REPORT ON THE FUNCTIONAL TESTS CARRIED OUT ON THE FOOD LAB INSTRUMENT

During the period of July-October 2001, 2500 tests were carried out to verify the operation of the FoodLab instrument supplied by CDR S.r.l. (Via degli Artigiani, 6 – 50020 Ginestra F.na – FIRENZE Tel. +39 055871431 Fax + 39 0558714322) for determining the presence of urea in milk.

The instrument is realized with spectrophotometric and microelectronic technology, with reading through solid-state devices and interferential filters. The instrument is portable (length 315 mm, width 190 mm, height 165 mm, weight 2500 gr.) and requires a normal 12 Vcc power supply outlet. The reading unit and the incubation unit (maximum 14 reading cells) are thermostated at 37°C. The system uses non-pretreated milk samples and specific ready-to-use microcuvettes, with previously dispensed reagents and non-returnable (disposable) bottles.

The reaction is based on the transformation of the urea into ammonia by the urease. The ammonia ions thus react with a phenolic derivative and form a blue-green colored complex whose intensity, measured at 700nm, is directly proportional to the concentration of urea in the sample. The reaction consists of a first phase of preheating of reagent R1 with the sample (5µl) that lasts for 5 minutes and of a second phase where the reaction goes to end point after 3 minutes from when reagent R2 is added. The system can be standardized through the use of three or more milk standard samples.

The tests were carried out in the Laboratorio Standard Latte (LSL) (Milk Standard Laboratory) of the Associazione Italiana Allevatori (A.I.A.) (Italian Breeders Association). The laboratory has been accredited since 1997 by Sinal with n° 0138 for six tests in the dairy industry, among which the determination of urea with the differential pH-metry instrument, EFA Instrument, Hamilton-Eurochem. Furthermore, the laboratory's quality system is UNI EN ISO 9001. 94 certified for the preparation and commercialization of reference material, reference material certified in the dairy industry and planning, organization and realization of inter-laboratory evaluation tests (1999).

A Project Plan (PdP) has been drawn up at the Laboratorio Standard Latte (Milk Standard Laboratory) in which the description of the operative protocol is given.

The tests were initially carried out for cow's milk and subsequently extended to sheep's milk and buffalo milk.

A 0.02% concentration of Technical Bronopol was added to the milk used supplied by the Laboratorio Standard Latte (Milk Standard Laboratory). It is not possible to analyze milk samples treated with Methylene Blue because the instrument measures in the blue-green spectrum.

The milk, coming from both single animals and from mass samples, was only heated to make the matrix homogeneous and did not undergo further preventive treatments.

The functional tests carried out are:

1. Determination of the testing speed
2. Check of the temperature of each individual well
3. Repeatability tests
4. Check of the instrument's calibration
5. Check of the instrument's linearity
6. Recovery and sensitivity tests
7. Check of the variation of the lots
8. Check for interference of the fat

These tests from n°3 to n°8 were repeated several times over the time span for the cow's milk and for the buffalo milk. The tests were repeated only once for the sheep's milk, which is considered intermediate between the buffalo milk and the cow's milk for the macrocomponents.

1. DETERMINATION OF THE TRUE TESTING SPEED

The true speed of the instrument is 48 tests/hour. This value was obtained by testing 14 samples at a time (maximum number of cuvettes for the instrument), keeping in mind the operator's speed and the reaction times preset by the instrument (5 minutes for the reaction mediated by the urease that transforms the urea into ammonia and 3 minutes for the colorimetric reaction).

The measurements were taken after the instrument, which arrives at the standard temperature after 10 minutes, was heated.

The instrument's nominal speed is 60 tests/hour.

2. CHECK OF THE TEMPERATURE OF EACH INDIVIDUAL WELL

It was possible, using some empty cuvettes, to determine the temperature of each individual well, using a second line thermometer calibrated for comparison with a reference sample, Delta Ohm digital thermometer with PT100 probe calibrated at the Sit center n° 20/M/E certificate n° 1341.

The cuvettes were filled with equal volumes of water (approximately 1 ml). Ten (10) measurements were taken on each individual well after the instrument was heated, after 30 minutes, an hour and at the end of the workday (ten readings total).

As shown in Table 1, the instrument maintains a temperature of 37°C ± 0.4°C in each individual well. The instrument's acceptability limit is 37°C ± 1.

Table 1: Check of the temperature of the reading cells. Ten (10) measurements were taken for each individual well.

SD: Standard deviation from the mean

vc: (variation coefficient) is the ratio between standard deviation and the mean of the measurements.

READING CELLS	MEAN VALUES (n = 10)	SD	vc	MAX	MIN
1	37.4	0.3	0.9	37.9	36.8
2	37.3	0.4	1.2	37.8	36.4
3	37.5	0.3	0.9	38.0	36.9
4	37.5	0.2	0.6	37.8	37.1
5	37.5	0.3	0.9	37.9	36.9
6	37.4	0.6	1.6	36.3	37.9
7	37.4	0.4	1.1	37.9	36.8
8	37.6	0.3	0.9	38.0	37.1
9	37.6	0.2	0.6	37.9	37.2
10	37.4	0.2	0.7	37.8	37.1
11	37.4	0.6	1.6	37.9	36.6
12	37.5	0.5	1.3	37.9	37.6
13	37.9	0.3	0.8	38.3	37.3
14	37.7	0.6	1.6	38.4	36.8
15	37.8	0.4	0.9	38.2	37.2

3. REPEATABILITY TESTS

- 10 REPETITIONS ON THE SAME SAMPLE

Analyzing five milk samples, 10 tests were carried out on each sample in 10 different cuvettes.

The total standard deviation (repeatability index), computed from the total deviations from the means of all of the measurements obtained, is 0.67 mg/dl for the cow's milk, 0.74 mg/dl for the buffalo milk and 0.72 mg/dl for the sheep's milk.

Table 2a: Repeatability tests on five different samples of cow's milk. The values given are expressed in mg/dl.

Readings	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1	27.3	26.0	39.7	36.0	32.0
2	27.3	27.8	39.8	35.3	32.5
3	28.6	26.6	40.0	36.7	32.0
4	28.0	25.4	39.1	37.0	32.0
5	28.7	25.9	37.8	36.6	32.3
6	27.4	25.3	40.0	36.4	32.4
7	27.8	26.0	39.1	36.6	32.5
8	28.2	25.8	39.7	36.6	32.8
9	27.4	25.9	39.5	35.6	32.2
10	27.6	26.2	38.5	36.0	32.4
MEAN	**27.83**	**26.09**	**39.32**	**36.28**	**32.31**
SD ±	0.53	0.70	0.71	0.54	0.26
vc %	1.89	2.7	1.81	1.48	0.82
MIN	27.30	25.30	37.80	35.30	32.2
MAX	28.70	27.80	40.00	37.00	32.8

Table 2b: Repeatability tests on five different samples of buffalo milk. The standard deviation, derived from the deviations from the mean of all the values, is 0.74 mg/dl

Readings	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1	44.9	49.3	46.0	44.6	43.6
2	43.2	49.8	46.6	44.3	42.5
3	42.9	49.0	45.6	44.3	43.6
4	43.7	48.8	44.9	43.9	43.3
5	43.2	50.6	46.1	43.7	42.5
6	43.1	48.8	44.7	44.1	41.8
7	41.9	48.5	45.2	45.6	42.3
8	42.7	49.5	46.9	44.1	44.1
9	42.5	48.6	45.4	44.0	43.1
10	43.1	48.9	44.3	_	45.1
MEAN	**43.12**	**49.18**	**45.57**	**44.29**	**43.19**
SD ±	0.79	0.64	0.84	0.56	0.97
vc %	1.83	1.31	1.83	1.25	2.25
MIN	41.90	48.50	44.30	43.70	41.80
MAX	44.90	50.60	46.90	45.60	45.10

Table 2c: Repeatability tests on five different samples of sheep's milk. Samples 1 and 2 are mass samples; the others are single animal. The total standard deviation is 0.72 mg/dl, obtained from the deviations of all of the values.

