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CENTRE D'EXPERTISE ET DE CONTROLE
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CECALAIT'S
NEWSLETTER

ENUMERATION OF SOMATIC CELLS IN GOATS' MILK

The objective of this work was to study and take on board the reference method for the enumeration of somatic cells in goats' milk (FDA method), and to study the methods of transition to automatic counters.

The following work was to be carried out :

- Taking into hand the reference method in the laboratory and comparison with the cows' milk method
- Determination of the characteristics of the reference method (repeatability)
- Reflection on the calibration method of automatic counters
- Specific calibration sample analyses
- Validation of the calibration (repeatability and accuracy)

The study was carried out from April 2004 to March 2005 by CECALAIT on request of the goats' milk interprofession (ANICAP) who has ensured the financial support of this project.

1/- STUDY OF THE REFERENCE METHOD

The retained method for the cellular counts is described in the FDA document from the publication of Packard and coll. 1992. It consist in the fixing of cells, and in the coloration using methyle-pyronine Y green, followed by a microscopic counting. The reference method currently used for the enumeration of somatic cells in cows' milk, is described in the standard IDF 148. It consists in the coloration of somatic cells using methylene blue, followed by microscopic counting.

A comparative test of cellular count between the FDA and IDF 148 methods was carried out at the beginning of April 2004 on 2 bulk goats' milk samples.

It appears that the results obtained on the 2 samples using the FDA method are globally inferior, by a third, to those obtained with the IDF method.

These lower results obtained with the FDA method compared to the IDF 148 method, are similar to previous observations and could be explained by the non-selectivity of methylene blue marking the cells as cellular fragments, and by the presence of numerous non-cellular particles in goats milk which are due to the type of milky apocrine secretion (damage of the gland in the form of cytoplasmic particles). The methyl-pyronine Y green colorant does not have this inconvenience, as it fixes only particles enclosing DNA and/or RNA and allows whole cells containing intact DNA/RNA (blue-green colour) to be differentiated from cellular fragments of RNA (pink colour).

Evaluation of repeatability

The samples used are bulk goats milk from the Rhône-Alpes and Poitou-Charentes regions of France. They were collected according to the practices of the dairy interprofession for milk payment during the period from May to September 2004. Colourless bronopol at 0.02% was added and the samples were kept at 4°C until analysis (with a limit of 5 days maximum following sampling).

The analyses were carried out at CECALAIT upon reception of samples. The samples for which the values were above $3000x10^3$/ml were eliminated. The results obtained are presented in the table below (10^3 cells/ml).

CLASS	0-3000	CLASS 0-1000	CLASS 1000-2000	CLASS 2000-3000
N	84	28	48	8
Sr	34	21	27	80
Sr (%)	2.8	2.8	2.0	3.3

Repeatability characteristics of the cellular enumeration (10^3/ml) in goats milk using the FDA method
N: number of results ; Sr et Sr (%): standard deviation of repeatability, absolute and relative

The results obtained present low standard deviations of repeatability of the same order of size as those obtained using the IDF 148 method with cows milk samples.

2/- STUDY OF AUTOMATED CELLULAR COUNTING

All the automated cell counting were carried out with an automatic counter, the Bentley SCC 150®,

previously calibrated using secondary reference material (SRM) cows milk samples. The technique used is flow cytometry with discrimination of the pulse-height according to a variable threshold.

The results obtained appear to be unsatisfactory, indeed the mean deviations are low (-5.10^3/ml), and the standard deviation of deviations and the residual standard deviation are high, which translates in a wide dispersion of results and an intercept significantly different from 0 (+80.10^3/ml). This dispersion is presumably linked to the accumulation of partial results over quite a long period of time (May to September 2004), as well as to the variations in calibration of the analyser over this period.

That is why, resorting to the preparation of specific goats milk SRM has been envisaged for the calibration of the analyser.

Calibration

In order to verify the usefulness of goats' milk SRMs, two series of 9 samples, of variable somatic cell content ranging from zero to 1800 10^3/ml, were realised by skimming and microfiltration of the bulk goats milk. Bronopol 0.1% was added to the samples. The series were realised in November 2004 and February 2005.

These samples were analysed in duplicate using the FDA method and by automatic counting (cows milk calibration).

