Department SERH 1985-05-31

REPORT 85.47 Pr.nr. 505.0290

Subject: Collaborative study on the determination of natamycin in cheese and cheese rind 1984.

Annexes: 5

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Project: Harmonisation of methods of analysis for additives

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Summary

A collaborative test on the determination of natamycin in cheese and cheese rind was carried out. In total 38 laboratories from 13 countries were participating. Eight samples, consisting of 4 duplicates were investigated by a spectrometric and an HPLC method.
The results are reported in Tables 1-5 and summarized in Table 6.
The overall results are as follows.

| | Detection method | Preconcentration applied | Mean mg/kg | $CV_r$ % | $CV_R$ % |
|---|---|---|---|---|---|
| Level A | Spectr. | no | 62,5 | 5,9 | 12,2 |
| | HPLC | | 60,8 | 9,3 | 20,6 |
| Level B | Spectr. | no | 15,2 | 6,2 | 11,9 |
| | HPLC | | 15,5 | 7,1 | 25,7 |
| Level C | Spectr. | yes | 1,19 | 16,5 | 35 |
| | HPLC | | 1,43 | 23,4 | 37 |
| Level D | Spectr. | yes | 0,27 | 42,5 | 60 |
| | HPLC | | 0,34 | 29 | 39 |

The quality of the results can be classified:

| | Level A | Level B | Level C | Level D |
|---|---|---|---|---|
| spectroscopic direct | good | good | not at all | not at all |
| spectroscopic after concentration | no need | no need | bad | not |
| HPLC-UV direct | reasonable | reasonable | not at all | not at all |
| HPLC-UV after concentration | no need | no need | bad | bad |

The method fulfills the requirements of the EEC. Based upon the method the ad hoc EEC working group adopted an unambiguous method for analysis of cheese rind and cheese.
The method is adopted by the Joint IDF/ISO/AOAC Group of Experts "Selected Food Additives" to eventually becom on IDF and ISO Standard Method.

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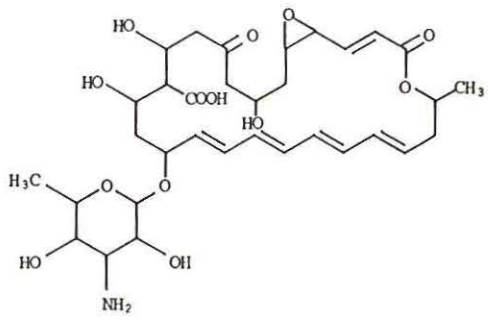
Rapporteur: dr W.G. de Ruig
Projectleader: ir P.C. Hollman

Contents

1. INTRODUCTION

Natamycin or pimaricin is a white to creamy-white almost tasteless and almost odourless, crystalline powder. It is a fungicidal antibiotic and antimycotic of the polyene macrolide group, and is produced by the actinomycete Streptomyces natalensis.

The chemical formula is $C_{33}H_{47}NO_{13}$, the molecular weight 665.74 and the structural formula




The fungus natamycin was discovered by A.P. Struyk in a soil sample originating from the environment of Pietermaritzburg in the province of Natal, Republic of South Africa. From its place of discovery the fungus got the names pimaricin and natamycin.

It has found wide application especially on cheese and sausages. In the dairy industry natamycin is applied in cheese coatings, and it has turned out to be effective in preventing mould formation without affecting the behaviour (taste, appearance) of the cheese. In these respects natamycin is superior to alternative products.

According to national legislation, official clearances for the use of natamycin as preservative for cheese have been granted by a large number of countries, including Argentina, Australia, Bahrein, Belgium, Canada, Chile, Czechoslovakia, Federal Republic of Germany, Finland, France, Ireland, Israel, Italy, Mexico, The Netherlands, Norway, Philippines, Poland, Saudi Arabia, South Africa, Spain, Sweden, Turkey, United States of America, Venezuela, Yugoslovia.

Natamycin is a matter of interest to international bodies such as Codex Alimentarius, the International Dairy Federation (IDF) and to the European Economic Commission (EEC).

The EEC Scientific Committee for Food (1) came to the following conclusions.

"1. Natamycin has a limited but important use in human medicine and is therefore not acceptable as food additive for general use in and on foodstuffs.

2. Its use for the surface treatment of the rind of whole pressed cheese (semi-hard) ripened under aerobic conditions for example Gouda and Edam, and on the casings of certain sausages requiring maturation before marketing is acceptable, provided that:
   (i) the substance is applied only to the final products;
   (ii) the residues of natamycin in food at the time of sale, expressed in relation to the surface area of the casing or rind, do not exceed 1 mg/dm$^2$ and that they will not be present at a depth greater than 5 mm.

3. The use of natamycin on the casings of these foods should be clearly indicated by suitable labelling.

4. The position should be reviewed if there is any significant increase in the range of therapeutic uses."

The Directive 64/54/EEC of the Council of the European Communities (2) last amended by the Directive 84/261/EEC (3), lays down a list of preservatives which may be used for the protection of foodstuffs intended for human consumption against deterioration caused by micro-organisms. In consequence of the opinion of the Scientific Committee the Permanent Representatives Committee has proposed to the Council of the European Committees to insert natamycin in this list as EEC no. E 235 for the surface treatment of the rinds of whole cheeses with a water content of not more than 69% by mass of the non-fatty matter, other than soft cheeses, provided that the natamycin is not present in the cheese at a depth greater than 5 mm and that at the time of sale to the ultimate consumer the residues of active natamycin do not exceed 1 mg/dm$^2$.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated natamycin (4) and concluded:

"New information was available on the effects of breakdown products and the development of microbial resistance to the antimycotic if it

is used for food preservation. While the Committee expressed general concern about the use of therapeutic agents in food, it agreed that the data on natamycin showed that problems were unlikely to arise from microbial resistance.
It appears therefore that the usual objects to the use of therepeutic antibiotics in foods have little relevance to natamycin.
Natamycine is used as a food additive to prevent the surface growth of moulds, which could in principle produce mycotoxins. This is an important advantage and one regarded by some experts as sufficient to offset any misgivings about the use of therapeutic antibiotics in food".

An acceptable daily intake of 0-0.3 mg/kg body weight was allocated. As its 11th session the Codex Committee on Food Additives accepted the conclusion of JECFA, classified natamycin as category A(1) additive and endorsed it for cheese with a limit of 2 mg/kg in the rind without plastic coating and 500 mg/kg in the plastic coating (5). Category A(1) additives are those who have fully been cleared by JECFA.