Readings	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1	36.0	36.0	38.3	37.6	41.1
2	35.2	34.2	37.3	38.1	40.7
3	35.3	36.3	38.0	36.0	41.8
4	36.0	35.1	37.6	38.1	40.2
5	35.6	35.5	36.2	40.0	41.1
6	36.0	35.4	37.2	39.0	40.1
7	35.9	35.2	36.4	38.4	40.9
8	36.1	35.3	38.3	38.3	41.7
9	35.3	35.4	38.3	39.8	41.1
10	36.3	36.0	38.4	39.0	41.9
MEAN	**35.77**	**35.44**	**37.60**	**38.43**	**41.06**
SD ±	0.39	0.59	0.81	1.15	0.62
vc %	1.09	1.66	2.16	2.98	1.52
MIN	35.20	34.20	36.20	36.00	40.10
MAX	36.30	36.30	38.40	40.00	41.90

- 10 REPETITIONS OF READING OF THE SAME CUVETTE

After having added 5µl of milk, and after the 5 minutes of reaction had passed, 10 readings of buffer R1 of the same cuvette were carried out. The second reagent (R2) was subsequently added and, after the 3 minutes of reaction time had passed, the 10 readings of the sample were carried out. Thus it was possible to verify the instrument's reading stability.

The readings, carried out in triple testing, show that there is no variation on the instrument's part in the measurement of the samples. In fact, the excellent repeatability of the data is highlighted by a standard deviation of the deviations from the mean of all of the values of 0.1 mg/dl for the cow's milk, 0.4 mg/dl for the buffalo milk and 0.15 mg/dl for the sheep's milk.

Table 3a: Reading tests on the same cuvette of a single sample of cow's milk, tested three times (1st test, 2nd test and 3rd test) to verify the instrument's stability.

Readings	1st test	2nd test	3rd test
1	29.6	30.8	29.8
2	29.5	30.5	29.7
3	29.5	30.6	29.8
4	29.4	30.3	29.7
5	29.5	30.5	29.5
6	29.5	30.5	30.0
7	29.5	30.2	29.9
8	29.6	30.6	30.0
9	29.4	30.5	29.8
10	29.5	30.4	29.7
MEAN	**29.5**	**30.49**	**29.79**
SD ±	0.067	0.166	0.152
vc %	0.225	0.545	0.511
MIN	29.40	30.20	29.50
MAX	29.60	30.80	30.00

Table 3b: Reading tests on the same cuvette of a single sample of buffalo milk, tested three times (1st, 2nd and 3rd tests).

Readings	1st test	2nd test	3rd test
1	43.3	45.2	44.4
2	43.4	45.3	44.9
3	43.5	44.9	44.7
4	43.2	44.8	44.8
5	43.4	44.8	44.8
6	43.2	44.8	45.0
7	43.3	44.9	44.9
8	43.3	44.6	44.7
9	43.0	45.0	44.6
10	43.1	45.1	44.8
MEAN	**43.27**	**44.94**	**44.76**
SD ±	0.15	0.21	0.17
vc %	0.35	0.47	0.38
MIN	43.50	45.30	45.00
MAX	43.00	44.60	44.40

Table 3c: Reading tests on the same cuvette of a single sample of sheep's milk, tested three times (1st, 2nd and 3rd tests).

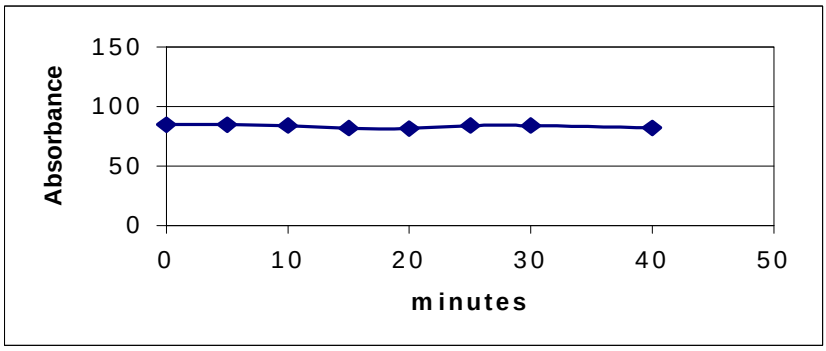
Readings	1st test	2nd test	3rd test
1	38.8	41.2	36.0
2	38.7	41.1	36.0
3	38.6	41.1	36.0
4	38.7	40.9	35.3
5	38.5	40.9	35.8
6	38.7	40.9	35.8
7	38.9	41.1	35.7
8	38.8	40.8	35.8
9	38.4	41.0	35.8
10	38.6	40.8	35.9
MEAN	**38.67**	**40.98**	**35.81**
SD ±	0.15	0.14	0.21
vc %	0.39	0.34	0.58
MIN	38.40	40.80	35.30
MAX	38.90	41.20	36.00

- VERIFICATION OF THE STABILITY TIME OF THE BUFFER AND OF THE COLOR

  The readings of the absorbance of buffer R1 (phenolic derivative that transforms urea into ammonia) were carried out from the moment in which the 5 µl of milk were added and after 5, 10, 15, 20, 25, 30 and 40 minutes.

Table 4: The mean values of the absorbance derived from 10 measurements, each with respect to the time expressed in minutes, are given.

MINUTES	ABSORBANCE n = 10
0	84.7
5	84.9
10	84.0
15	81.8
20	81.6
25	84.1
30	84.0
40	82.2



Graph 1: Progress of the buffer stability curve. The time expressed in minutes is given on the abscissas and the mean values of the absorbance on the ordinates.

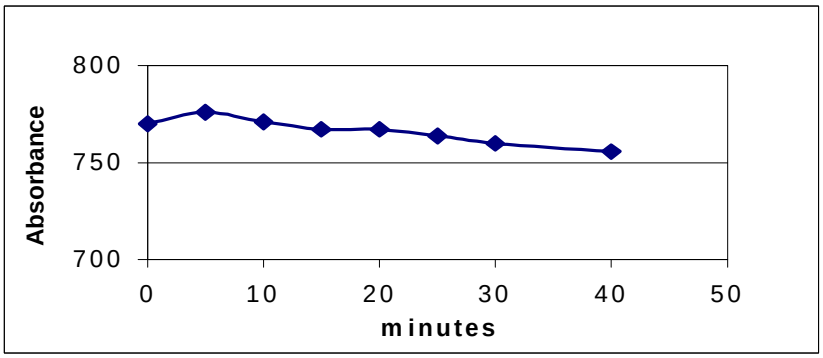
We can see from the graph that the curve remains constant from when the milk sample is added until 15 minutes later, after which the absorbance values begin to decrease slightly.

