

<b>Guarantee/quality Control Milk and Foodstuffs Division</b>		<b>TITLE: CDR FoodLab Validation Test for the Determination of Lactic Acid (L) in Whole Milk and Skim Milk</b>
<b>ANALYSIS METHODS</b>	<b>CODE/file name</b>	Lactic.doc
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## THE PRINCIPLE OF THE CDR INSTRUMENT

In the presence of oxygen, lactic acid is transformed from lactate oxidase enzyme into pyruvic acid. The pyruvic acid obtained reacts with a phenolic derivative in the presence of the enzyme peroxidase to produce Chinoneimina (violet compound), the intensity of which, measured at 545 nm is directly proportional to the concentration of lactic acid present in the sample.

## THE PROCESS

The reaction consists in a first phase of pre-heating of the **R1** reagent with the sample (100 µl) with the addition of **R1<sub>a</sub>** reagent (1 drop) for five minutes and a second phase where the reaction goes to end-point in two minutes from the addition of the **R2** reagent (1 drop). The spectrophotometric reading is carried out on the white (colour) at the end of the first phase. The final spectrophotometric reading is successively effectuated at the end of the second phase.

**Table 1.** Reagents utilised in the analysis of lactic acid in samples of milk.

Reagent	Component
R1 (in cuvette)	Phenolic derivative Phosphate buffer
R1 <sub>a</sub>	Enzyme
R2	Lactate oxidase Peroxidase

The system utilises samples of non pre-treated milk and disposable micro-cuvettes containing the **R1** reagent.

*NB: agitate thoroughly after each addition of sample and reagent.*

## FUNCTIONAL TESTS CARRIED OUT

The tests were carried out both on samples of whole milk and on samples of skim milk.

The following parameters were checked:

- Homogeneity of the variance
- Linearity
- Analysis of the variance (Anova)
- Precision (inter-day repeatability)

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## REFERENCE MEASUREMENT

A specific enzymatic test for the analysis of lactic acid in multiple foodstuff products, produced by Boehringer Mannheim was used to obtain a reference measurement of the lactic acid. This test is based on the oxidation of L-lactic acid (the only one of two isomers that are available naturally) into pyruvate by the Nicotinamide Adenine Dinucleotide (NAD<sup>+</sup>) in the presence of L-lactate dehydrogenase (L-LDH):

The pyruvate that originates reacts with the L-glutamate in the presence of the glutamate-pyruvate transaminase (GTP) enzyme that gives origin to L-alanine and 2-oxoglutarate.

The measurement is effectuated by a spectrophotometer with a wavelength of 340 nm.

This analysis requires a laborious preparation of the sample:

- The addition of 10 ml of milk sample to 20 ml of distilled water.
- The addition of 5 ml of potassium hexacyanoferrate(II) trihydrate solution (Carrez 1)\*.
- The addition of 5 ml of heptahydrated zinc sulphate (Carrez II)\*\*.
- The addition of 10 ml of soda (0.1N).
- The addition of 100 ml of distilled water.
- Leave the sample to rest for 30 minutes.
- Filtration via a paper filter. Elimination of the first fraction and collection of the following fraction.

\* Carrez = 3.60g of potassium hexacyanoferrate(II) trihydrate in 100 ml of distilled water.

\*\* Carrez II = 7.20g of heptahydrated zinc sulphate in 100 ml of distilled water.

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- Preparation of the cuvettes for spectrophotometric reading:

Reagent	White (ml)	Sample (ml)
L-glutamic acid (Solution 1)	1000	1000
NAD (Solution 2)	0.200	0.200
GTP (Suspension 3)	0.020	0.020
Sample solution	-	1000
Distilled water	1000	-
L-LDH (Solution 4)	0.020	0.020

- Delicately agitate the cuvettes after being sealed with Parafilm.

The first reading is made five minutes after the addition of the solutions 1, 2, 3 and the sample, whilst the final spectrophotometric reading is made after 35 minutes from the addition of solution 4. To pass from the spectrophotometric reading to the concentration of lactic acid in samples of milk, it is necessary to utilise the following formula:

$$c = \left\{ \left[ \frac{(V * MW)}{(\epsilon * d * v * 1000)} \right] * \ddot{A}A \right\}$$

where V is the final volume [ml], v represents the volume of the sample [ml], MW is the molecular weight of the lactic acid [ $g * mol^{-1}$ ], d is the length of the cell (cms) and  $\epsilon$  represents the coefficient of molar extinction [ $l * mmol^{-1} * cm^{-1}$ ]. The concentration obtained is expressed in g of L-lactic/l acid of sample solution.