Results

The results obtained are presented in the table below:

PARAMETER	SERIES 1	SERIES 2
d	3	19
Sd	31	30
b(b')	0.974 (0.997)	0.982 (0.974)
a	20	-3
Sy,x (S'y,x)	28 (30)	30 (28)

Standardisation parameters of the automatic cell counter Bentley SCC150® using goats milk SRM. d and Sd: mean and standard deviation of deviation instrument-reference ; b et b': single linear regression gradient (Y = b.X + a) or forced by a=0 (Y = b'.X); a: intercept of the single linear regression; Sy,x et S'y,x: residual standard deviation of the single or forced linear regression by a=0.

The mean deviations are low. The regression gradients are close to 1 (non significant difference) and the intercepts are close to 0. The residual standard deviations are low.

It can be noted that the relation between the two methods is stable over the two periods tested.

Globally, the deviations being low, the calibrations proposed using goats milk samples do not appear to be statistically different from those in vigour using cows milk samples.

3/- VALIDATION OF STANDARDISATION

A comparative analysis of goats milk samples by the reference method and the automated method calibrated with cows milk samples, was carried out in order to evaluate the counting accuracy with this type of calibration.

The samples used were obtained as previously described over the period of February and March 2005.
These samples were analysed in duplicate by the FDA method and by automated counting, the instrument having previously been calibrated using cows milk SRM ranging from zero to 1800 10^3/ml.

Results

Evaluation of repeatability

The results obtained are presented in the table below (10^3 cell /ml).

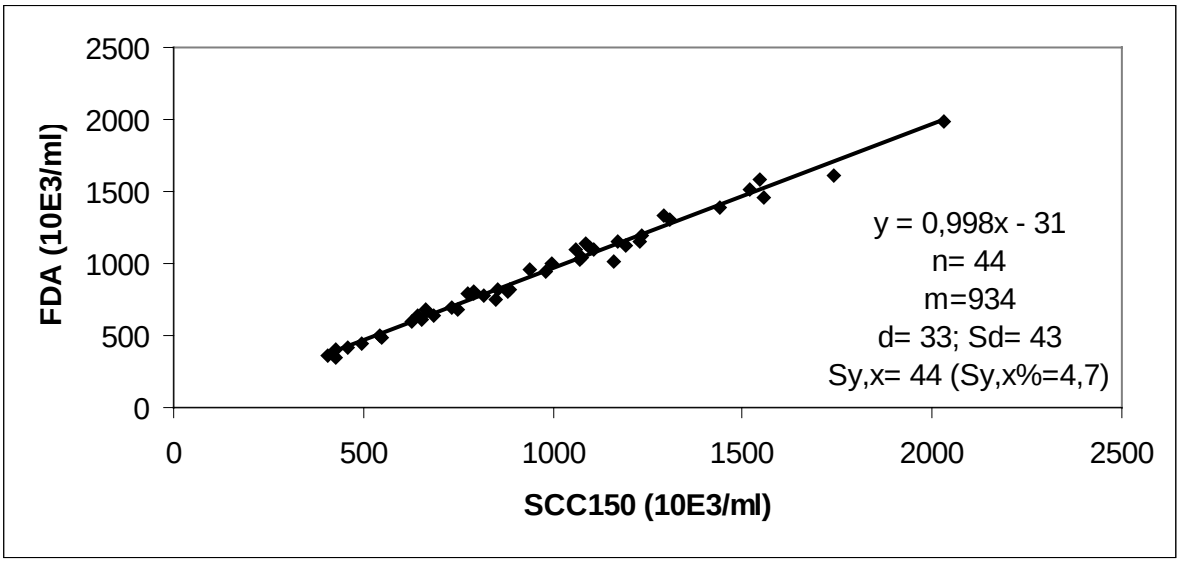
CLASS	0-2000	CLASS 0-1000	CLASS 1000 - 2000
N	44	24	20
Sr	13	9	17
Sr(%)	1.4	1.3	1.3

Repeatability characteristics of cellular enumeration (10^3/ml) of goats milk by automated counting (Bentley SCC150®)
N: number of results ; Sr et Sr(%): standard deviation of repeatability, absolute and relative.