After having added the 5 µl of milk and having allowed the first 5-minute reaction, reagent R2 is added (alkaline solution that determines the colorimetric reaction) to the sample and we proceed directly to the reading of the color according to the previously described modalities. The zero time corresponds to the 3 minutes following the

addition of R2 (reaction time in which the ammonia reacts with the alkaline solution, forming a green complex).

Table 5: The mean values of the absorbance derived from 10 measurements each are given. The zero time corresponds to the end of the three-minute colorimetric reaction.

MINUTES	ABSORBANCE n = 10
0	770.1
5	775.9
10	771.0
15	767.2
20	767.2
25	763.8
30	760.0
40	755.7



Graph 2: Progress of the color stability curve. The zero time corresponds to the end of the colorimetric reaction. The mean values of the absorbance are given on the ordinates and the time expressed in minutes on the abscissas.

In this case the curve initially ascends and, after 20 minutes have passed, the slope begins to decrease.

However, the variation of the curve does not significantly influence the final result since the value of the concentration of urea is given by the difference between the color and the buffer, multiplied by coefficient K of the straight line and added to coefficient q (according to the equation y = Kx + q).

This test, for buffalo and sheep's milk, did not show differences from cow's milk in the progress of the curve. Obviously, the absorbance values are higher for both since the milk of these two species has much more fat than cow's milk.

- INFLUENCE OF THE SORTING

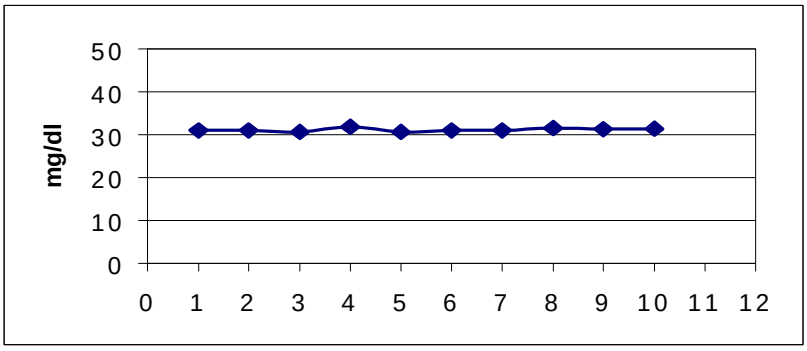
The milk sample was equally divided into 10 different Eppendorfs (a, b, c, d, e, f, g, h, i, l) and some random readings were carried out on the aliquots in double testing, to make sure that any lack of homogeneity of the milk does not influence the measurement by the instrument.

The results obtained showed a standard deviation from the mean of ± 0.50 μg/dl.

The mean values of the measurements and the mean, with the relative standard deviation, of the total readings are given in the table.

Table 6: Random readings of a single milk sample sorted in 10 Eppendorfs. The mean values are given for each aliquot. The mean and the relative standard deviation refer to the total readings.

RANDOM READINGS	MEAN VALUES (n = 2)
a	31.0
g	31.0
i	30.6
e	31.8
l	30.6
b	31.0
d	31.0
h	31.5
c	31.3
f	31.4
MEAN	**31.1**
SD	0.5
vc	1.5
MIN	30.6
MAX	31.8



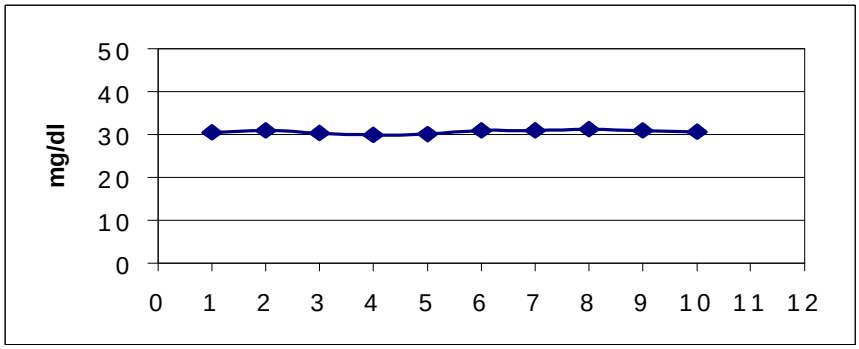
Graph 3: Progress of the sorting of a milk sample equally divided into 10 Eppendorfs. The values of the concentration of urea expressed in mg/dl are given on the ordinates and the 10 measurements on the abscissas.

The same milk sample was equally divided into five 100 ml test tubes (A, B, C, D and E) also, on which some random measurements were conducted in double testing.

The results obtained showed a standard deviation from the mean of 0.60 mg/dl.

Table 7: Random readings of a single milk sample sorted in five 100 ml test tubes. The mean values are given for each aliquot. The mean and the relative standard deviation refer to the total readings.

RANDOM READINGS	MEAN VALUES (n = 2)
A	30.5
E	30.9
C	30.3
D	29.9
B	30.1
C	30.9
B	30.9
C	31.2
E	30.9
D	30.6
MEAN	**30.6**
SD	0.5
vc	1.7
MIN	29.9
MAX	31.2



Graph 4: Progress of the sorting of a milk sample equally divided into five 100 ml test tubes. The values of the concentration of urea expressed in mg/dl are given on the ordinates and the 10 measurements on the abscissas.

- THE SAME SAMPLE TESTED BY DIFFERENT OPERATORS

  Two operators tested the same milk sample in order to determine how manual ability interferes in a test carried out by different operators.

Table 8: Two different operators tested the same milk sample. The concentrations are expressed in mg/dl.

Readings	Operator 1	Operator 2	Differences
1	21.6	20.9	0.7
2	20.6	21.4	0.8
3	21.0	21.0	0.0
4	20.9	21.0	0.1
5	21.1	21.9	0.8
Mean	**21.0**	**21.2**	**0.2**
SD ±	0.4	0.4	--
vc %	1.7	2.0	--
Min	20.6	20.9	--
Max	21.6	21.9	--

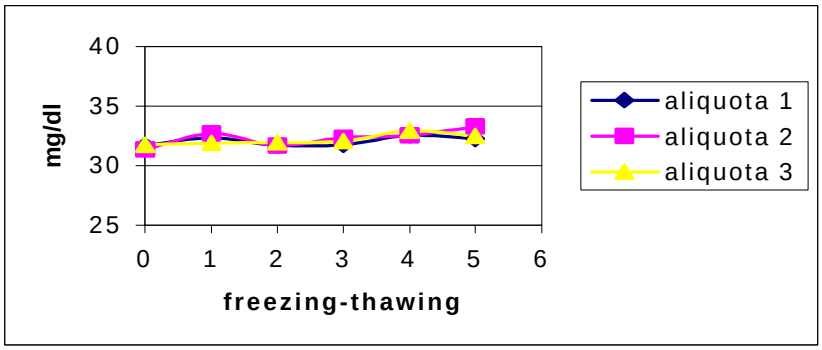
- DIFFERENCES BETWEEN FROZEN AND THAWED OUT SAMPLES

FREEZING IN LIQUID NITROGEN: the milk sample was tested at 37°C and subsequently frozen in liquid nitrogen (the freezing process was carried out as rapidly as possible). The sample was then thawed out in a bath thermostated at 40°C and tested. The entire freezing and thawing out process, carried out five times during the course of the day, was carried out on three samples, each read in double testing.

None of the samples shows important variations (the standard deviation from the mean is comparable to the standard deviation determined by the repeatability tests).

Table 8a: The mean values of each aliquot (1, 2 and 3) of cow's milk during the 5 procedures of freezing in liquid nitrogen and thawing out at 40°C are shown in the table.

