816:1 method.

For this, it was decided to involve in the activities organized at national level by NRL-MMP and RC-CMQ, also laboratories outside the net of IIZZSS as fruitfully just made , in occasion of the project for the conversion line for the total bacterial count in milk with cytometric instruments (2012).

The present trial required the preparation of samples with homogeneity and stability characteristics in compliance with ISO 13528:2005. In the light of resources optimization, it was also the occasion for field experiments of a Current Research Project (007/2012 financed by the Italian Ministry of Health) finalized to the preparation of reference materials in lyophilized matrix with assigned values and relative uncertainties. Finally, the organization of this trial was also an opportunity to start a discussion with

EURL- MMP (ANSES in Maisons Alfort) concerning the standardization of samples preparation for interlaboratory trials.
As prosecution of this study, other activities are just planned at national level, to consider the performances of laboratories using alternative methods in compliance with Reg. (EC) n°1664/2006 for the determination of the activity of alkaline phosphatase.

Besides the technical and statistical results, the main value of this work relies on the start of a coordinated and shared activity in Italy also for the fluorimetric method for the determination of the phosphatase activity in milk. In fact, although this analysis is a regular and frequent task in a milk laboratory for the importance of this parameter from hygienic and technologic points of view, yet no specific interlaboratory trials had been implemented at national level in Italy.

In line with the activities of EURL-MMP and with the scope of a better standardization of the application of the Reference Method, other dairy matrices will be considered in a near future.

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General informations

Participant laboratories

The PT involved 19 laboratories , 2 of which could not send results in time for instrumental problems and another 1 send the results so late that it was not possible to include it in the statistical elaboration (tab. 1)

Table 1: participant laboratories

Laboratorio	Paese	Accreditamento
EURL M-MP A.N.S.E.S	Maisons Alfort (F)	x
Associazione Allevatori del Friuli Venezia Giulia	Codroipo (UD)	x
Associazione Regionale Allevatori Lombardia	Crema (CR)	
Bio-Lat S.n.c.	Lusciano (CE)	x
Caseificio Sociale di Manciano S.c.a.	Manciano (GR)	
CASTALAB di Bussolati & Miti	Fidenza (PR)	x
Centrale del latte di Firenze Pistoia Livorno S.p.a.	Firenze (FI)	
CHELAB - SILLIKER S.r.l.	Resana (TV)	
ECO-LAT S.r.l.	Lodi (LO)	x
EPTA-NORD S.r.l.	Conselve (PD)	
Francia Latticini S.p.a.	Sonnino (LT)	
I.Z.S. Lazio Toscana	Roma (RM)	x
I.Z.S. Lombardia Emilia Romagna - CRNQLB	Brescia (BS)	x
I.Z.S. Piemonte Liguria Valle d'Aosta	Torino (TO)	x
Latteria Soresina S.c.a.	Peschiera Borromeo (MI)	
Marino S.r.l.	Santa Maria a Vico (CE)	x
Trentingrana - CONCAST S.c.a.	Trento (TN)	x
Università degli Studi di Milano - DEFENS	Milano (MI)	
Veneto Agricoltura	Thiene (VI)	x

Organisation of the proficiency testing trial (PT)

Laboratories equipped with Fluorophos® Test System, Advanced Instruments, Norwood, US were invited by a circular mail to the PT and informed of the method to apply, the number of samples and the relevant dates for samples reception, analysis and data transmission. Participant laboratories received the following documents:

- "acknowledgement of receipt " to fill upon receipt
- "operative protocol " with all the relevant instructions for the test performance
- "results form" to fill with the analyses results

Samples were shipped in the second week of July 2014. All participants laboratories received the samples free of charge. The deadline for running the analyses was indicated within the 30 July 2014. No precise day to run the analyses was required due to the stability of the lyophilized samples.

SAMPLES

Each laboratory received 4 vials. Each vial had to be analyzed three times in repeatability conditions, starting from the sampling. Vial samples were randomly coded:

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

One additional vial of lyophilized/"ALP free"/semi skimmed cow milk , labelled "calibrator" was enclosed in the shipment to verify the calibration ratio of each instrument used for the analyses.

According to the operative protocol, all the vials had to be reconstituted with 2.0 g of demineralized water on a technical balance, throughly mixed (in case, vortexed) till complete solution and immediately analyzed after reconstitution.