The results obtained are good. Indeed, the standard deviations of repeatability are low and inferior to those obtained by the reference method. They are inferior to those observed when using individual cows milk samples (approximately 2.5%) and inferior to the recommended 5%.

Evaluation of accuracy

The figure below presents the results obtained:



Linear relation between the FDA method and the Bentley SCC150® on bulk goats milk samples
n : number of results, m: mean reference results ; d et Sd: mean and standard deviation of deviations instrument-reference ; Sy,x et Sy,x%: residual standard deviation of linear regression, absolute and relative

Taking into mind these results, it appears that the mean deviation of $33x10^3$/ml is superior to 0 (non-significant difference at 5%) which shows a good global concordance between results.

The gradient of 0.998 is very close to 1 (non significant difference) and the negative intercept is –31 (statistically non significant difference). This last parameter can be explained by the absence of low cell content samples (inferior to 300) which does not allow a good adjustment of the intercept.

The absolute residual standard deviation of 44 (45 for a=0) 10^3/ml can appear to be high, but when related the mean content, it is only 4.6 % (4.7% for a=0) and therefore inferior to that of 6 to 7% observed during the evaluation of the instrument with cows milk.

In conclusion, the calibration carried out using cows milk standards (wide range 1800 10^3/ml) allows a satisfactory accuracy to be assured for the enumeration of cells in goats milk.

4/- CONCLUSION AND PERSPECTIVES

Concerning the use of goats milk SRM, the results obtained do not show the use in using such specific preparations. In effect, the study showed that the calibration using cows milk standards according to the protocol in vigour, is satisfactory for the enumeration of cells in goats milk. The results can be explained for the following reasons:

- The colorant used for automatic counting (ethidium bromide) only fixes DNA in the same way as the colorant used in the reference method for goats milk (methyl-pyronine Y green). The two colorants are therefore specific to cells enclosing DNA and do not fix non cellular particles (present in goats milk). Conversely, the methylene blue used in the reference method for cows milk, colours all the particles and its usage for cows milk is possible only thanks to the quasi-absence of non cellular particles.

- The selection of pulses by the variable threshold technique allows only the strong pulses, predominantly originating from the somatic cells excluding cellular fragments, to be retained.

Thus, by extrapolation of the results obtained with the Bentley SCC150® analyser, it can be considered that the enumeration of goats milk by automated counting necessitates, a calibration of the analysers by means of cows milk samples over an approximate range of 0 to 1800 10^3/ml (including a control of linearity within this range), as well as, presumably, the use of a variable threshold mode (differentiation of pulse height). It can be thought that the results obtained are adaptable to other analysers presenting the same functionality and performances.

Finally, due to the evolution in the types of cells present in goats milk throughout lactation, it would be interesting to verify the stability of the relation between the results obtained by the goats milk reference method and the automated counts calibrated using cows milk.

BIBLIOGRAPHY

QUERVEL X., TROSSAT Ph. **Study report on the enumeration of somatic cells in goats' milk**, CECALAIT, May2005

PACKARD VS & Coll. 1992, **"Direct microscopic methods for bacterial or somatic cells"** in Standards method for the examination of dairy products. American Public Health Association, Washington, DC, p. 309-325

COEPT THE INTER-COMPARISON OF INTER-COMPARISONS

Summary of the talk presented by Dr Piotr ROBOUCH (Institute for Reference Materials and Measurements, IRMM, Geel, Belgique) at CECALAIT's AGM 2005

The participation of laboratories in proficiency tests is obligatory because of the requirements of accreditors, legislative authorities, customers. They often participate in several proficiency tests which involve extra-cost s(time and participation fees), and restrictions concerning free trade.
COEPT (the Comparability of the Operating and Evaluation Proficiency Testing schemes) is an European program of evaluation and comparison of protocols used by providers of proficiency tests. 17 partners participated in this European project in 2003-2005. It regrouped about 30 proficiency test providers in 4 sectors (water, soil, food, and hygiene & safety). 3 working seminars have been held in Berlin (February 2003), Geel (October 2003) and Eden (February 2005), and 2 inter-comparisons have been realised.