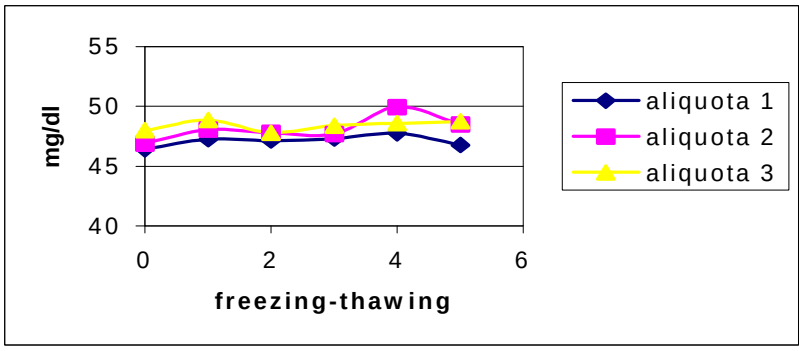
Freezing - thawing	**Aliquot 1 (n = 2)**	**Aliquot 2 (n = 2)**	**Aliquot 3 (n = 2)**
0	31.70	31.35	31.75
1	32.35	32.70	31.90
2	31.70	31.70	31.95
3	31.75	32.35	32.05
4	32.55	32.55	32.95
5	32.25	33.30	32.50
SD±	**0.77**	**0.83**	**0.52**
MIN	31.70	31.35	31.75
MAX	32.35	33.30	32.95



Graph 5a: We can see from the graph that there is no variation of concentration of urea expressed in mg/dl (shown on the ordinates) following the 5 processes of freezing in liquid nitrogen and thawing out at 40°C (on the abscissas).

Table 8b: The mean values of each aliquot (1, 2 and 3) of buffalo milk during the 5 processes of freezing in liquid nitrogen and thawing at 40°C are shown in the table.

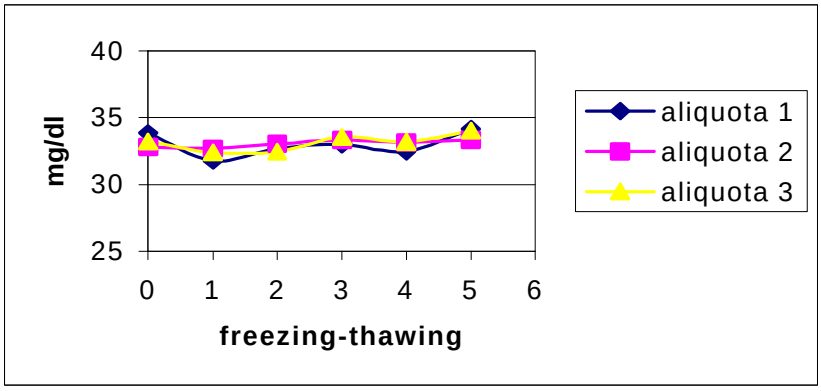
Freezing - thawing	Aliquot 1 (n = 2)	Aliquot 2 (n = 2)	Aliquot 3 (n = 2)
0	46.40	46.90	48.00
1	47.25	48.05	48.85
2	47.15	47.80	47.80
3	47.30	47.70	48.40
4	47.75	49.95	48.60
5	46.75	48.50	48.75
SD±	**0.47**	**1.02**	**0.42**
MIN	46.40	46.90	47.80
MAX	47.75	49.95	48.85



Graph 5b: No variations were detected for the buffalo milk either following freezing and thawing out of the three aliquots.

Table 8c: The mean values of each aliquot (1, 2 and 3) of sheep's milk during the 5 processes of freezing in liquid nitrogen and thawing out at 40°C are given in the table.

Freezing - thawing	Aliquot 1 (n = 2)	Aliquot 2 (n = 2)	Aliquot 3 (n = 2)
0	33.85	32.80	33.25
1	31.80	32.70	32.40
2	32.70	33.05	32.45
3	33.00	33.35	33.55
4	32.50	33.15	33.20
5	34.15	33.35	34.05
SD±	**0.87**	**0.27**	**0.64**
MIN	31.80	33.35	32.40
MAX	34.15	32.70	33.55

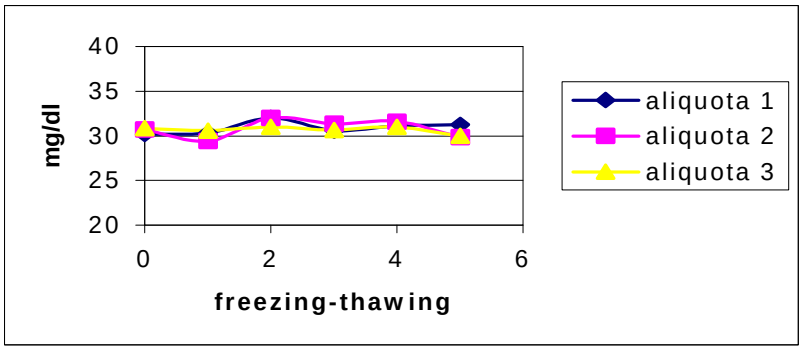


Graph 5c: The sheep's milk does not undergo variations due to the freezing and thawing out of the three aliquots.

FREEZING IN A DOMESTIC FREEZER: the freezing process was carried out in a normal domestic freezer also, always on a milk sample equally divided into 3 Eppendorfs analyzed in double reading. The thawing out was carried out in a bath thermostated at 40°C.

Table 9a: The mean values of each aliquot (1, 2 and 3) of cow's milk during the 5 processes of freezing in a domestic freezer are given in the table

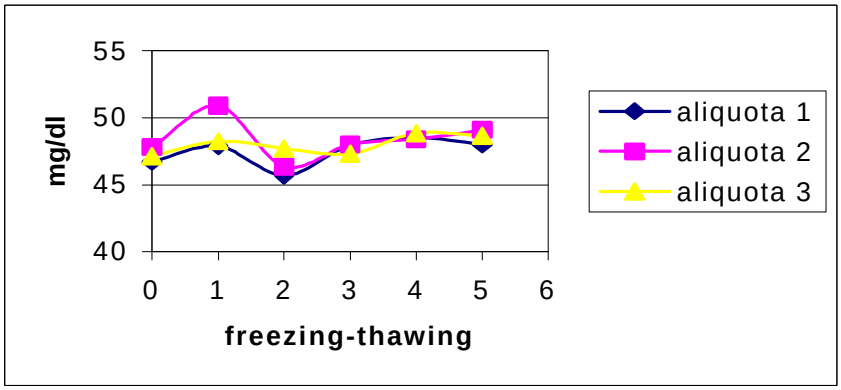
Freezing - thawing	Aliquot 1 (n = 2)	Aliquot 2 (n = 2)	Aliquot 3 (n = 2)
0	30.20	30.70	30.85
1	30.30	29.40	30.60
2	32.00	32.00	31.00
3	30.60	31.35	30.65
4	31.10	31.60	31.00
5	31.25	29.85	30.00
SD±	**0.80**	**1.03**	**0.47**
MIN	30.20	29.40	30.00
MAX	32.00	32.00	31.00



Graph 6a: Even the 5 freezing processes (on the abscissas) carried out in a domestic freezer do not involve variation of the concentration of urea (on the ordinates, expressed in mg/dl) on the cow's milk.