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 of the samples

The homogeneity was preliminarily tested according to ISO guide 35 (Reference materials — General and statistical principles for certification) and , in addition and only for the sample "6000" , also using the results of the inter laboratory test on both vials of the "6000" sample.

Before the interlaboratory test, a number of randomly chosen samples equivalent to the 5% the total samples prepared for each level of alcaline phosfatase was analysed in duplicate under repeatability conditions, starting from the sampling.

The between bottles standard deviations (Sbb) obtained by ANOVA analysis were summarized in the box below. According to the criteria defined in ISO 13528: 2005 B.2, the level of homogeneity observed was deemed satisfactory for the purposes of use.

Between Bottles Standard Deviation (Sbb):
sample "60" = 2,20 mU/L
sample "600" = 14,78 mU/L
sample "6000" = 173,37 mU/L
sample "6000" = 95 mU/L (evaluated during the PT)

Note: The homogeneity value calculated for each level of determination was successively used for the evaluation of the expected uncertainty measurement of the samples.
Due to the lyophilized status of the samples the complete series of stability tests is scheduled over a long term period (5-7 years). A first short-term evaluation was performed in the period elapsing between the controls on each batch at the production time and the trial, according to ISO 13528:2005 (Statistical methods for use in proficiency testing by interlaboratory comparisons): 4 samples for each level of alkaline phosphatase were randomly selected and analysed in duplicate under repeatability conditions, starting from the sampling. Results obtained were fully satisfactory respect to ISO 13528:2005 limits for homogeneity.

RESULTS AND ELABORATION

References:
ISO 13528:2005,
ISO 11816-1:2013
ISO guide 35, 2006
ISO 5725-2:1994

(Excel®)spreadsheets and statistics procedures in code R were used for the elaboration of results.

Data received

14 laboratories fully observed the operative indications supplied.

- laboratory 12 sent samples results (with a marked delay), but no data for control of the instrumental functionality (laboratory 12 results are reported in table 2, but were not considered in the statistical evaluations)
- laboratory 13 did not run the analyses for prolonged instrument problems
- 2 laboratories did not submit data for the instrument set-up; one of them did not report the value of the calibration ratio (however both labs were kept in the final elaboration)
- 1 laboratory reported difficulties in the dissolution of 2 samples pointing out particulate matter in suspension
- No one submitted comments on transportation or reception of the material.

ELABORATION

The test results are summarized in Table 2. The calculation of the expected values at all the levels of alkaline phosphatase was made by robust estimation, as specified by ISO 13528: 2005 5.6.1 and 5.6.2.