Methods of analysis for the determination of natamycin on cheese are published based upon microbiological, spectrometric, TLC and HPLC-UV detection (6-11).
The behaviour of natamycin and its determination has been thouroughly studied by the Netherlands State Institute for Quality Control of Agricultural Products (RIKILT), in cooperation with the Netherlands Institute for Dairy Research (NIZO) and the Netherlands Inspection Institute for Milk and Milk Products (ZCI) (12,13).
Two methods of analysis were developed, one based upon spectrometric (14) and one upon HPLC detection (15). A series of national collaborative studies have been carried out, which enhanced the methods. These studies made clear, that the microbiological method does not fit quantitative measurements. The method tested in this collaborative study is in fact merged from the spectrometric and the HPLC method.
Internationally, methods of determination were discussed by the EEC Working Group Additives and by the Joint IDF/ISO/AOAC Group of Experts on Additives (E 43). Both groups felt the desirability of a collaborative study.

In the United Kingdom, the Food Science Division of the Ministry of Agriculture, Fisheries and Food was also planning a collaborative study on a national level. It was concluded that one collaborative study would be preferable, to be organized by RIKILT. In 1983 as pilot collaborative study was carried out, with 9 laboratories in 4 countries (16). On account of the results of this pilot study, minor alterations in the method have been made.

In the collaborative study, carried out in 1984 and reported here, 38 collaborators from 13 countries were participating.

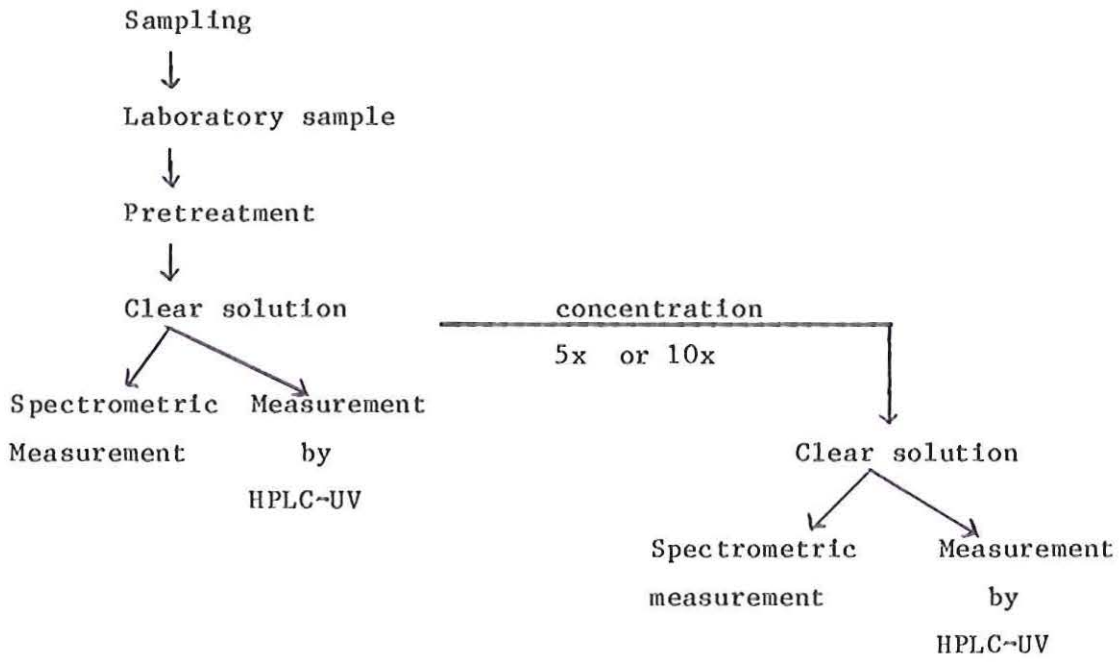
The cheeses from which the samples were prepared were obtained from the Netherlands Institute for Dairy Research, which has produced and stored the cheeses and treated them with natamycin-containing cheese coating.

For calibration natamycin reference samples were kindly made available by Gist Brocades N.V.

The study was organized by RIKILT without grants from national or international bodies.

## 2. METHOD

The method under investigation in this collaborative study consists of a sampling procedure, pretreatment of the laboratory sample, and detection by either spectrometric or HPLC-UV measurement. When the natamycin concentration is low, and its quantification is still required, a concentration step has to be applied prior to measurement. The method can be represented schematically as follows.



This study concerns the determination and detection steps only. It does not include the sampling procedure and the preparation of the laboratory sample. These can add a substantial contribution to the inaccuracy of the method, but are beyond the scope of this study.

3. AIMS OF THE COLLABORATIVE TEST

Two methods of measuring the amount of natamycin were tested: a spectrometric and an HPLC-UV method. As to these methods the following items had to be investigated.

- The applicability of both methods, for various concentration.
- The reliability of both methods, for various concentration levels, in terms of statistical parameters.
- Whether or not both methods will give corresponding results.
- Whether or not false results may be obtained by interference of degradation products of natamycin.
- The recovery of the method.

4. SAMPLES

Each participant received eight samples, consisting of lyophilized cheese rind or cheese, packed in brown bottles under nitrogen. Each sample was about 15 grams, that is sufficient for one analysis. It was advised to store the samples in a refrigerator.

The samples were blind duplicates on four concentration levels and dispatched under code numbers. Participants were not informed whether duplicates or split level samples were present.

The samples have been prepared in May 1984.

The following materials were used for the preparation of the samples.

Level A = sample 1 and 4

Cheese rind, high level = above EEC limit.

Date of production cheese: 1984-04-10.

Treated 4 times with cheese coating containing 0.005% natamycin in the period 1984-05-09 to 1984-05-16.

Level B = sample 2 and 8

Cheese rind, low level = about EEC limit.

Date of production cheese: 1982-07-15.

Treated 3 times with cheese coating containing 0.0125% natamycin in the period 1982-07-15 to 1982-08-31, and 2 times with 0.005% natamycin during 1983.

Level C = sample 3 and 6
Cheese, inner part, treated with natamycin.
Level above detection limit.

Level D = sample 5 and 7
Cheese, inner part, treated with natamycin.
Very low level.

From earlier investigations it could be expected that level A and B could be determined by direct determination without concentration, that level C had to be concentrated, and that level D was at or below the detection limit.
Although no blank samples have been dispatched, the results for level D may give an impression of the appearance of false positive results.