Table 9b: The mean values of each aliquot (1, 2 and 3) of buffalo milk during the 5 processes of freezing in a domestic freezer are shown in the table

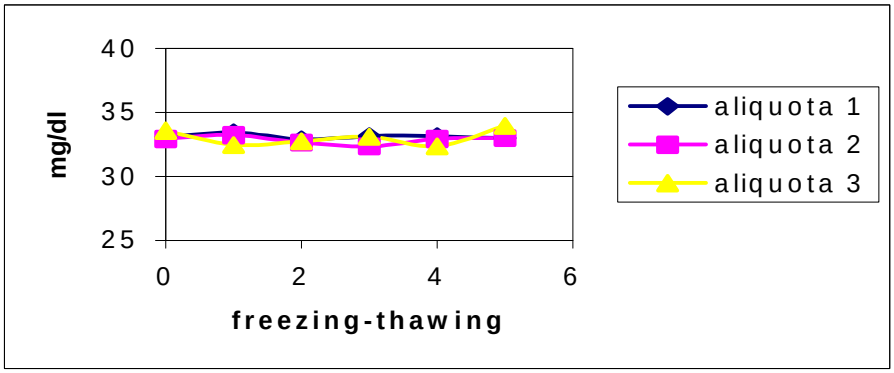
Freezing – thawing	Aliquot 1 (n = 2)	Aliquot 2 (n = 2)	Aliquot 3 (n = 2)
0	46.70	47.80	47.10
1	47.85	50.90	48.25
2	45.70	46.35	47.70
3	47.95	48.00	47.30
4	48.50	48.40	48.85
5	48.00	49.10	48.65
SD±	**1.04**	**1.51**	**0.72**
MIN	45.70	46.35	47.10
MAX	48.50	50.90	48.85



Graph 6b: The 5 freezing processes (on the abscissas) carried out in a domestic freezer do not involve variation of the concentration of urea (on the ordinates, expressed in mg/dl) on the buffalo milk either.

Table 9c: The mean values of each aliquot (1, 2 and 3) of sheep's milk during the 5 processes of freezing in a domestic freezer are shown in the table

Freezing - thawing	Aliquot 1 (n = 2)	Aliquot 2 (n = 2)	Aliquot 3 (n = 2)
0	33.05	32.95	33.60
1	33.45	33.25	32.45
2	32.90	32.65	32.75
3	33.15	32.35	33.10
4	33.15	32.95	32.35
5	33.00	33.00	33.95
SD±	**0.19**	**0.31**	**0.64**
MIN	32.90	32.35	32.35
MAX	33.45	33.25	33.95



Graph 6c: The 5 freezing processes (on the abscissas) carried out in a domestic freezer do not involve variation of the concentration of urea (on the ordinates, expressed in mg/dl) on the sheep's milk either.

- VERIFICATION OF POSSIBLE EVAPORATION OF THE AMMONIA IN MILK SAMPLES KEPT OPEN (WITH AND WITHOUT BRONOPOL)

In order to establish whether or not there may be ammonia evaporation from the milk (with consequent underestimation of the measurement of the concentration of urea), four milk samples were prepared: 0.02% Bronopol was added to two samples (A and B), while the other two (C and D) were kept without Bronopol.

A and C were maintained at 4°C, closed and tested, in triple reading, every day over the course of the week..

B and D were kept open (covered with gauze) at 4°C and tested, in triple reading, every day over the course of the week.

We can affirm that no ammonia evaporation occurred in the aliquots left open, given the considerable overlapping of the points on the respective curves.

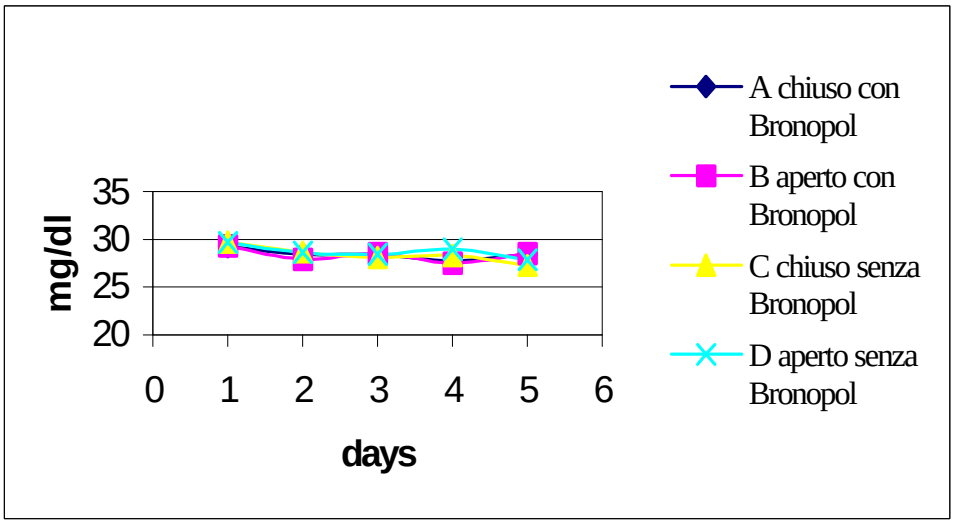
Table 10: A = milk sample with 0.02% Bronopol kept closed for 5 days at 4°C

B = milk sample with 0.02% Bronopol kept open for 5 days at 4°C

C = milk sample without 0.02% Bronopol kept closed for 5 days at 4°C

D = milk sample without 0.02% Bronopol kept open for 5 days at 4°C

	1st DAY	2nd DAY	3rd DAY	4th DAY	5th DAY	MIN	MAX
A(n=3)	**29.20**	**27.83**	**28.43**	**27.73**	**28.40**	**27.73**	**29.20**
SD_A	0.10	0.55	0.15	0.40	0.50		
B(n=3)	**29.30**	**27.93**	**28.50**	**27.50**	**28.53**	**27.50**	**29.30**
SD_B	0.45	0.57	0.53	0.75	0.25		
C(n=3)	**29.73**	**28.67**	**28.10**	**28.33**	**27.27**	**27.27**	**29.73**
SD_C	0.32	0.32	0.35	0.76	0.15		
D(n=3)	**29.73**	**28.63**	**28.40**	**28.97**	**27.87**	**27.87**	**29.73**
SD_D	0.32	0.06	0.46	0.15	0.25		



Graph 7: Comparison between samples A (blue curve), B (pink curve), C (yellow curve) and D (sky-blue curve).

- INTERMEDIATE REPEATABILITY

The same sample sorted in different aliquots and kept at 4°C ± 2°C, was tested daily (this test was associated with the preceding “ammonia evaporation test”).

It is evident from Table 10 that no variations in the concentration of urea are found over the course of the week.

4. INSTRUMENT CALIBRATION

The instrument is calibrated with three different reference materials that fall in the concentration range of approx. 15-27-40 mg/dl. These reference materials were obtained by testing milk samples 10 times with the differential pH-metry instrument, EFA Instrument, Hamilton-Eurochem.

The instrument is aligned on a straight line having equation $y = Kx + q$ (passing through the three points corresponding to the three values of the reference materials), where K and q are the coefficients of the straight line and, specifically, K = slope and q = bias.

- After the instrument has memorized coefficients K and q following the standardization, the 3 reference materials were tested as simple samples, and the respective values were compared to those known, to verify the curve's linearity and stability.