1st inter-comparison

The objectives were:

- to evaluate the similarities, or differences, of statistical protocols applied by the proficiency test providers and the influence on the reference value, the uncertainty, the deviation…
- to evaluate the capacity of the protocols to meet difficulties concerning a set of data (bimodality or dissymmetry for example)
- to compare the evaluation of performance

Concerning the food sector, 5 proficiency test providers participated in this inter-comparison. 11 data sets on fat, protein, moisture, lactose and ash were transmitted to providers for statistical treatment. The evaluations realised were returned to COEPT for comparison (mean, standard deviation of the reference value, evaluation).
Taking into mind the results transmitted, it appeared that there was a good concordance between the 5 proficiency test providers of this sector. However, the estimation of the uncertainty associated to the assigned value is a point which requires close attention. Indeed, even though 3/5 providers supply this parameter, the range observed between the estimations is high.

2nd inter-comparison

It was carried out between February and November 2004. The objectives were:

- to study the performance of the statistical protocols of the proficiency test providers in a real case,
- to see if the results of the 1st inter-comparison were confirmed when a reference material in a proficiency test is used,
- to study the evaluation made by the proficiency test providers.

5 proficiency test providers participated in this inter-comparison (Relacre, Muva, Cecalait, Fapas, QM). A sample of reference material (dry milk produced by a provider of the study) was sent to the laboratories participating in the inter-comparison (172 laboratories). The inter-comparison has been organised on 5 criteria : fat, dry matter, lactose, protein, and ash.

After analysis and comparison of the results by COEPT, it appeared that globally, there was a good agreement between the assigned values determined by the providers, as well as the reference values of the reference material supplied.

	Certified Uncertainty Value	Reported Reference standard Deviation Value							
		F01	F02	F03	F04	F05	Mean	s	RSD
Ash	5.64 0.04	5.67 0.05	5.66 0.07	5.64 0.04		5.64 0.05	5.65	0.01	0.3%
Dry matter	96.11 0.11	96.3 0.2	96.2 0.2	96.1 0.2	96.2	96.1 0.1	96.2	0.1	0.1%
Fat	26.03 0.11	26.0 0.1	25.8 0.5	26.0 0.2	26.0	26.0 0.1	26.0	0.1	0%
Lactose	37.64 0.45	23.8 0.6	35.4 1.1	37.6 0.5	36.7	37.6 0.8	34.8	5.5	15.7%
Protein	26.65 0.09	26.09 0.30	26.60 0.85	26.65 0.17	26.87	26.65 0.28	26.58	0.26	1.0%

However, during the examination of the raw results, deviations, due to the definition and comprehension of the criteria, were observed:

- moisture and dry matter
- nitrogen and protein
- lactose monohydrate and anhydrous

The expression of the results in the correct units permitted to obtain a total concordance on all the criteria between the assigned values determined. Only in the case of the determination of lactose, the assigned value of a provider has been identified as non-concordant with the other assigned values and the reference value of the reference material supplied. After study, it appeared that this difference was due to the results of only one laboratory.

Conclusion

To conclude, it appears that there is a good concordance between the participants concerning the assigned values on artificial data sets or during a real inter-comparison. Nevertheless, it appears that it was necessary to improve and harmonise the definition and the comprehension of criteria in order to have results expressed in the same units. Finally, the set up of the uncertainty estimation on the assigned values seems indispensable.

IN THE PRESS – ON THE WEB

Classification in alphabetical order of keywords

BACTERIA / *LISTERIA*

Scientists develop fast bacteria detector

http://www.foodnavigator.com/news/news-ng.asp?n=61072-scientists-develop-fast

► A new machine using flow cytometry, which can analyse and identify *Listeria monocytogenes* and *Candida albicans* accurately has been developed.

STANDARDISATION

The Codex Alimentarius Commission adopts over 20 food standards

http://www.nutritionhorizon.com/prntnews.asp?n=y&sendid=8594

► The Codex Alimentarius Commission adopted more than 20 new and amended food standards during its annual session.