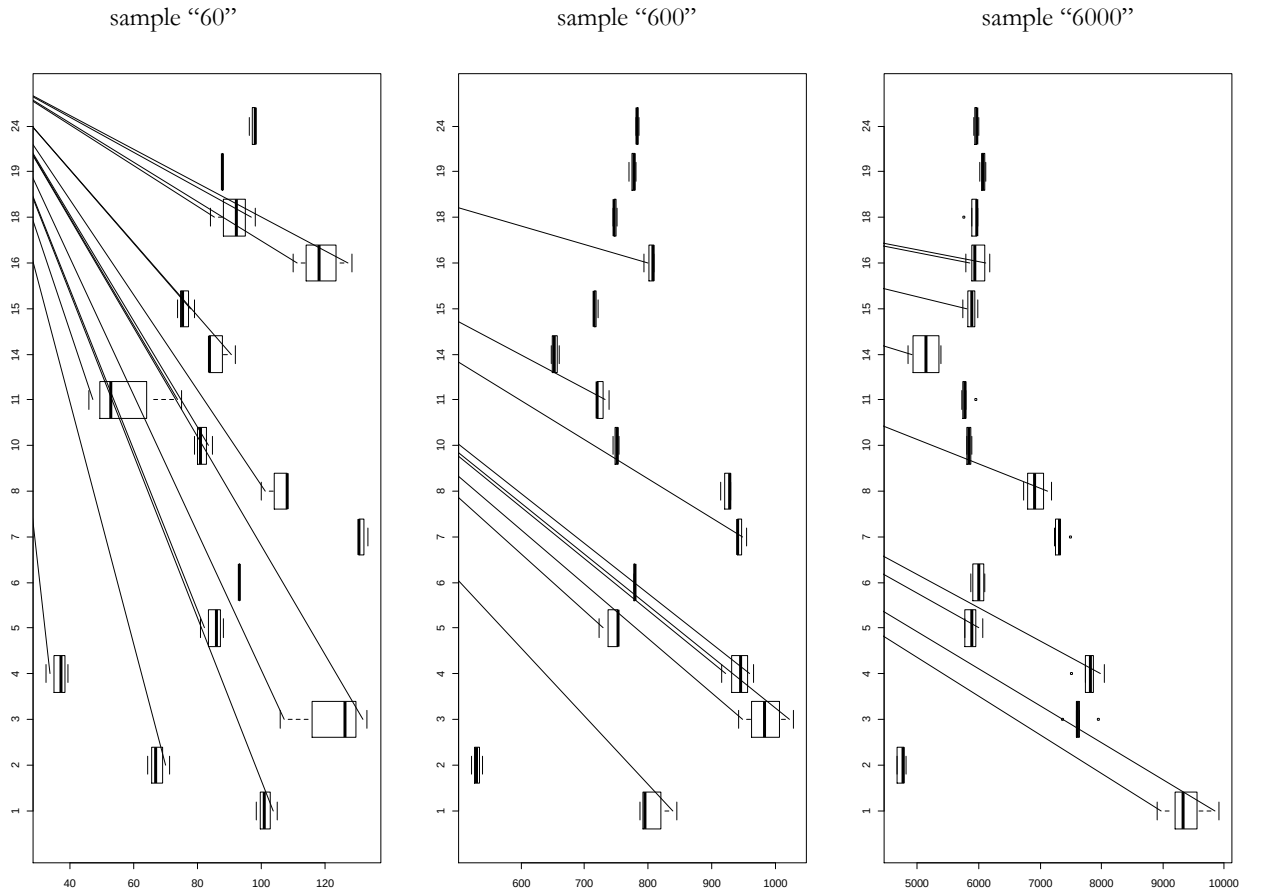
Table 2: -Results of the analyses (expressed in mU / L)

LAB.	Sample 60			Sample 600			Sample 6000 A			Sample 6000 B		
code	Rep. A	Rep. B	Rep. C	Rep. A	Rep. B	Rep. C	Rep. A	Rep. B	Rep. C	Rep. A	Rep. B	Rep. C
1	104,8	98,4	100,7	845,4	795,3	786,5	9361	9202	9285	9913	8915	9552
2	66,7	64,4	71,3	530	539,7	523,1	4735	4675	4675	4785	4808	4785
3	132,9	105,7	126,0	942,4	982,8	1027,9	7608	7599	7364	7617	7640	7948
4	39,5	32,6	37,2	965,4	945,6	914,8	7829	7861	7732	7511	7815	8040
5	81	86	88	752	723	752	5953	6059	5953	5811	5778	5769
6	93	93	93	779	777	779	6040	6105	6086	5953	5898	5866
7	133,3	130,6	130,6	954,3	939,6	939,6	7328	7231	7309	7498	7318	7249
8	108	108	100	913	930	928	6997	7185	7052	6836	6804	6730
10	80,9	79,1	84,6	754,4	750,7	745,2	5870	5820	5801	5811	5870	5880
11	52,9	74,9	46	719,4	739,2	719,4	5958	5778	5789	5737	5774	5732
14	83,7	83,7	91,9	647	653	661	5346	5241	5384	4850	4925	5038
15	79,1	73,6	75,4	720,8	715,3	715,3	5829	5939	5985	5930	5834	5737
16	118,1	128,3	109,9	793,4	809,5	807,7	5958	6100	6178	5893	5788	5921
18	84	98	92	745	747	751	5889	5760	5990	5958	5962	5962
19	87,8	87,8	87,8	780,1	778,3	770,5	6109	6063	6050	6017	6063	6096
24	96,1	97,9	97,9	784,7	781,0	782,9	5972	5939	5958	5999	5926	5976
12	96,2	81,3	83,1	642,8	567,6	633,9	4981	4540	4602	n.e.	n.e.	n.e.

n.a.= not analyzed

The overall evaluation of the PT is illustrated in Fig 1 in which, for each participating laboratory and for each level of alkaline phosphatase , the distribution of the results , the difference between replicas and the number of eventual "abnormal"results are evidenced