Table 11: Variation of coefficients K and q in the six calibrations carried out with the reference materials

r^2 linear correlation coefficient

STD known reference material

ANL reference material analyzed after the calibration

Calibration date	**K**	**q**	**r^2**	**STD**	**ANL**	**Diff**	**SD± of the deviations**
07/24/01	44.36	-0.44	0.99	• 19.6 • 24.4 • 40.8	• 19.6 • 24.8 • 39.2	• 0.0 • 0.4 • 1.6	**0.83**
07/25/01	44.31	-0.36	0.99	• 19.6 • 24.4 • 40.8	• 18.6 • 24.7 • 39.7	• 1.0 • 0.3 • 1.1	**0.43**
07/27/01	44.76	-0.77	0.99	• 19.6 • 24.4 • 40.8	• 19.6 • 25.6 • 41.3	• 0.0 • 1.2 • 0.5	**0.60**
07/30/01	47.85	-1.52	0.99	• 19.8 • 25.5 • 44.5	• 20.6 • 26.0 • 43.1	• 0.8 • 0.5 • 1.4	**0.46**
08/06/01	50.13	-4.57	0.99	• 19.8 • 25.5 • 44.5	• 19.2 • 25.9 • 45.5	• 0.6 • 0.4 • 1.0	**0.30**
08/20/01	43.24	-1.1	0.99	• 19.8 • 25.5 • 44.5	• 20.1 • 26.3 • 44.9	• 0.3 • 0.8 • 0.4	**0.26**

- The instrument was calibrated and the calibration was not modified for an entire week during which the three reference materials of known concentration were tested daily:

  STD 1: 19.8 mg/dl

  STD 2: 25.5 mg/dl

  STD 3: 44.5 mg/dl

  K=41.27 ; q= -0.95

Table 12 : Reading of the 3 reference materials keeping fixed coefficients K = 41.27 and q = -0.95

STD 1: 19.8 mg/dl

STD 2: 25.5 mg/dl

STD 3: 44.5 mg/dl

Day	SAMPLE 1	SAMPLE 2	SAMPLE 3
I	19.5	25.1	43.5
II	20.3	25.2	43.1
III	20.6	24.4	43.8
IV	20.3	25.2	43.5
V	19.9	24.8	43.0

- After having memorized coefficients K and q, the three reference samples of known concentration were frozen:

  STD1:19.8 mg/dl;

  STD2: 25.5 mg/dl;

  STD3: 44.5 mg/dl.

  After approx. one month, the previously obtained values of K and q were reset and the three reference materials relative to the corresponding calibration were tested, verifying in this way the stability of the instrument’s calibration over time.

Table 13: The three reference materials were tested again, inserting the relative coefficients K and q.

Known sample 1: 19.8 mg/dl

Known sample 2: 25.5 mg/dl

Known sample 3: 44.5 mg/dl

K and q	Sample 1	Sample 2	Sample 3
K 44.95 **Q –1.42**	20.75	26.55	48.00
K 49.32 **Q –1.66**	20.75	27.25	46.75
K 42.54 **q 0.14**	19.55	24.50	42.20
SD±	0.57	1.42	3.05

5. LINEARITY

A milk sample was tested and once the concentration of urea was established, it was diluted with distilled water in order to obtain a final limit concentration of urea. Subsequently some fat was added in order to restore the initial matrix.

The latter sample was considered the “diluent”. Mgs of urea were added, per weighing, to part of the “diluent” in order to obtain a final maximum concentration (approximately 100 mg/dl of urea).

Then a series of dilutions were carried out between the concentrated sample and the diluent.

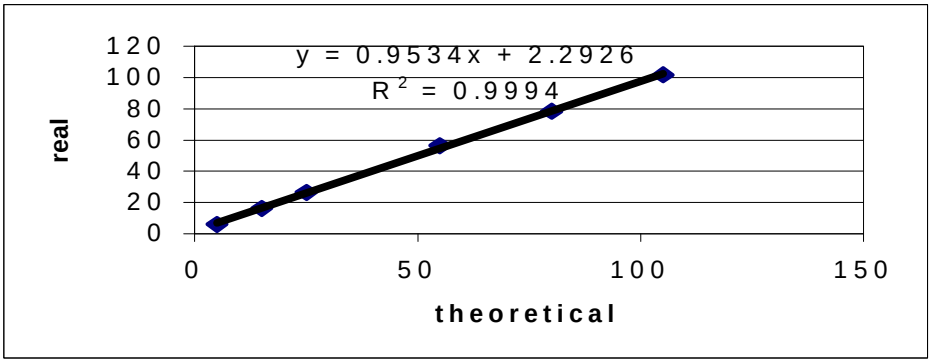
The results obtained were put displayed graphically with the theoretical ones and the regression line, indicator of the instrument’s linearity, was computed.

Table 14a: The sample of cow's milk was diluted in order to obtain a limit concentration of 5 mg/dl of urea. The concentrated sample was obtained by adding 100 mg/dl of urea per weighing.

The dilutions were carried out according to the following plan:

1. 3 parts of the concentrated sample + 1 part of the diluent sample (concentration of urea 80 mg/dl)
2. 1 part of the concentrated sample + 1 part of the diluent sample (concentration of urea 55 mg/dl
3. 1 part of the concentrated sample + 4 parts of the diluent sample (concentration of urea 25 mg/dl
4. 1 part of the concentrated sample + 9 parts of the diluent sample (concentration of urea 15 mg/dl

THEORETICAL mg/dl	REAL mg/dl (n=3)
5	6.13
15	16.37
25	26.6
55	56.33
80	78.37
105	101.67



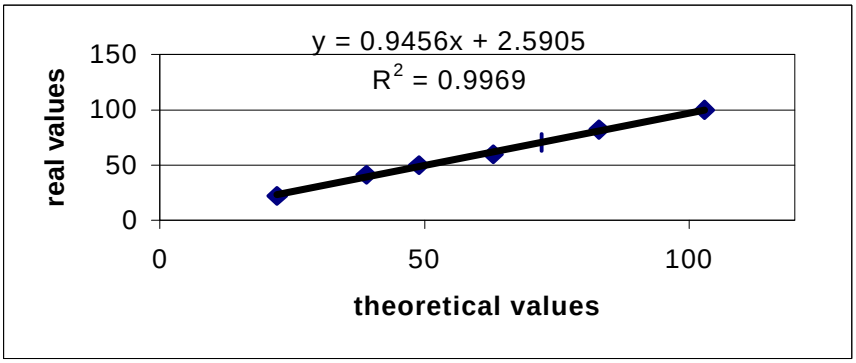
Graph 8a: The theoretical values are shown on the ordinates and the real values obtained on the abscissas. The equation of the regression line that is derived from it is shown on the graph itself.

Table 14b: The sample of buffalo milk was diluted in order to obtain a limit concentration of 22.15 mg/dl of urea. The concentrated sample was obtained by adding 80 mg/dl of urea per weighing.