NOTE ON THE ACTIVITY OF THE "TBX MEDIUM" WORK GROUP OF THE AFNOR COMMISSION V08B "FOOD MICROBIOLOGY"

PART 1: REPORT

Tryptone-bile-glucuronide (TBX) and peptone-tergitol-glucuronide (PTX) agars are two chromogenous media which allow the enumeration of *E. coli* by their positive character for the β-D-glucuronidase, present in about 95% of *E. coli* found in food products. *E. coli* 0157:H7 for example, is deprived of this character.
Since 1993, the medium PTX is described in a routine standard at a national level (NF V 08-053). However, following the non-commercialisation of tergitol 7, a selective agent in this medium, it was replaced, at the end of 2002, by the medium TBX, which is standardised at an international level since 2001 (ISO 16649 parts 1 and 2).
TBX had been selected following the ISO SC9 meeting in 1997, where comparisons had been presented on various matrices, with various methods and chromogenous media.
However, taking into mind these results, TBX did not seem to possess much higher performances than the other chromogenous media.
With the use of this new medium, some laboratories met difficulties, particularly for the analysis of dairy products. These observations are described in this first article.

1) PROBLEMS MET IN DIFFERENT LABORATORIES

1.1 Comparison between PTX and TBX media

At the beginning of 2003, following the set up of the new standardised medium, TBX, in place of PTX, 2 laboratories, analysing dairy products, alerted us on discordant results between these agars.

- Results obtained in naturally contaminated samples:

The laboratory L1 compared results obtained from the analysis of 25 raw milk samples on TBX and PTX of a same supplier (E). Globally, this laboratory observed a strong inhibition with the medium TBX in raw milk (-0.7 log on average). However, the colonies were not identified.

The laboratory L2 compared enumerations obtained with 5 suppliers of ready-to-use TBX in relation to a medium PTX on a majority of raw milk cheese samples. Globally, few differences were observed (on average +/- 0.1 log according to the supplier). However, on a batch from a supplier of TBX (B), colonies were uncountable because they were only weakly, or not, visible, whereas important enumerations were obtained on the medium PTX and on 2 other TBX media. It should be noted that this batch was close to the use-by-date.

- Results obtained on the samples from CECALAIT's proficiency tests:

Proficiency test *E. coli* in milk in February 2003 (5 samples):

The laboratory L1 tested various suppliers of the TBX and PTX media. The 2 PTX media gave results located within the target of conformity (+/- 0.3 log in relation to the reference), whereas 3 TBX media, on average, largely underestimated the populations (-0.4 to –0.8 log).

The laboratory L2 compared 4 different suppliers of TBX and one of PTX: only one TBX (supplier C) was out of range (-0.4 log on average).

CECALAIT's laboratory, by using dehydrated TBX and PTX media from the same supplier (A), obtained identical results, within the target of conformity. With the TBX from this supplier, our laboratory has always obtained good results in the proficiency tests.

Proficiency test *E. coli* in cheese in December 2002 (5 samples):

The laboratory L1 once again compared TBX and PTX media from the same supplier (E): PTX was within the target of conformity, whereas TBX underestimated on average 0.9 log in relation to the reference

1.2 Other problems met in CECALAIT's proficiency tests

Thereafter, other laboratories related to us their difficulties of enumeration using TBX in the proficiency tests. For example, the phosphate plug diluent used with TBX can have an inhibiting effect. This effect is not observed with the Ringer diluent or on other matrices.
These problems are more often met with the ready-to-use than with the dehydrated media. They can concern all the media proposed by the different suppliers, although they appear punctually according to the batches used.

2) TOTAL INTERPRETATION OF CECALAIT'S PROFICIENCY TESTS

Note: Proficiency test allows to evaluate the performances of a laboratory, but not the performance of a method or a supplier. However, this vision can alert us on the general tendencies.

From June 2003, when the use of TBX in the laboratories increased, we noted that, globally, a more substantial dispersion of the results of *E. coli* proficiency tests, and more significantly in cheese than in milk. Generally, the results were more closely grouped with the medium PTX.

So, we tried to interpret in more detail the *E. coli* proficiency tests in cheese. We observed, for the medium TBX, a more important percentage of results off target, generally underestimations, than for the other media.

By locating the suppliers on the target, it was not possible to show a constant supplier effect with time. In the same way, the underestimation of the ready-to-use media compared to the dehydrated formula was not clearly demonstrated. However, recently the results have tended to improve.

Following these reports, the AFNOR commission V 08B decided to create a work group, which must gather and study these observations and try to provide solutions. Organised by AFNOR and animated by P. ROLLIER of CECALAIT, this work group is composed of about 10 suppliers or users. The group has met 3 times between 2004 and 2005. The activities and conclusions of this work group will be presented in the second part of this article in the next Lettre de CECALAIT.