Fig 1. Box-plot



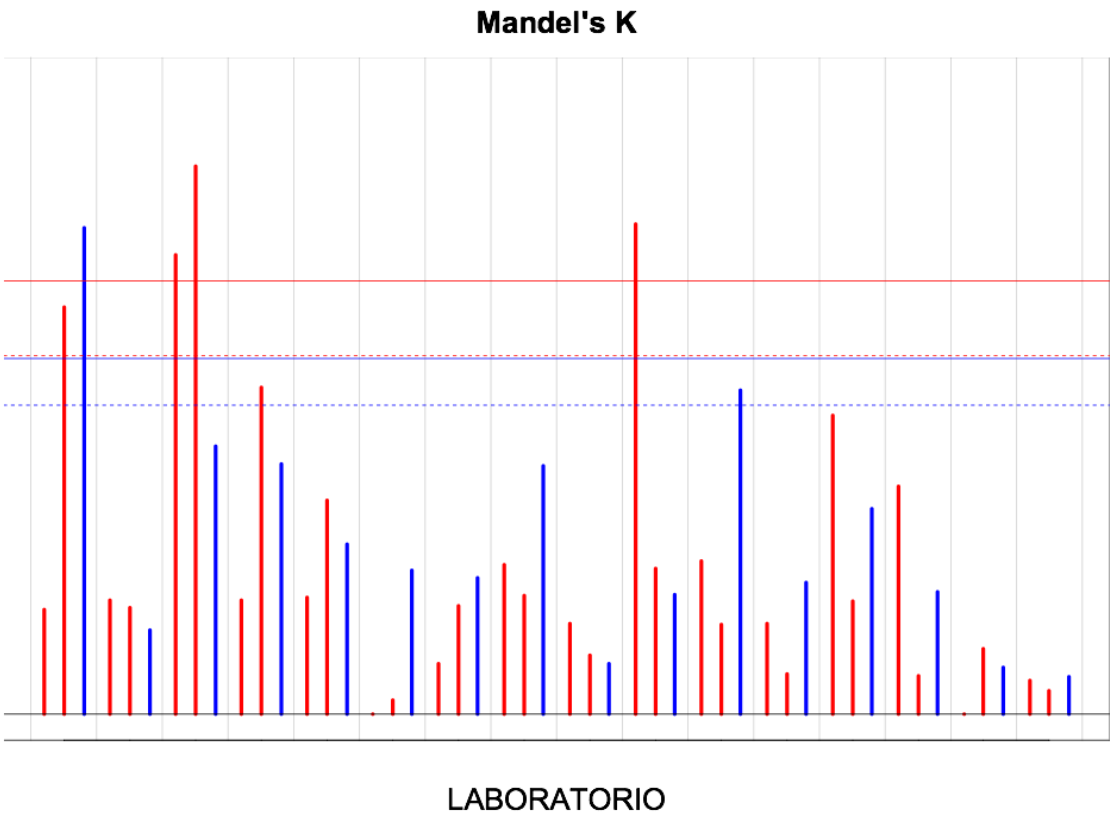
A further overall evaluation of the PT results was made according to the indication of ISO 13528:2005, 8.4. In particular, the repeatability of measurements was evaluated by the Mandel's k factor (Fig.2) (ISO 5725-2:1994). The sample "6000" with 6 determinations (2 samples in 3 replicates) is in blue whereas samples "60" and "600" with 3 determinations each, are reported in red. The horizontal lines represent the limits of the expected deviation, respectively, with P = 99% in whole line and P = 95% in dotted line.

Note:

- Laboratory 1: sample "600" and sample "6000"
- Laboratory 3: sample "60" and sample "600"
- Laboratory 11: sample "60"
- Laboratory 14: Sample "6000"

On the contrary, the laboratories 6 and 19 for the sample "60" obtained 2 extremely low indicators, due to numerically identical replicas.

Fig 2 Mandel's K.