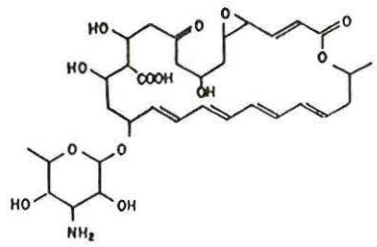
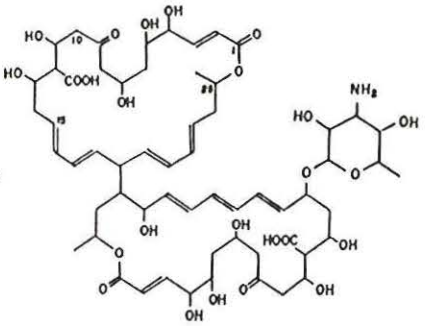
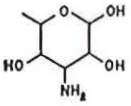
## 5. SAMPLE PREPARATION

The main problem in getting samples was that neither the cheese as such nor natamycin on the rind is stable. In the Netherlands intercomparisons it had already been found that no comparable results are obtained when there are differences in duration and conditions of storage before analysis. For a worldwide intercomparison the samples have to be stable under normal conditions. From prior investigations it turned out that in cheese rind, which is homogenized and lyophylized and then packed in brown glass bottles under nitrogen, natamycin is stable for a longer period. Such samples have been used succesfully in the pilot-international study.

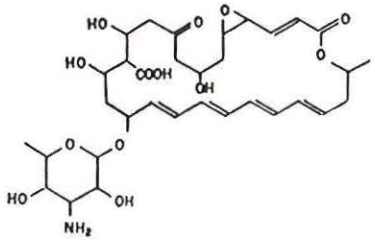
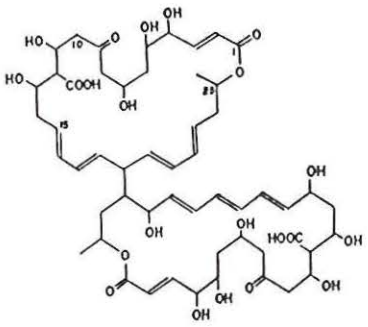
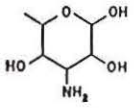
## 6. DEGRADATION PRODUCTS

Natamycin degrades (17), under mild acid conditions, into a biologically inactive substance, called aponatamycin, a substance consisting of one natamycin- and one natamycinolide-moiety.
In the case when natamycin is degraded under more drastic acid circumstances the resulting, biologically inactive substances are mycosamine, the natamycinolidediol-dimer and its decarboxyanhydro-analogue.

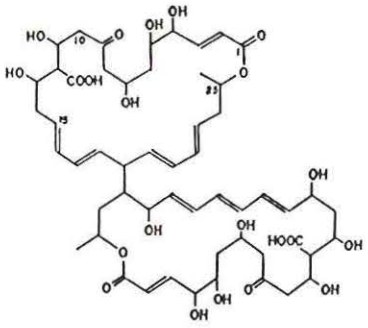
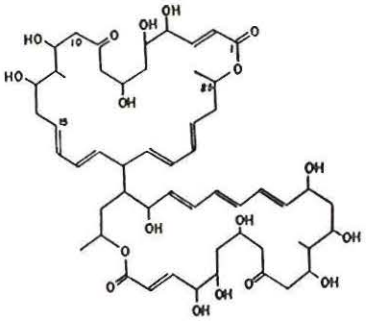
The reactions are given below:

2  + $2H_2O$ --->  + 

natamycin aponatamycin mycosamin

2  + $2H_2O$ --->  + 2 

natamycin di-natamycinolidediol mycosamin

 ---->  + $2H_2O$ + $2CO_2$

di-natamycinolidediol decarboxyanhydro-analogue of di-natamycinolidediol

In case of measurements by HPLC, degradation products turned out to have a shorter retention time, so that two separate peaks are obtained. The spectrometric method is less specific, and degradation products may seemingly enhance the results for natamycin.
One aim of this study was to test whether this occurs in practice when the described method is applied. If so, the spectrometric results will be higher than the HPLC results. This will be the case particularly for older cheeses where more degradation products can be expected. Therefore, one of the levels, namely level B = sample 2 and 8, was prepared from an extremely old cheese, treated with natamycin throughout two years. Especially for this level remarkable difference between the two methods has been observed.

7. HOMOGENEITY TESTS

Natamycin content of 5 lots of each sample, as dispatched to the participants (mg/kg).

| | | | | |
|---|---|---|---|---|
| Sample A | 61.7 | | | |
| | 65.3 | | | |
| | 60.0 | | | |
| | 61.2 | | | |
| | 59.3 | mean: 61.4 | s.d.: 2.22 | CV: 3.6% |
| Sample B | 14.5 | | | |
| | 15.2 | | | |
| | 14.3 | | | |
| | 14.5 | | | |
| | 14.5 | mean: 14.6 | s.d.: 0.35 | CV: 2.4% |
| Sample C (5x conc.) | | | | |
| | 1.5 | | | |
| | 1.8 | | | |
| | 1.3 | | | |
| | 1.8 | | | |
| | 1.5 | mean: 1.52 | s.d.: 0.28 | CV: 17.8% |

Sample D (10x conc.)

0.35
0.43
0.47
0.42
0.41 mean: 0.42 s.d.: 0.04 CV: 9.5%

8. RECOVERY TESTS

For determination of the recovery, the participants were asked to analyse two other samples, prepared by themselves, as follows.

Cut a piece of rind to a thickness of about 5 mm from a half-hard type of domestic cheese.

Grind the rind, and homogenize.

Weigh 10 g of the ground rind into a 200 ml conical flask, according to paragraph 6.1. Dissolve 100 mg of the natamycin reference sample (91.6% natamycin) in 50 ml of methanol.

Dilute 1:10 with methanol. Add 1 ml of this solution to the content of the conical flask. Continue the procedure starting at paragraph 6.1.

The concentration in the sample is thus 0.916 x 20 = 18.32 mg/kg.

9. RESULTS

The results of the participants as reported are collected in Tables 1 and 2:

1.1 Direct determination, spectrometric detection

1.2 Direct determination, HPLC detection

2.1 After concentration, spectrometric detection

2.2 After concentration, HPLC detection.

The recoveries of the spectrometric and HPLC detection are reported in Tables 3.1 and 3.2.

In these tables the concentration in mg/kg only is given. According to the method also the amount of natamycin in mg/dm$^2$ can be calculated, taking into account the surface (Y) and the mass (X) of the laboratory sample. As the participants did not make this laboratory sample themselves, these values were given in the protocol, namely,

X = 15 g, Y = 25 cm$^2$, so that

$C'(mg/dm^2) = 0.1 \times \frac{15}{25} \; C = 0.06 \; C \; (mg/kg)$.

The results obtained were sent in a provisional form to all participants to check the correctness of the data. In some cases, where obviously something seemed to be wrong, the institute in question was contacted. It turned out that a number of institutes had not corrected for the standard natamycin, being 91.6%, without reporting that. After a questionnaire some participants corrected their results afterwards. The data in this report have been corrected where necessary. As the method was not carried out as described the following laboratories have not been included in the evaluation of this collaborative study:

Spectrometric: 8, 22 (after concentration only)

HPLC: 11, 15, 21, 30, 43.