STANDARDS, DRAFT STANDARDS

Classification in alphabetic order by theme

ISO published standards

MILK AND CANNED EVAPORATED MILK		
MILK / CANNED EVAPORATED MILK TIN / SPECTROMETRY	ISO/TS 9941: 2005 June 2005	MILK AND CANNED EVAPORATED MILK Determination of tin content - Spectrometric method
MILK AND DAIRY PRODUCTS		
MILK / DAIRY PRODUCTS / QUALITY	ISO 14461-1: 2005 May 2005	MILK AND DAIRY PRODUCTS Quality control in microbiological laboratories Part 1: Analyst performance assessment for colony counts
MILK AND MILK POWDER		
MILK / MILK POWDER AFLATOXIN / CHROMATOGRAPHY	ISO 14674: 2005 May 2005	MILK AND MILK POWDER Determination of aflatoxin M1 content - Clean-up by immunoaffinity chromatography and determination by thin-layer chromatography
QUALITY		
QUALITY / CALIBRATION / TESTING	ISO 17025: 2005 May 2005	General requirements for the competence of testing and calibration laboratories
YAOURT		
YOGURT / SOLIDS CONTENT	ISO 13580: 2005 May 2005	YOGURT Determination of total solids content (Reference method)

NEW EU STANDARDS AND REGULATIONS

Classification is established in alphabetical order of the first keyword

FOOD INGREDIENT

O.J.E.U. L 160, 23rd June 2005 - Commission Decision of 4 April 2005 authorising the placing on the market of isomaltulose as a novel food or novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council

http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/oj/2005/l_160/l_16020050623en00280030.pdf

PESTICIDE / RESIDUE

O.J.E.U. L 177, 9th July 2005 - Commission directive 2005/46/EC of 8 July 2005 amending the annexes to Council directives 86/362/EEC, 86/363/EEC and 90/642/EEC as regards maximum residue levels for amitraz

http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/oj/2005/l_177/l_17720050709en00350041.pdf

PROTECTED DESIGNATION OF ORIGIN

O.J.E.U. L 214, 19th August 2005 - Commission Regulation (EC) No 1357/2005 of 18 August 2005 supplementing the Annex to Regulation (EC) No 2400/96 as regards the entry of a name in the "Register of protected designations of origin and protected geographical indications" Chevrotin (PDO)

http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/oj/2005/l_214/l_21420050819en00060008.pdf

VETERINARY MEDICINAL PRODUCTS / RESIDUE / FOODSTUFFS

O.J.E.U. L 185, 16th July 2005 - Commission Regulation (EC) No 1148/2005 of 15 July 2005 amending Annex I to Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, as regards penethamate

http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/oj/2005/l_185/l_18520050716en00200021.pdf

O.J.E.U. L 206, 9th August 2005 - Commission Regulation (EC) No 1299/2005 of 8 August 2005 amending Annexes I and III to Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, as regards phenoxymethylpenicillin, phoxim, norgestomet and thiamphenicol

http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/oj/2005/l_206/l_20620050809en00040007.pdf

O.J.E.U. L 214, 19th August 2005 - Commission Regulation (EC) No 1356/2005 of 18 August 2005 amending Annex I to Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, as regards oxolinic acid and morantel

http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/oj/2005/l_214/l_21420050819en00030005.pdf

O.J.E.U. L 244, 20th September 2005 - Commission Regulation (EC) No 1518/2005 of 19 September 2005 amending Annexes I and III to Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, as regards acetylisovaleryltylosin and fluazuron

http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/oj/2005/l_244/l_24420050920en00110012.pdf

BOOKSHOP: LATEST PUBLICATIONS

The classification in alphabetic order of the first keyword allows you to consult the references according to your interests. The web site allows you to know more, or to order the book.

TECHNOLOGY / MILK / DAIRY PRODUCTS

WALSTRA P.; WOUTERS J.T.M.; GEURTS T.J. – **Dairy Science and Technology, second edition –** CRC Press Edition – ISBN 0-8247-2763-0 – 808 p.

This book provides information on the composition and properties of milk. It also shows the characteristics of milk and dairy products during processing and storage. http://www.crcpress.com

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