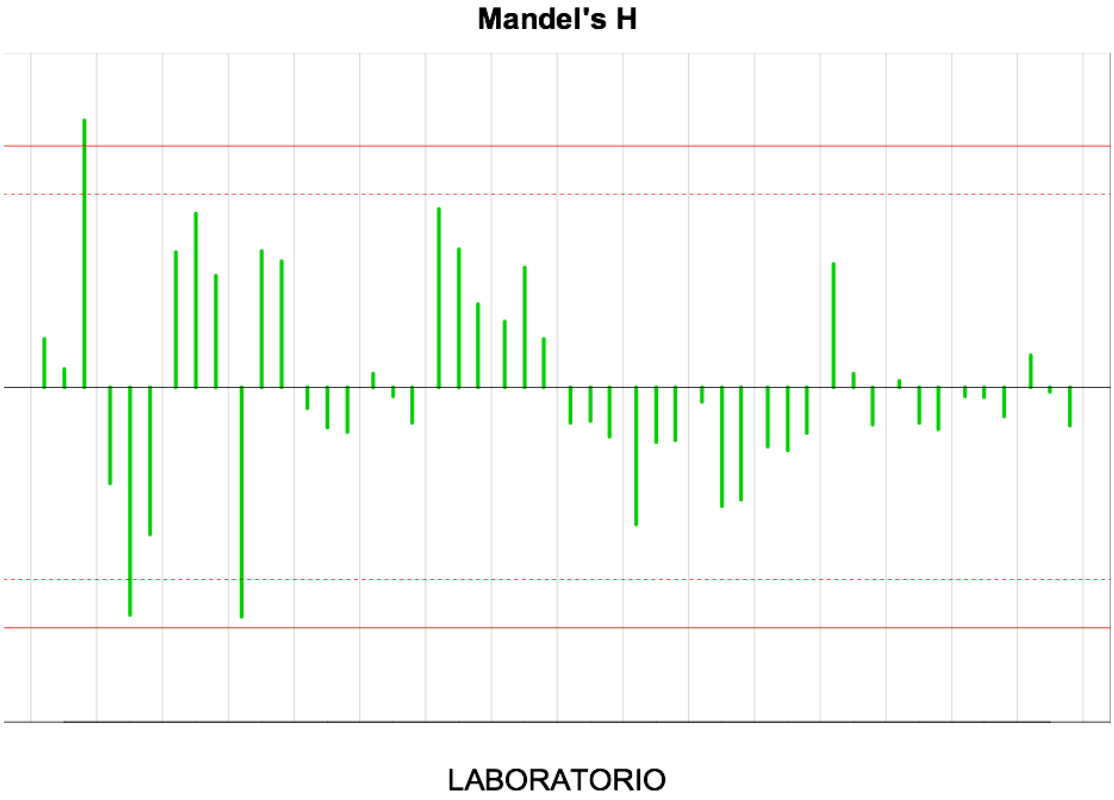


The dispersion between laboratories was analyzed through the parameter Mandel's h (Fig 3) , that represents, for each laboratory, the means of the three samples analyzed also in this case , according to the limits of significance respectively: P= 99% in whole line and P = 95% in dotted line.

To note:

- Laboratory 1 for the average value of the sample "6000" (P = 99%)
- Laboratory 2 and 4, respectively, for samples "600" and "60" (P = 95%)

Fig. 3: Mandel's h



Note: it should be noted that these statistical analyses confirm the level of homogeneity of the sample results (Mandel's k) and 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.
This, after all, well suits the specific purposes of this PT, being the first of its kind at our national level for this method and being the participant laboratories characterized by no completely uniform experiences and skill.

Definition of the standard deviation assigned for the test:

In the absence of criteria available for all the 3 chosen levels of alkaline phosphatase activity of the samples for the reference method (ISO 11816-1: 20136), the standard deviation of the observations obtained in the test, was estimated by the " robust standard deviations of the averaged mean values of each laboratory, as shown in ISO 13528: 2005 point 6.6.1.

Assigned standard deviation for the PT:
sample "60" = 23.86 mU/L
sample "600" = 117.62 mU/L
sample "6000"= 1058.81 mU/L

Definition of the assigned value X and of its uncertainty

Expected values of each level of alkaline phosphatase were fixed as the robust means "X" according to ISO 13528:2005 C.1.

The statistical software R (package MetRology, function algA) was used for the application of the algorithms

Assigned value X:
X sample "60"= 90.77 mU/L
X sample "600"= 792.65 mU/L
X sample "6000"= 6297.20 mU/L

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

$$\frac{}{}$$

Where:
s * = robust standard deviation of the averages of each laboratory (23,86 for the sample "60", 117.62 for the sample "600", 1058.81 for sample "6000").
p = 16, number of laboratories involved in the estimate.