Deviations consisted of use of other HPLC column, mobile phase or flow rate, deviations in pretreatment, results of a second experiment with less sample. In the tables 1.1 to 2.2 the results of the laboratories are inserted in parentheses.

As examples of primary data in figures 1-4 some results are given:

Figure 1. Spectrometric detection. UV spectra of standard solutions.

Figure 2. Spectrometric detection. UV spectra of samples.

Figure 3. HPLC detection. Chromatograms of standard solutions.

Figure 4. HPLC detection. Chromatograms of samples.

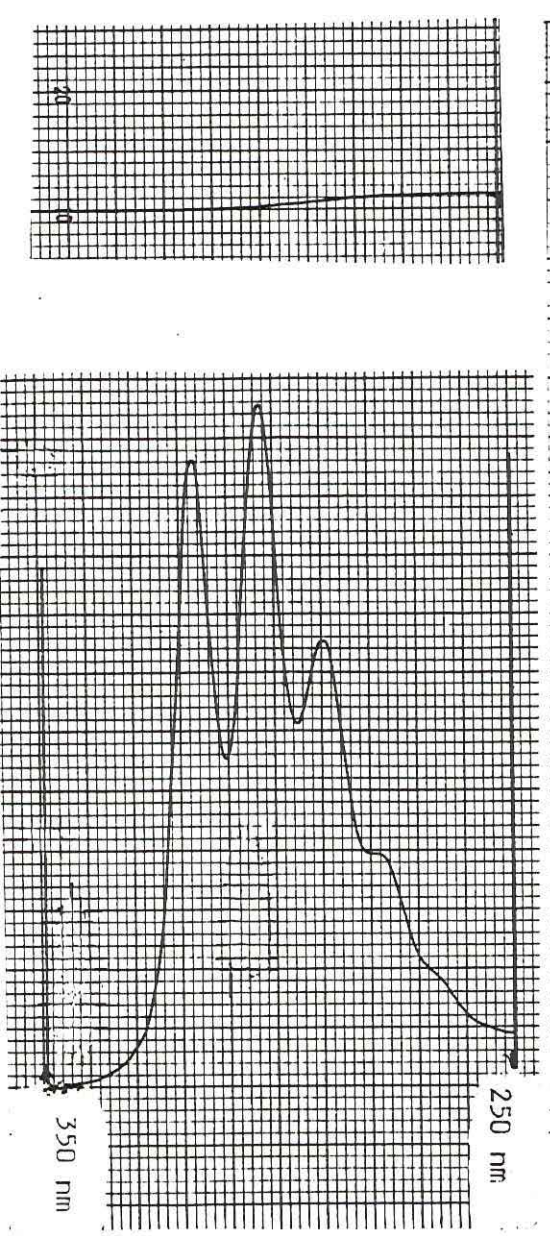



Figure 1 Spectrometri detection
Standard 5 µg/ml.

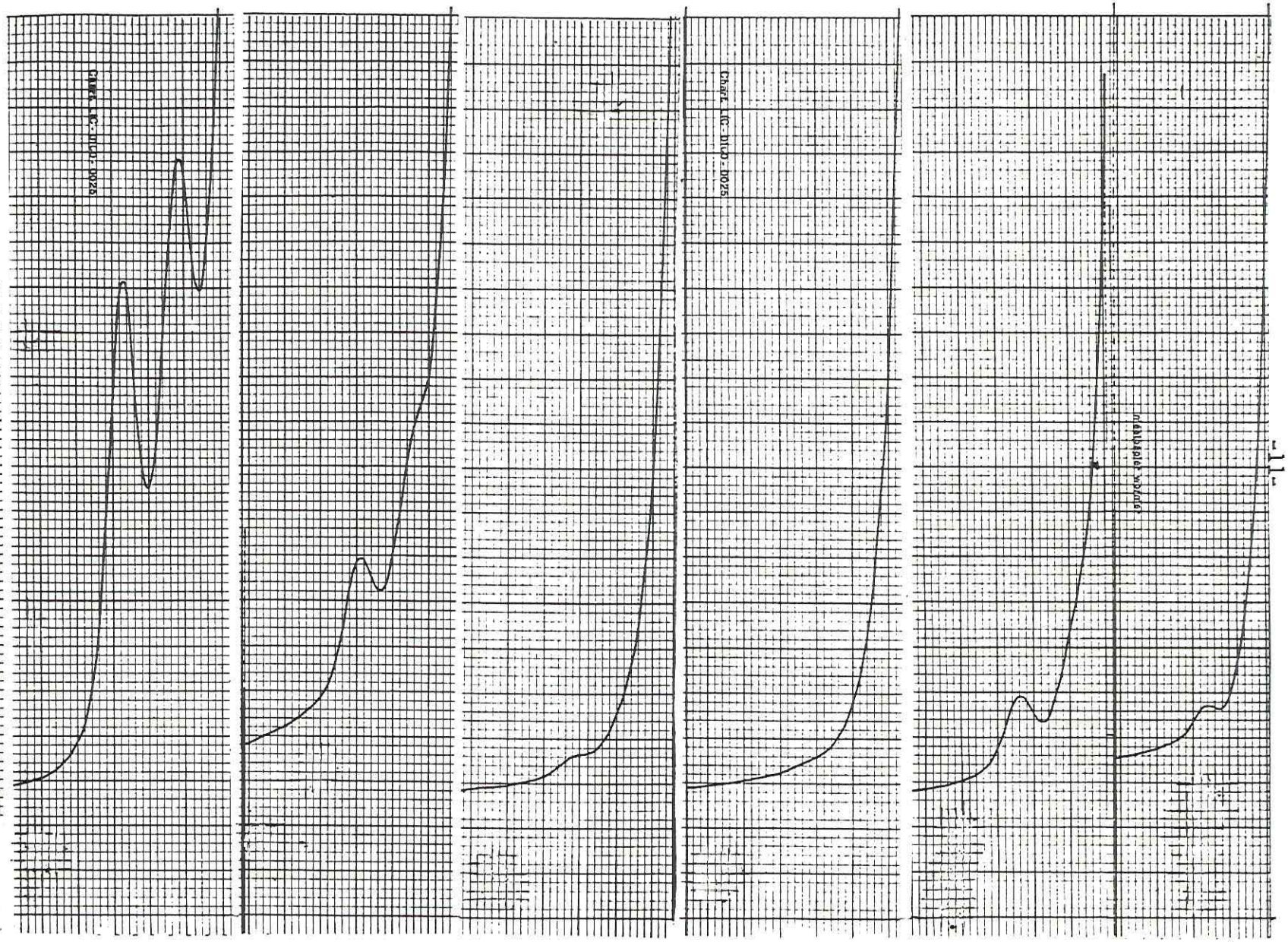



Figure 2 Spectrometric detection. Samples

Level A | Level B | Level C | Level D | Level C 5x conc. | Level D 10x conc.