From which

$$c = \left\{ \left[ \frac{(2,240 * 90.1)}{(6.3 * 1 * 1 * 1000)} \right] * \ddot{A}A \right\}$$

From the moment that the final datum comes in g/l, a conversion becomes necessary into mg/l (ppm) with object of supplying a homogenous result to that supplied by the CDR instrument:

$$C_{final} = c * 1000$$

The corresponding value to  $C_{final}$  is the concentration in ppm of lactic acid present in the sample of milk.

A preliminary study was effectuated to establish the time necessary to bring to end-point the enzymatic reaction that brought about the registration of the absorbance variation in respect of the reaction time.

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## CALIBRATION OF THE INSTRUMENT

The instrument was calibrated via repeated analyses on five different levels of concentration covering a range of 12.16 ppm to 50.56 ppm inclusive.

The reference standards were obtained by additions of a standard solution of lactic acid of known concentration of analyta, obtained to start from a base solution at 5000 ppm to the sample of milk.

The measurements of these reference standards were effectuated via the utilisation of a specific enzymatic test produced by Boehringer Mannheim (the UV-visible spectrophotometer utilised for measuring was the JASCO Model 7800).

The calibration straight line for the sample of milk is shown below.

The straight lines obtained were evaluated also in relation to the errors associated to each one of the coefficients, intercepted and inclination, as well as the significance of the intercept (via an e-test).

$$Y = 53.737 (\pm 0.001) X + 5.6820 (\pm 0.0003)$$

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It is advisable to repeat the calibration each time the production lot of cuvette tests and the R2 reagent are changed.

## HOMOGENEITY OF THE VARIANCE

Before checking that the experimental points obtained are interpolated linearly, it is necessary to carry out a homogeneity test of the variance. One of the homogeneity of the variance that is not homogenous could, apart from being completely imprecise, create inaccuracy caused by the possible change of the inclination of the curve and therefore the sensitivity. Via the minimum variance test, it is possible to compare the relative variance at all points.

As can be observed in Table 2, the value of the Fcalc is superior to the value of the Ftab. Therefore, the homogeneity of the variances is satisfactory.

**Table 2.** Homogeneity of the variance

Fcalc	Ftab <sup>a</sup>
0.000165	0.00000160
<sup>a</sup> Significance = 5% degrees of freedom = 1.4	

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A preliminary study was also conducted on the linearity: particular importance was attributed to the analysis of residues. By residues it is meant the difference between the true value and the estimated value via the model. If the model is adequate, the residues must derive exclusively from the experimental error. Therefore it is necessary to wait for the said residues to be distributed normally around zero. Anomalous residues signify that there is probably a system error and therefore the model is not adequate. In particular, when the distribution of the residues assume a parabolic progression, the model is probably missing a quadratic term.

Finally, the Mandel mathematic test was used for the statistical checks of the linearity. For this reason, calibration functions of the first order were used ( $y = b_0 + b_1x$ ) and the second order ( $y = b_0 + b_1x + b_2x^2$ ), including the corresponding residual variances ( $S_y$ ). This test foresees the use of an F-test where, if the value of F is less or equal to that tabulated, the calibration function of the second order do not supply a model significantly better than the linear model and therefore the calibration function is linear. In the case of levels superior to those tabulated, the function of the second order is that which best interpolates the points.

Comparing the calculated results ( $F_{calc}$ ) with those obtained from the table ( $F_{tab}$ ) for the data of the calibration obtained via the use of the CDR spectrophotometric technique (Table 3), it is possible to affirm that the calibration function of the second order does not supply a significantly better model of a linear function (with  $\bar{U} = 5\%$ ).

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**Table 3.** Mandel test for the analysis conducted on samples of milk.

Fcalc	0.1495
Ftab <sup>a</sup>	4.75
Ftab <sup>a</sup>	9.33
<sup>a</sup> Significance= 5% degrees of freedom – 1.12	
<sup>a</sup> Significance = 1% degrees of freedom – 1.12	

### THE VARIANCE ANALYSIS (ANOVA)

The study conducted on the merits of adaptation of the linear model to the data relative to the sample of whole milk revealed how this is sufficiently adequate to describe the relationship between X and Y ( = 1%). This result is in line with the results obtained by the analysis of the residues and by the check on the linearity of the model (Mandel test).

**Table 4.** Analysis of the variance

Flof	2.9496
Ftab <sup>a</sup>	4.75
Ftab <sup>a</sup>	9.33
<sup>a</sup> Significance= 5% degrees of freedom – 1.12	
<sup>a</sup> Significance= 1% degrees of freedom – 1.12	

### PRECISION

The precision was evaluated in terms of inter-day repeatability on five levels of concentration and carrying out three repeats for each level. A 12.16ppm – 50.56ppm interval of concentration was taken on the milk under examination. The parameters evaluated were: the variance of the repeatability, standard deviation of the repeatability, repeatability, the variation coefficient (VC%) and the confidence interval (CI).