The dilutions were carried out according to the following plan:

5. 200µl of the concentrated sample + 800µl of the diluent sample (concentration of urea 39 mg/dl)
6. 400µl of the concentrated sample + 800µl of the diluent sample (concentration of urea 49 mg/dl)
7. 500µl of the concentrated sample + 500µl of the diluent sample (concentration of urea 63 mg/dl)
8. 800µl of the concentrated sample + 500µl of the diluent sample (concentration of urea 72 mg/dl)
9. 750µl of the concentrated sample + 250µl of the diluent sample (concentration of urea 83 mg/dl)

THEORETICAL mg/dl	REAL mg/dl (n=3)
22.15	22.15
39	41.40
49	50.15
63	60.05
72	70.35
83	81.95
103	99.80

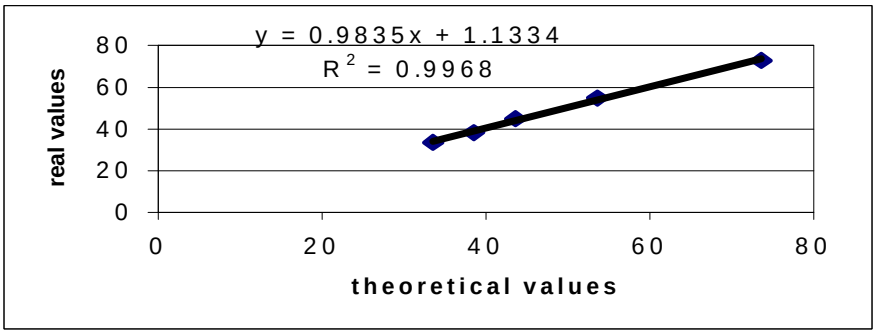


Graph 8b: Linearity for buffalo milk

A recovery-linearity test was carried out for the sheep's milk since difficulties were encountered in the dilutions with the matrix of this species' milk.

Table 14c: The linearity, for sheep's milk, is seen from the comparison between the theoretical values obtained for urea added per weighing and the results actually obtained.

mg/dl urea	Real (n=2)	Theoretical	Differences
0	33.57	33.57	0.0
5	38.57	38.37	0.2
5	43.57	44.87	1.3
10	53.57	54.87	1.3
20	73.57	72.83	0.74



Graph 8c: Linearity tests for sheep's milk obtained by adding urea (recovery)

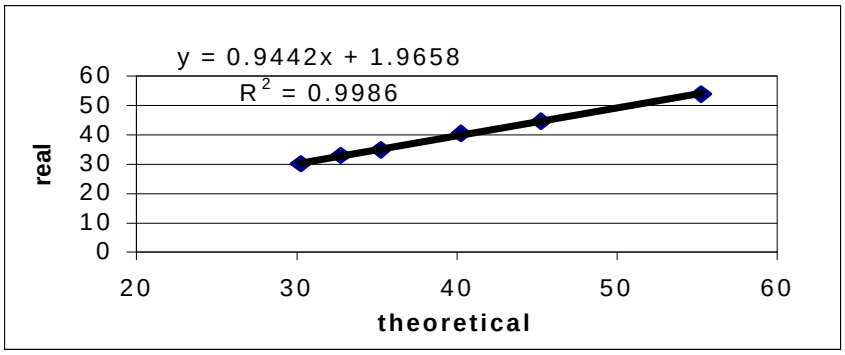
6. SENSITIVITY AND RECOVERY

The recovery is the instrument's capability to measure exactly the amount of urea that is added per weighing.

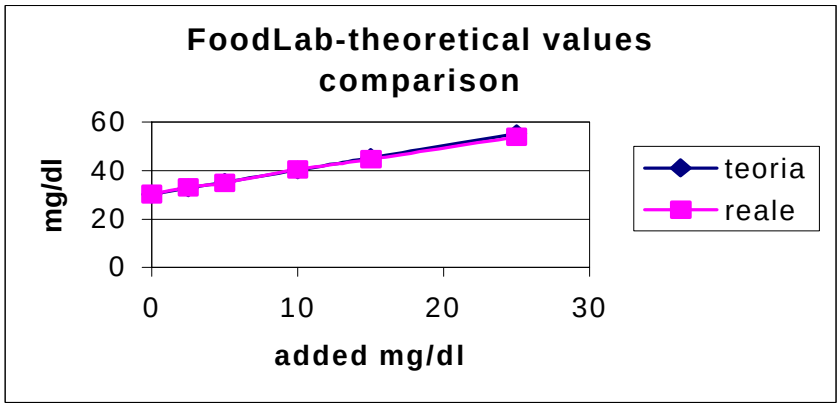
The instrument, besides recording an almost total recovery, was capable of distinguishing up to 2.5 mg/dl of added urea (sensitivity) for the cow's milk.

Table 15: Mgs/dl of urea per weighing were added sequentially.

mg/dl urea	Real (n=2)	Theoretical	Differences
	30.25	30.25	0.00
2.5	33.10	32.75	0.35
2.5	34.95	35.25	0.30
5	40.50	40.25	0.25
5	44.70	45.25	0.55
10	53.95	55.25	1.30



Graph 9a: The theoretical values are shown on the abscissas and the real values obtained on the ordinates. The straight line, whose equation is shown in the graph, is the linearity index.



Graph 9b: Comparison between the real measurements and the theoretical ones. The mgs/dl of urea added are shown in abscissa.

Table 15b: Sensitivity and recovery tests for buffalo milk

mg/dl urea	Real (n=2)	Theoretical	Differences
0	48.65	48.65	0.00
2.5	51.15	50.20	0.95
5	56.15	56.20	0.05
5	61.15	59.50	1.65
10	71.15	69.80	1.35
20	91.15	84.70	6.45

Graph 9c: Progress of the linearity for buffalo milk. The regression line equation is shown on the graph

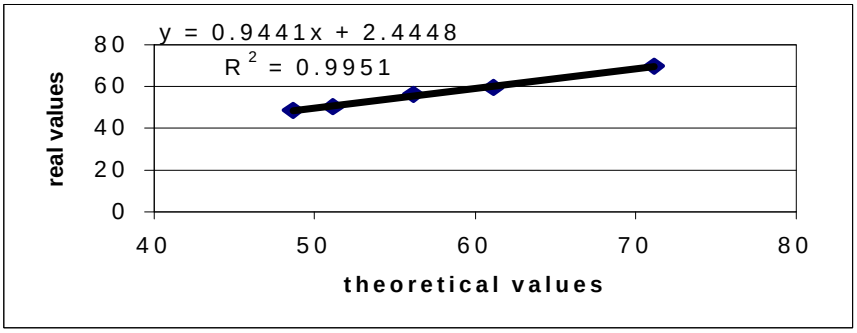
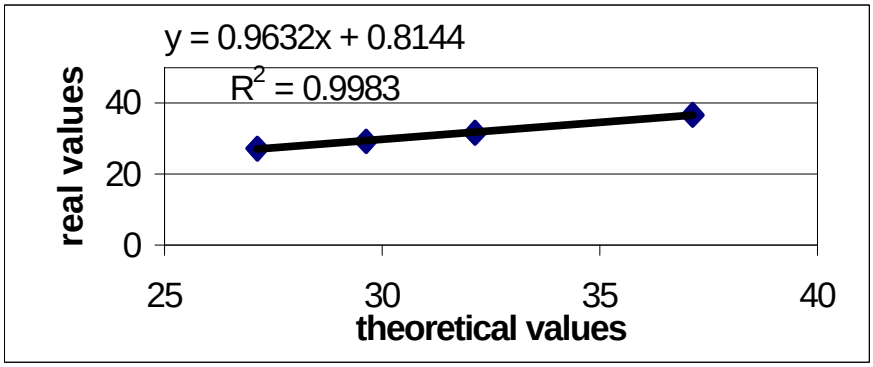


Table 15c: Sensitivity tests for sheep's milk

mg/dl urea	Real (n=2)	Theoretical	Differences
0	27.13	27.13	0.0
2.5	29.63	29.17	0.0
2.5	32.13	31.67	1.3
5	37.13	36.67	0.6



Graph 9d: Progress of the regression line, which is linear, for sheep's milk, following the addition of mgs/dl of urea. The equation of the straight line is shown on the graph.