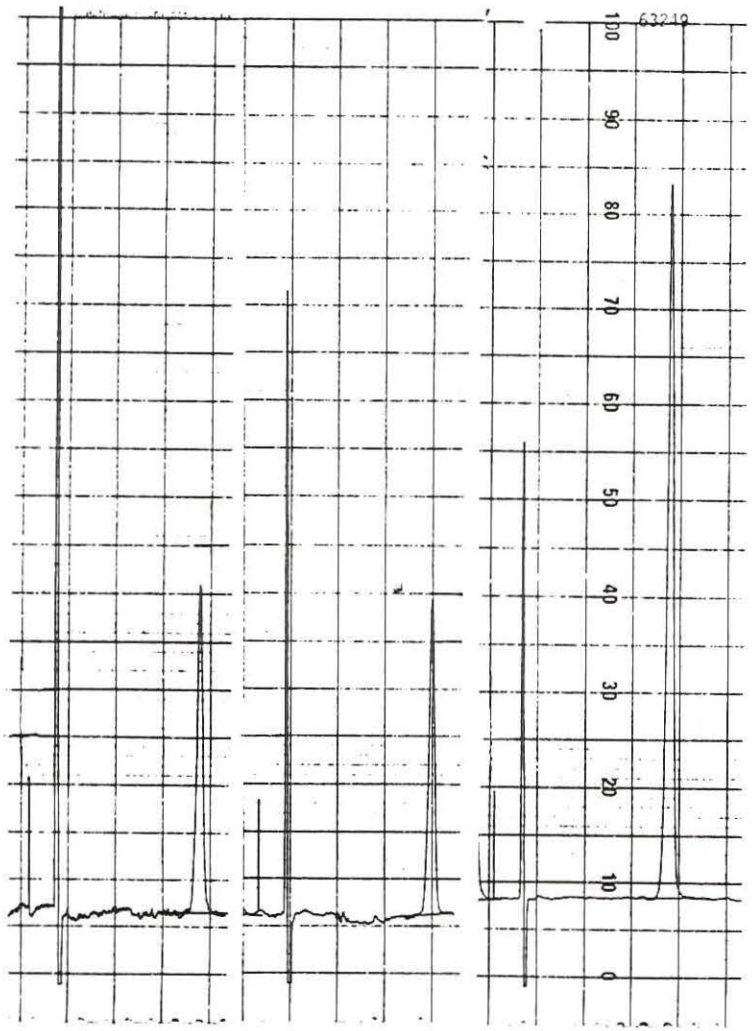



Figure 3 HPLC detection
Standard solutions

| | 0.5 µg/ml | 1 µg/ml | 5 µg/ml |
|---|---|---|---|
| AUFS: | 0.005 | 0.01 | 0.02 |

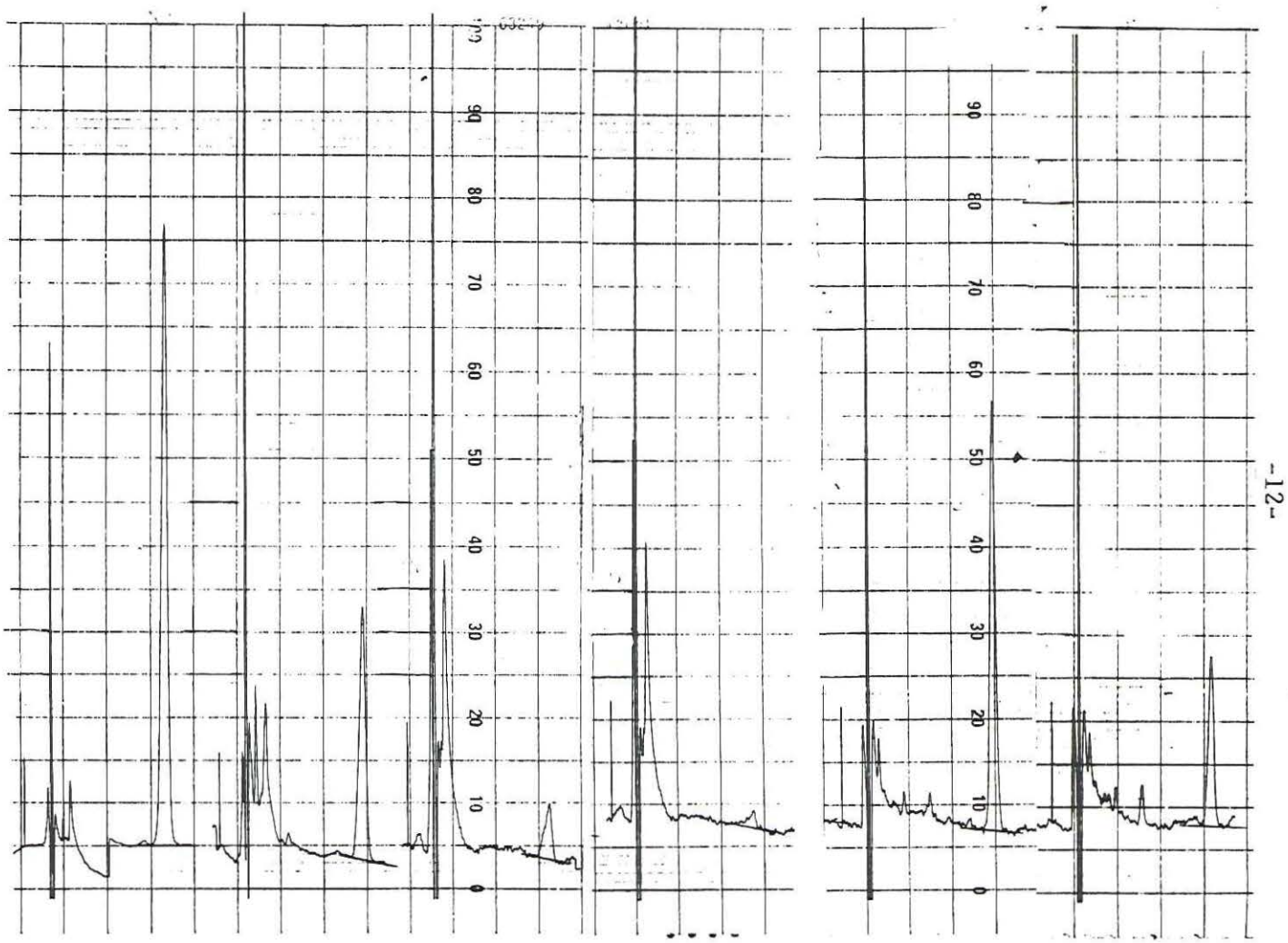



Figure 4 HPLC detection
Samples

| | Level A | Level B | Level C | Level D | Level C 5x conc. | Level D 10x conc. |
|---|---|---|---|---|---|---|
| AUFS: | 0.02 | 0.01 | 0.005 | 0.005 | 0.005 | 0.005 |

## 10. EVALUATION OF RESULTS

The results of laboratories which applied the method as described have been statistically evaluated according to ISO 5725. According to this standard, Cochran's maximum variance test is used to test the precision under repeatibility conditions in the laboratories, and Dixon's outlier test, to test the precision between laboratories. Outliers were rejected, stragglers were kept in.