Standard measurement uncertainty $_X$:
sample "60"= 7.46 mU/L
sample "600"= 36.76 mU/L
sample "6000"= 330. 88 mU/L

The estimated uncertainties appear reasonable compared to the criteria defined in ISO 13528: 2005 4.2

ID		
Sample "60"	7,46	7,15
Sample "600"	36,76	35,28
Sample "6000"	330,88	317,64

The obtained uncertainty of measurement resulted slightly higher than the expected limits, but acceptable and could therefore be considered negligible in the calculation of the z-score.

Statistics calculation of performances

Laboratory performance was evaluated in terms of z-score:

$$z = \frac{x - X}{\sigma}$$

where :
"x" is the laboratory mean for each level of alkaline phosphatase and "X" the robust mean obtained from all the results for that level

The performance of each laboratory was estimated according to the following criteria:

- $|z| \leq 2$: satisfactory
- $2 < |z| \leq 3$: questionable. The lab should verify its procedures and their implementation
- $|z| > 3$: unsatisfactory. Controls and corrective actions must be taken

z-score values are reported in tab 3, 4 e 5 (and fig 4, 5 e 6)

Table 3: Sample "60"

laboratory	Averaged values	n°data	
1	101,30	3	0,44
2	67,46	3	-0,97
3	121,53	3	1,28
4	36,43	3	-2,27
5	85,00	3	-0,24
6	93,00	3	0,09
7	131,50	3	1,70
8	105,33	3	0,61
10	81,53	3	-0,38
11	57,93	3	-1,37
14	86,43	3	-0,18
15	76,03	3	-0,61
16	118,76	3	1,17
18	91,33	3	0,02
19	87,80	3	-0,12
24	97,30	3	0,27

Table 4: sample "600"

laboratory	Averaged values	n°data	
1	809,06	3	0,13
2	530,93	3	-2,22
3	984,36	3	1,62
4	941,93	3	1,26
5	742,33	3	-0,42
6	778,33	3	-0,12
7	944,50	3	1,29
8	923,66	3	1,11
10	750,10	3	-0,36
11	726,00	3	-0,56
14	653,66	3	-1,18
15	717,13	3	-0,64
16	803,53	3	0,09
18	747,66	3	-0,38
19	776,30	3	-0,13
24	782,86	3	-0,08

Table 5: sample "6000"

laboratory	Averaged values	n°data	
1	9371,28	6	2,90
2	4743,83	6	-1,46
3	7629,33	6	1,25
4	7798,00	6	1,41
5	5887,16	6	-0,38
6	5991,33	6	-0,28
7	7322,16	6	0,96
8	6934,00	6	0,60
10	5842,00	6	-0,42
11	5794,66	6	-0,47
14	5130,66	6	-1,10
15	5875,66	6	-0,39
16	5973,00	6	-0,30
18	5920,16	6	-0,35
19	6066,33	6	-0,21
24	5961,66	6	-0,31

Fig 4: grafical representation of z-scores for sample "60"

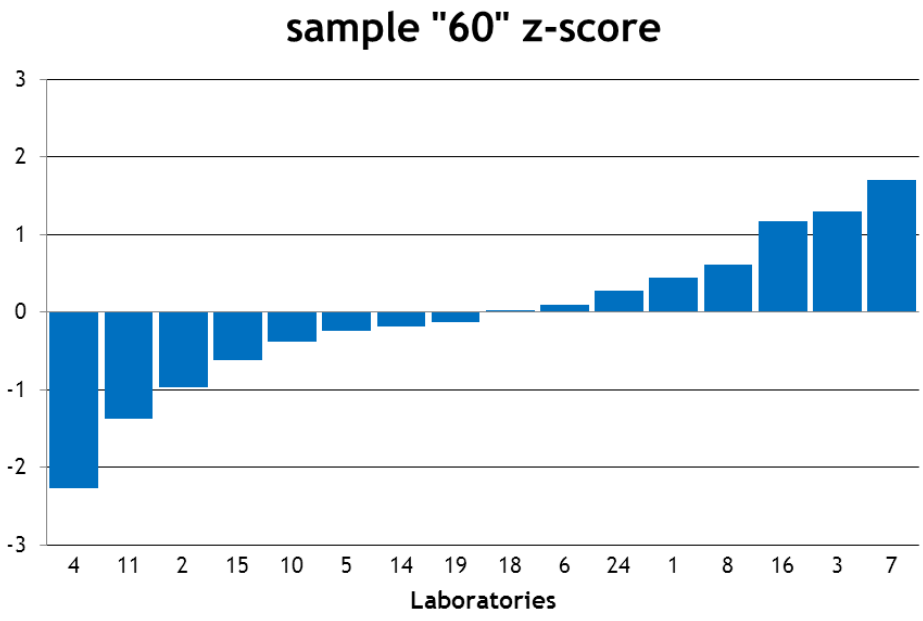


Fig 5: grafical representation of z-scores for sample "600"