7. VARIATION OF THE LOTS

Possible differences between blisters of different lots were verified, testing the same milk sample, in 10 readings for each lot, to make sure that any production defect does not influence the measurements.

Table 16: The mean values of the 10 readings are shown in the table, with the relative standard deviations, of each sample for each lot, and the differences between them.

Sample	Lot 0107_b	Lot 0108	Differences between lots
1 (n=10)	**18.49**	**18.90**	0.41
SD	0.27	0.31	_
2 (n=10)	**24.06**	**24.66**	0.60
SD	0.37	0.46	_
3 (n=10)	**38.62**	**39.32**	0.70
SD	0.44	0.42	_

8. VERIFICATION OF THE INTERFERENCE OF THE SODIUM AZIDE USED AS PRESERVATIVE IN ASSOCIATION WITH THE BRONOPOL

Two different milk samples were tested in triple reading both with sodium azide and without sodium azide. The differences between the samples treated in the two different ways are of no importance.

Table 17: The mean values of the three measurements of two different milk samples, each of which was subdivided into a sub-sample treated with sodium azide (SA) and in a sub-sample without sodium azide (No SA) are shown in the table.

Samples	No SA	SA	Diff.
1 (n =3)	26.70	25.93	-0.77
2 (n =3)	30.20	30.27	0.07

9. DILUTION TESTS

Some dilution tests were also carried out to verify the influence of the milk fat on the instrument.

The milk sample was diluted by 50% with distilled water and, after having carried out 10 measurements, the fat was replaced to restore the initial matrix. The measurements were carried out both on 5µl and on 10µl.

Table 18a: Dilution tests to verify the possible influence of the fat in the cow's milk.

Readings	Fat removed	Diluted 50%	10µl diluted	Fat replaced	10µl fat replaced
1	23.8	13.6	23.7	13.6	24.4
2	23.9	14.0	23.7	14.3	24.7
3	25.1	13.2	23.8	13.8	24.2
4	24.5	14.8	24.3	13.8	24.2
5	24.2	14.1	23.6	14.1	25.1
6	24.2	14.0	24.2	14.5	25.6
7	23.8	13.6	23.8	13.9	24.8
8	24.2	13.9	24.2	14.1	25.2
9	24.2	14.1	24.2	14.2	24.8
10	24.1	14.1	23.8	14.3	24.6
MEAN	**24.2**	**13.9**	**23.9**	**14.1**	**24.8**
SD	0.4	0.4	0.3	0.3	0.4
vc	1.6	3.0	1.1	2.0	1.8
MIN	23.8	13.2	23.6	13.6	24.2
MAX	25.1	14.8	24.3	14.5	25.6

Table 18b: Dilution tests for buffalo milk. Some variances are obvious from the table for the milk with the fat replaced, probably due to a matrix effect.

Readings	Fat removed	Diluted 50%	10µl diluted	Fat replaced	10µl fat replaced
1	51.1	28.1	51.3	31.5	61.6
2	51.6	27.8	50.6	31.9	64.1
3	52.7	27.4	51.3	31.6	62.9
4	54.8	27.7	51.1	31.8	63.2
5	50.1	27.8	53.2	31.9	62.9
6	53.2	28.0	51.2	31.7	61.4
7	49.8	27.7	51.9	32.0	62.9
8	52.5	27.7	51.5	31.6	62.7
9	50.8	28.0	51.1	30.3	61.1
10	51.3	27.8	50.9	32.1	60.6
MEAN	**51.8**	**27.8**	**51.4**	**31.6**	**62.3**
SD	1.5	0.2	0.7	0.5	1.1
vc	2.9	0.7	1.4	1.6	1.8
MIN	49.8	27.4	50.6	30.3	60.6
MAX	54.8	28.1	53.2	32.1	64.1

Table 18c: Dilution tests for sheep's milk. In this case also some conflicting values are found for the sample with the fat replaced due to a matrix effect.

Readings	Fat removed	Diluted 50%	10µl diluted	Fat replaced	10µl fat replaced
1	36.7	20.3	37.4	21.2	41.4
2	35.8	19.6	36.1	20.8	41.5
3	36.0	19.6	36.4	21.0	41.5
4	36.5	19.8	--	21.7	40.8
5	36.2	19.3	36.7	22.6	39.6
MEAN	**36.2**	**19.7**	**36.7**	**21.5**	**41.0**
SD	0.4	0.4	0.6	0.7	0.8
vc	1.0	1.9	1.5	3.4	2.0
MIN	35.8	19.3	36.1	20.8	39.6
MAX	36.7	20.3	37.4	22.6	41.5

CONCLUSIONS

Tests were carried out on the FoodLab instrument during the period July – October 2001 for a total of 2400 assessments.

The following conclusions can be established based on the results obtained:

- It is possible to determine the concentration of urea using the FoodLab system without preventive treatment of the sample.
- Total reading cells: 15 (1 for reading and 14 for incubation)
- The thermostating of the 15 reading cells (37°C ± 0.4°C) was homogeneous and the differences recorded are fully within the stated tolerance.
- The nominal speed is 60 tests/h.
- The true speed, measured during the normal work routine, is 48 tests/h.
- The tests were carried out on cow's milk, buffalo milk and sheep's milk.

INTERFERENCES

- The milk frozen in liquid nitrogen and thawed out in a bath thermostated at 40°C shows no variation of the concentration of urea.
- The milk frozen in a domestic freezer and thawed out in a bath thermostated at 40°C shows no variation of the concentration of urea.
- The presence of 0.02% technical Bronopol does not cause any interference.
- The association of sodium azide with the Bronopol does not cause variations of the concentration of urea.
- The sorting of the milk, both in 1.5 ml Eppendorfs and in 100 ml test tubes, does not cause lack of homogeneity in the sample.
- The influence of the manual ability of the operator was not found.

REPEATABILITY

- The repeatability is on the whole good. The total standard deviation computed from the deviations from the means of each measurement is 0.67 mg/dl for cow's milk, 0.74 mg/dl for buffalo milk and 0.72 mg/dl for sheep's milk.

- The reading repeatability of each single test tube is excellent and has been expressed by the standard deviation of the deviations from the mean of all of the values and is equal to 0.1 mg/dl for cow's milk, 0.4 mg/dl for buffalo milk and 0.15 mg/dl for sheep's milk.
- The intermediate repeatability (testing of the same sample repeated for several days) shows no variation.

RECOVERY

- Recovery is almost total for all three species.

SENSITIVITY

- The instrument is capable of distinguishing up to 2.5 mg/dl

CALIBRATION AND STABILITY

- The instrument is calibrated with three different reference materials that fall within the concentration range of 15-27-40 mg/dl. The instrument is thus aligned on a straight line having the equation $y = Kx + q$ (passing through the three points corresponding to the three values of the reference materials), where K and q are the coefficients of the straight line and, specifically, K = slope and q = bias.
- The calibration remains stable and aligned over time.
- The measurement of the instrument is perfectly aligned with the calibration curve, which always shows an r^2 >0,99.

CARRY OVER

- No carry over effect is found because the samples are inoculated in individual cuvettes and read separately.

MALFUNCTIONS

The instrument showed no operation problems of any type for the entire testing period.

MAINTENANCE

The instrument requires no maintenance.