Values reported as "non detectable" were also considered to be outliers, and not included in the calculations of results. The outliers are labelled in Tables 1.1 to 2.2 with an asterisk *.

As was expected, levels A and B could be determined without concentration, but level C could hardly be and level D not at all be determined without preconcentration. Therefore only the direct determinations for levels A and B, and the determinations after concentration for levels C and D were evaluated, and reported in Table 4.

In this table the following values are also included.

$n$ = number of evaluated laboratories

$\bar{\bar{x}}$ = total mean value = mean of the mean value

$s_{\bar{x}}$ = standard deviation of the mean values

$s_{\bar{\bar{x}}}$ standard deviation of the total mean value

$$s_{\bar{\bar{x}}} = \frac{s_{\bar{x}}}{\sqrt{n'}}$$

$= \bar{\bar{x}}_{Sp} - \bar{\bar{x}}_{H}$ = difference between spectrometric and HPLC method.

$s_{\Delta} = s\ (\bar{\bar{x}}_{Sp} - \bar{\bar{x}}_{H})$ = standard deviation of the difference.

$$s_{\Delta} = s\ (\bar{\bar{x}}_{Sp} - \bar{\bar{x}}_{H}) = \sqrt{(s_{\bar{\bar{x}}})^2_{Sp} + (s_{\bar{\bar{x}}})^2_{H}}$$

$t$ = t test $\qquad t = \frac{\Delta}{s_{\Delta}}$

The results are summarized in Table 5.

Applying the t test for random samples on the spectrometric and the HPLC results turns out that there is no significant difference in the results of both methods on all four levels investigated; $t < 1.96$ in all cases. It could be expected that in the spectrometric method degradation products would contribute in the measuring signal, thus

giving rise to significant higher values. Especially for level B (samples made from very old cheese, thus a high level of degradation products expected) this could be the case. But obviously such on interference does not occur; the spectrometric method gives not too high values.
The repeatability and the reproducibility of the spectrometric method is better than those of the HPLC method for levels A and B. For levels C they are comparible, and for level D the HPLC is the better one.

11. COMMENTS OF THE PARTICIPANTS
Most of the comments of the participants were concentrated on the following items (see Annex 2).

1. Turbid extracts in 6.4.
Reply: To obtain a clear filtrate will become harder, when the suspension is warmed up. Filtration has to be carried out rapidly when the suspension is still cold.

2. Degradation products in the measuring solution.
Reply: Natamycin is unstable in a MeOH/$H_2O$ solution. You have therefore to proceed as rapidly as possible.

3. MeOH/$H_2O$ in 8.6 + 8.7.1 (spectrometric) and MeOH in 8.6 + 8.8.1 (HPLC). Some participants suggest to use MeOH in both cases, others, to use MeOH/$H_2O$ in both cases.
Reply: In case of spectrometric determination it is advantageous to reduce the amount of interfering substances by precipitation with water and filtration. In case of HPLC, interference is overcome by chromatographic separation, so there is no need for an extra purification step.

4. Difficulties with HPLC.
Some participants report big problems with the prescribed column and mobile phase. Others, however, did not have any problem at all.

5. Spectrometric calculation.
Some participants calculated their results in 7.1.1.2 using the absorptions at exact 317 and 311 nm although their maximum and minimum were slightly besides these values.

In the Joiunt IDF/ISO/AOAC Group of Experts was referred to the difficulties encountered due to interference from paprika and pepper in the analysis of fresh cheeses. The occurrence of this phenomenon will be obvious from the complete spectrum, which is altered, but can give rise to misinterpretation when the three relevant wavelengths only are measured.
Concerning these comments some remarks have been made in the prescription of the method, to prevent lack of clearness.
In case of spectrometric determination measurement of the complete spectrum has been made obligatory.
No further essential alterations in the method have been introduced.

12. CONCLUSIONS

1. The "true values" of the samples can be estimated to be:

| | | |
|---|---|---|
| Level A | 61.7 mg/kg | 3.71 mg/dm$^2$ |
| Level B | 15.4 " | 0.92 " |
| Level C | 1.31 " | 0.08 " |
| Level D | 0.30 " | 0.018 " |

were these figures are the arithmetric means of the mean values of the spectrometric and the HPLC detection.

2. More collaborators were able to carry out the spectrometric determination than the HPLC. Moreover more difficulties were reported for the HPLC determination.
So it seems that the spectrometric determination is more rigid and straightforward, and easier to carry out than the HPLC determination.

3. At level A natamycin can be determined directly by both methods. However, for the spectrometric method the coefficients of variation, $CV_r$ and $CV_R$, are less than half of those for the HPLC method.
There is no need to apply the concentration step for this level.

4. Level B can be measured directly by both methods too. The within-laboratories coefficient of variation ($CV_r$) is about the same, but the among-laboratories coefficient of variation ($CV_R$) of the HPLC is twice that of spectrometric.
For this level too, concentration is unneccessary.

5. About one third of the laboratories were not able to determine level C by direct determination (spectrometric: 11 out of 37, HPLC: 13 out of 32). After concentration all participants could obtain measurable results. $CV_r$ is slightly better for the spectrometric detection, $CV_R$ is comparable.

6. Level D cannot be measured directly by spectrometric or HPLC detection: "Not detectable" by 28 out of 37 and 17 out of 29 laboratories. After concentration for spectrometry 7 out of 27 and for HPLC 1 out of 24 laboratories did not report measurable results. For the remaining results the coefficients of variation for HPLC is better (or: less bad) than for spectrometry.

7. To judge the applicability of the method we can apply, arbitrarily, the following classification.

$CV_R$ 0-15% good
$CV_R$ 16-30% reasonable
$CV_R$ 31-45% bad
$CV_R$ >45% not detectable.