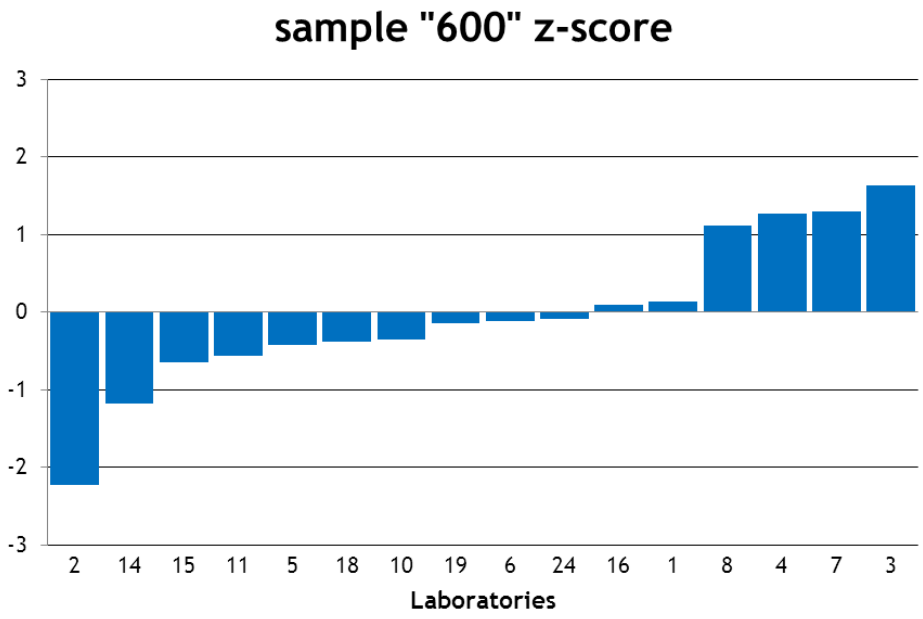
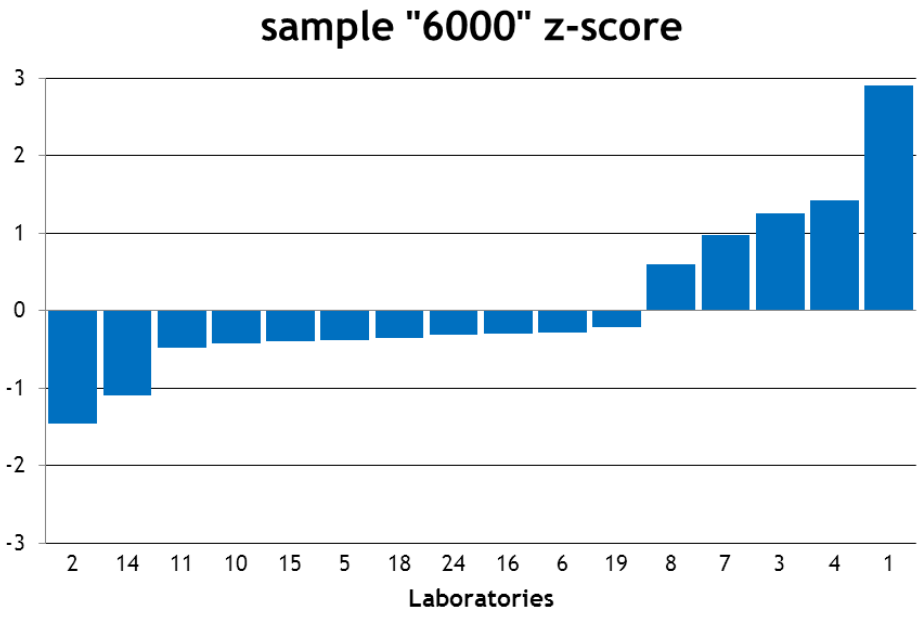


Fig 6: grafical representation of z-scores for sample "6000"



Evaluation of

Overall, the PT outcome was considered satisfactory. For each level of contamination the results of 16 laboratories were available and, for each level, "only" 1 of 16 laboratory reported 1 of 3 values of z-score > 2: Laboratory 4 for sample "60", Laboratory 2 for sample "600", and Laboratory 1 for the sample "6000". However the performances of these laboratories for the remaining 2 tests were z-scored in the range of full acceptability (Tab. 3-5).