It can be observed from Table 6 how the variation coefficient value remains sufficiently low for all levels of concentration under consideration.

**Table 5.** Inter-day repeatability

Level (ppm)	Variance of the repeatability	Standard deviation of repeatability	Repeatability (a = 5%)	Repeatability (a = 1%)
12.16	3.03333E-05	0.0055	0.03358	0.0773
22.08	0.001046333	0.0323	0.1968	0.4540
31.68	0.000726333	0.0269	0.1640	0.3783
44.16	0.000566333	0.0238	0.1448	0.3340
50.56	0.001906333	0.0436	0.2657	0.6128

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**Table 6** Inter-day repeatability

Level (ppm)	VC% standard deviation repeatability	Maximum CI	Minimum CI
12.16	5.1154	0.12	0.09
22.08	10.2149	0.39	0.23
31.68	5.1961	0.58	0.45
44.16	3.4775	0.74	0.62
50.56	5.2394	0.94	0.72

<sup>a</sup>The VC% was calculated with an  $\alpha = 5\%$ .

### EVALUATION OF THE REPEATABILITY OF THE TWO TECHNIQUES

Repeated tests were made on the entire procedure of preparation of the sample and the spectrophotometric reading for both techniques. As can be observed in Table 7 and 8, the analysis effectuated via the Boehringer Mannheim enzymatic kit brought about a greater variability in the results certainly caused by the various handling procedures to which the sample underwent.

**Table 7.** Readings effectuated on one sample treated by both techniques.

CDR reading	Jasco spectrophotometer reading
13.26	10.24
14.33	10.24
14.01	10.88
13.74	11.85
14.71	11.21
14.06	13.45

**Table 8.** Repeatability of the two techniques

	CDR spectrophotometer	Jasco spectrophotometer
Average	14.02	11.31
Standard deviation	0.50	1.21
VC (%)	3.54	10.72

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The value of 13.45 ppm noted in Table 7 was considered a suspect outlier and therefore underwent the Dixon test. On the basis of the test, the datum does not appear to be anomalous with a level of significance of 95% and it was therefore included in the statistical evaluation.

## 11 – NOTE

A comparison was effectuated between the analysis conducted using the Boehringer Mannheim enzymatic kit with the relative spectrophotometric analysis and the CDR instrument used for analysis of lactic acid in samples of milk. In particular, the costs of each analysis were evaluated and also the time necessary to effectuate the said analysis.

**Table 9.** Cost of components necessary for the analysis of lactic acid in milk.

BOEHRINGER MANNHEIM KIT		CDR KIT	
Components	Cost (Euro)	Components	Cost (Euro)
Kit (for 25 analyses)	90,00	Cuvette test (pack of 10)	20,20
Solution 1	Included in price	Reagent R1	Included in price
Solution 2	Included in price	Reagent R1 <sub>a</sub>	Included in price
Solution 3	Included in price	Reagent R2	Included in price
Solution 4	Included in price		
Cuvette (pack of 500)	17,34		

**Table 10.** Cost of each single analysis of lactic acid in milk.

BOEHRINGER MANNHEIM KIT		CDR KIT	
Components	Cost (Euro)	Components	Cost (Euro)
Cuvette	0.03	Cuvette test	2.02
Single analysis solutions	3.60	Reagents (R1, R1?, R2)	0
Total	3.63	Total	2.02

**Table 11.** Time required for each single analysis of lactic acid in milk.

BOEHRINGER MANNHEIM KIT	CDR KIT
<b>Time for analysis (minutes)</b> >80	<b>Time for analysis (minutes)</b> 10

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**Table 12.** Cost of one person for each single analysis of lactic acid in milk

BOEHRINGER MANNHEIM KIT		CDR KIT	
<b>Time of analysis (minutes)</b>	<b>Cost of analysis (Euro)</b>	<b>Time of analysis (minutes)</b>	<b>Cost of analysis (Euro)</b>
>80	34.17	10	4,27

The main aspect is the practicality in the overall aspects connected to the use of the two analytical instruments for the analysis of lactic acid in samples of milk.

In fact, the CDR instrument requires less handling in respect of the addition of the R1<sub>a</sub> And R2 reagents, does not require preliminary treatments of the sample and supplies a result expressed directly in concentration terms (ppm) of lactic acid present in milk.

Instead, the analysis carried out via the Boehringer Mannheim enzymatic kit for L-lactic acid foresees a more consistent use of handling, glassware, reagents and analysis time. This analysis, in fact, requires the preparation of solutions (Carrez I and Carrez II) with the consequent utilisation of glassware (beaker, pipette, etc.) and long waiting times between one step and another of the determination.

However, the necessity of effectuating dilutions, additions, and filtration of the sample can create systematic errors that could affect the final result of the determination of lactic acid (Table 8).