Thus, the results obtained by this collaborative study are as follows. (See table 6.)

| Level<br>mg/kg<br>mg/dm2 | A<br>60<br>3.8 | B<br>15<br>0.9 | C<br>1.7<br>0.1 | D<br>0.3<br>0.02 |
|---|---|---|---|---|
| Spectrometric direct | good | good | not at all | not at all |
| Spectrometric after concentration | no need | no need | bad | not |
| HPLC direct | reasonable | reasonable | not at all | not at all |
| HPLC after concentration | no need | no need | bad | bad |

8. The recovery at level 20 mg/kg is approximately 100%.

9. The results of the spectrometric and the HPLC method are not significant different. A contribution of degradation products of natamycin in the results of the spectrometric method is not observed.

13. EEC OFFICIAL METHOD

Based upon the results of this collaborative study the EEC ad hoc working group on natamycin adopted a method which consists of two parts, i.e.

- spectrometric without concentration for the determination of natamycin in the cheese rind, i.e. the outer 5 mm layer of the cheese, to be expressed in mg/dm$^2$;
- HPLC after 10x concentration for the determination of natamycin in the inner part of the cheese, to be expressed in mg/kg.

This method shall be presented to the Council as official method to an EEC Directive on natamycin (18).

14. IDF AND ISO STANDARD METHOD

The method is adopted by the Joint IDF/ISO/AOAC Group of Experts E 43 "Selected Food Additives" to eventually become both an IDF and an ISO Standard Method (19).

15. ACKNOWLEDGEMENTS

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G. van den Berg and C.B.G. Daanen of NIZO were involved in manufacturing, storage and treatment of the cheeses. The comprehensive statistical evaluation was carried out by Ms G.A. Werdmuller of RIKILT. The analyses prior to the intercomparison and the preparation of the samples were carried out by J.J. van Oostrom and H.J. Korbee of RIKILT.

Finally all participants are thanked for their contribution.

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Table 1.1 RESULTS, direct determination, spectrometric (mg/kg)

| LAB | Level A | | Level B | | Level C | | Level D | |
|---|---|---|---|---|---|---|---|---|
| | sample 1 | 4 | 2 | 8 | 3 | 6 | 5 | 7 |
| 1 | 63.20 | 59.40 | 16.00 | 13.20 | 0.80 | 0.40 | nd | nd |
| 5 | 61.99 | 55.88 | 14.75 | 12.85 | nd | nd | nd | nd |
| 6 | 61.37 | 56.79 | 14.01 | 13.83 | 1.01 | 1.19 | nd | nd |
| 8 | - | (31.70) | (3.30) | (20.28) | (0.25) | - | - | (0.58) |
| 9 | 62.10 | 60.90 | 16.50 | 15.90 | 1.50 | 1.35 | nd | nd |
| 11 | 59.40 | 60.20 | 15.90 | 16.00 | 1.17 | 1.17 | nd | nd |
| 12 | 64.36 | 65.42 | 15.29 | 14.02 | nd | 0.85 | nd | nd |
| 14 | 66.79 | 64.21 | 17.30 | 16.93 | nd | nd | nd | nd |
| 15 | 63.03 | 69.10 | 17.10 | 19.20 | nd | nd | nd | nd |
| 16 | 59.45 | 44.47 | 14.52 | 14.12 | 2.37 | nd | nd | nd |
| 17 | 57.08 | 47.46 | 12.82 | 13.83 | nd | nd | nd | nd |
| 18 | 70.60 | 65.20 | 15.90 | 16.80 | nd | nd | nd | nd |
| 19 | 63.90 | 56.47 | 18.80 | 17.75 | 1.60 | 1.71 | 1.30 | 0.40 |
| 20 | 75.90 | 67.10 | 18.70 | 15.50 | 2.90 | 2.60 | nd | nd |
| 21 | 59.53 | 57.13 | 14.63 | 14.61 | nd | nd | nd | nd |
| 22 | 62.30 | 61.20 | 13.60 | 13.20 | 0.76 | nd | nd | nd |
| 23 | 74.70 | 78.20 | 16.30 | 16.30 | 1.60 | 1.90 | 0.50 | 0.50 |
| 24 | 67.14 | 66.59 | 13.92 | 15.85 | 1.29 | 1.00 | nd | nd |
| 26 | 63.80 | 63.80 | 12.80 | 15.00 | 1.10 | 0.90 | nd | nd |
| 27 | 73.50 | 70.80 | 14.80 | 15.70 | 0.40 | 0.90 | nd | nd |
| 29 | 61.90 | 57.40 | 14.90 | 15.30 | 2.90 | 3.00 | 1.70 | nd |
| 30 | 60.66 | 56.56 | 15.40 | 14.53 | 0.84 | 1.25 | nd | nd |
| 31 | 54.06 | 51.34 | 10.68 | 12.15 | 2.10 | 2.30 | 8.38 | 0.42 |
| 32 | 47.50 | 40.50 | 18.10* | 10.10* | nd | nd | nd | nd |
| 33 | 73.50 | 63.90 | 15.60 | 15.80 | 1.30 | 0.80 | nd | nd |
| 34 | 68.25 | 63.42 | 16.85 | 18.26 | 0.63 | 0.63 | 0.39 | 0.38 |
| 35 | 75.90 | 70.70 | 17.60 | 16.40 | nd | 1.20 | nd | nd |
| 37 | 64.83 | 62.50 | 14.50 | 14.66 | 1.66 | 1.66 | nd | nd |
| 38 | 62.82 | 63.47 | 15.63 | 15.27 | nd | nd | nd | nd |
| 39 | 65.81 | 67.67 | 15.68 | 15.06 | 1.23 | nd | nd | nd |
| 40 | 65.50 | 61.10 | 17.00 | 16.20 | 0.70 | nd | nd | nd |
| 41 | 68.93 | 66.37 | 14.21 | 15.37 | nd | nd | nd | nd |
| 43 | 65.95 | 59.55 | 14.65 | 16.50 | 2.80 | 2.70 | nd | nd |
| 44 | 66.58 | 60.72 | 14.45 | 16.12 | 0.62 | 1.47 | nd | nd |
| 45 | 44.00 | 43.17 | 10.67 | 9.50 | 0.22 | nd | nd | nd |
| 47 | 65.49 | 67.44 | 15.81 | 13.60 | nd | nd | nd | nd |
| 48 | 67.12 | 65.12 | 15.67 | 15.30 | nd | nd | nd | nd |

nd = not detectable
- = missing
( )= method not carried out as described
* = outlier