The overall analysis of the z-scores obtained, given that all laboratories applied the same method, could evidence that the majority of the laboratories were grouped around very low and fairly homogeneous values. Hence, the overall situation of uniformity of the laboratories involved in this first experience can be regarded as an element of robustness for the evaluation of the title definition for the reference material tested in the course of this trial (see Appendix 1).

We thank all the laboratories that with their participation allowed us to get a very representative picture of the Italian situation in the realization of this PT. Considering the importance of the meaning of the parameter alkaline phosphatase in milk, it is worth to evaluate in the short future, the performance of laboratories that use alternative methods for this parameter

IL DIRIGENTE RESPONSABILE

Dr. G. Bolzoni

ADDITIONAL CONCLUSION

For information purposes, in Annex 1 are reported some statistical key points that RC-CMQ of IZSLER of Brescia obtained in the course of the Italian Project (007/2012) for the determination of the titles and the relative uncertainties of experimental batches of reference material in lyophilized matrix. In the last table, differences, in terms of mean value and uncertainty of measurement, resulting from this second step of the work done, are reported.

It results that the contribution of the PT was more than satisfactory. It is in our intention to further develop the process to determine the title of the reference material produced (and of those currently in the experimental phase in cream, butter, cheese matrices) through raising the number of collaborating laboratories.

With this in mind, besides the involvement of much more Italian laboratories for future activities, preliminary contacts with the EURL-MMP ANSES Paris (who took part in this test) were taken to try to involve in similar future activities other reference laboratories with clear experience in the application of the fluorimetric method and comunity interlaboratory trials.

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ANNEX I

SUMMARY OF EVALUATIONS CONDUCTED FOR THE DEFINITION OF THE TITLE OF THE REFERENCE MATERIALS

The statistics procedure applied, in addition to the already described basic controls of stability and homogeneity of the batch of material produced, provided a more selective approach for the individuation of "outliers" aimed, on the one hand, to select "most reliable information" and, on the other, to reduce the uncertainty of the title (ISO Guide 35).

For this purpose Cochran test and Grubbs were applied (Table 1).

Table 1. Outliers from Cochran e di Grubbs test (P 99%, P 95%)

	Test di Cochran		Test di Grubbs	
Sample	P 99%	P 95%	P 99%	P 95%
"60"	Lab. 3	Lab. 11		Lab. 4, 7
"600"	Lab. 3, 4	Lab. 1		
"6000"	Lab. 1	Lab. 14		

After elimination of outlier and "straggled data" between 95 and 99%, the analysis of variance was runned on the valid data to characterize the precision parameters sr, sL and sR (Table 2)

Table 2. Estimation of the components of the dispersion (ANOVA)

Sample	**N° of Labs**	**N° observations**	**Mean value**	**Standard Deviation**		
				sr	sL	sR
"60"	12	36	90,94	4,4	13,5	14,1
"600"	13	39	759,77	8,0	104,5	104,8
"6000"	14	84	6267,09	112,4	839,8	847,3

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The final estimate of the uncertainty of the reference materials was then estimated taking into account the uncertainties derived from the test of homogeneity preliminarily performed and applying the coverage factor 2 (Table 3).

Table 3. Estimation of measurement uncertainty (U)

Sample	N° of Labs	N° of observations	Reference Value	u char	u homog.	u char + u homog.	U (P95, K=2)	U%
60	12	36	90,94	3,95	2,19	4,52	9,04	9,9%
600	13	39	759,77	28,99	14,78	32,55	65,09	8,6%
6000	14	84	6267,09	224,77	173,37	283,87	567,74	9,1%

Table 4. Comparison of results obtained from the procedure to assign the title (value and uncertainty) and respectively from PT elaboration

Assegnazione Titolo			Interlaboratory test	
U	Assigned value	Sample	Assigned value	U
9,04	90,94	**60**	90,77	14,92
65,09	759,77	**600**	792,65	73,52
567,74	6267,09	**6000**	6297,20	661,76

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