Table 1.2 RESULTS, direct determination, HPLC (mg/kg)

| LAB | Level A | | Level B | | Level C | | Level D | |
|---|---|---|---|---|---|---|---|---|
| | sample 1 | 4 | 2 | 8 | 3 | 6 | 5 | 7 |
| 1 | 58.74 | 53.43 | 14.69 | 12.15 | 1.85 | nd | nd | nd |
| 4 | 63.00 | 60.20 | 15.10 | 15.60 | nd | nd | nd | nd |
| 5 | 52.58 | 49.83 | 15.66 | 12.63 | nd | nd | nd | nd |
| 6 | 58.62 | 54.04 | 15.57 | 14.56 | 1.65 | 2.47 | nd | nd |
| 9 | 62.43 | 61.66 | 16.57 | 13.88 | 1.93 | 2.31 | 0.77 | 0.58 |
| 11 | (61.20) | (61.80) | (16.20) | (15.90) | (1.70) | (1.90) | (0.80) | (0,50) |
| 12 | 63.76 | 68.04 | 17.10 | 17.66 | 1.77 | 1.93 | 0.48 | 0.46 |
| 15 | (67.50) | (71.20) | (16.80) | (17.30) | (2.00) | (2.00) | (nd) | (nd) |
| 16 | 54.37 | 31.04 | 13.54 | 13.14 | nd | nd | nd | nd |
| 17 | 54.96 | 35.04 | 7.56 | 14.29* | nd | nd | nd | nd |
| 18 | 69.40 | 67.00 | 17.80 | 16.40 | 2.60 | 1.90 | 0.60 | 0.50 |
| 19 | 85.10* | 17.09* | 25.90* | 12.55* | 3.13 | 2.43 | 1.90 | 0.14 |
| 20 | 77.60 | 85.00 | 18.00 | 19.80 | 1.80 | 2.50 | nd | nd |
| 21 | (52.05) | (54.02) | (14.73) | (14.49) | (nd) | (nd) | (nd) | (nd) |
| 22 | 39.80 | 34.20 | nd* | nd* | nd | nd | nd | nd |
| 23 | 63.80 | 63.60 | 14.10 | 14.70 | 1.80 | 1.80 | nd | nd |
| 24 | 62.56 | 67.42 | 14.38 | 14.38 | nd | nd | nd | nd |
| 26 | 56.50 | 57.80 | 10.50 | 12.20 | nd | nd | nd | nd |
| 27 | 64.58 | 50.70 | 13.95 | 13.95 | 2.06 | 2.79 | nd | nd |
| 30 | (45.30) | (43.10) | (11.90) | (10.00) | (1.10) | (1.20) | (nd) | (nd) |
| 32 | 54.30 | 49.20 | 15.70 | 13.80 | nd | nd | nd | nd |
| 33 | 81.70 | 69.60 | 17.20 | 18.50 | nd | nd | nd | nd |
| 35 | 68.60 | 61.90 | 9.90 | 9.64 | nd | 1.00 | nd | nd |
| 37 | 66.33 | 60.33 | 12.17 | 12.50 | 1.33 | 1.33 | 0.33 | 0.33 |
| 38 | 53.15 | 53.52 | 13.50 | 13.38 | 1.50 | 1.04 | 0.15 | 0.15 |
| 39 | 64.84 | 65.87 | 15.77 | 14.87 | 2.63 | 2.32 | 1.18 | 1.20 |
| 40 | 87.00 | 92.00 | 23.00 | 25.00 | 8.00 | nd | nd | nd |
| 41 | 68.70 | 66.64 | 8.11 | 9.25 | nd | nd | nd | nd |
| 43 | (64.10) | (59.55) | (18.30) | (17.40) | (2.75) | (2.75) | (0.90) | (nd) |
| 45 | 42.67 | 42.33 | 13.50* | 4.73* | nd | 2.00 | nd | nd |
| 47 | 65.10 | 69.00 | 23.00 | 23.60 | nd | nd | nd | nd |
| 48 | 71.00 | 66.20 | 21.10 | 24.40 | nd | nd | nd | nd |

nd = not detectable
( )= method not carried out as described
* = outlier

Table 2.1 RESULTS, after concentration, spectrometric (mg/kg)

| LAB | Level B | | | | Level C | | | | Level D | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | conc. | sample 2 | conc. | sample 8 | conc. | sample 3 | conc. | sample 6 | conc. | sample 5 | conc. | sample 7 |
| 1 | - | - | - | - | 10 | 1.26 | 10 | 1.18 | 10 | 0.14 | 10 | 0.18 |
| 5 | - | - | - | - | 10 | 0.84 | 5 | 1.37 | 10 | nd | 10 | 0.26 |
| 6 | - | - | - | - | 10 | 0.92 | 10 | 1.10 | 10 | 0.18 | 10 | 0.27 |
| 9 | - | - | - | - | 5 | 1.32 | 5 | 1.56 | 10 | 0.31 | 10 | 0.30 |
| 12 | - | - | - | - | 10 | 0.87 | 10 | 0.94 | 10 | 0.21 | 10 | 0.25 |
| 16 | - | - | - | - | 5 | 1.09 | 10 | 0.85 | 10 | 0.24 | 10 | 0.37 |
| 17 | 5 | 10.60 | - | - | 5 | 1.07 | 10 | 1.21 | 10 | 0.42 | 10 | 0.05 |
| 18 | - | - | - | - | 10 | 1.50 | 10 | 1.10 | 10 | nd* | 10 | nd* |
| 19 | - | - | - | - | - | - | 5 | 1.82 | 5 | 0.52 | 5 | 0.38 |
| 20 | - | - | - | - | - | - | - | - | 10 | nd* | 10 | nd* |
| 21 | 5 | 11.88 | 5 | 5.31 | 10 | 0.61 | 10 | nd | 10 | nd* | 10 | nd* |
| 22 | 10 | (12.90) | 10 | (12.90) | 10 | (0.74) | 10 | (0.85) | 10 | (0.21) | 10 | (0.14) |
| 23 | - | - | - | - | 10 | 1.20 | 10 | 1.50 | 10 | 0.30 | 5 | 0.30 |
| 24 | - | - | - | - | 5 | 1.58 | 10 | 1.25 | 5 | 0.16 | 5 | 0.28 |
| 26 | - | - | - | - | 10 | 0.60 | 10 | 0.30 | 10 | nd* | 10 | nd* |
| 27 | - | - | - | - | 10 | 1.17 | 10 | 0.66 | 10 | 0.09 | 10 | nd |
| 30 | - | - | - | - | 10 | 1.04 | 10 | 1.15 | 5 | 0.16 | 10 | 0.15 |
| 31 | - | - | - | - | 10 | 4.35* | - | -* | - | - | 10 | 0.64 |
| 32 | - | - | - | - | 10 | 1.50 | 10 | 1.42 | 10 | 0.28 | 10 | nd |
| 33 | - | - | - | - | 5 | 1.60 | 5 | 1.60 | 5 | 0.70 | 5 | 0.50 |
| 35 | - | - | - | - | 10 | 1.26 | 10 | 1.64 | 10 | 0.38 | 10 | 0.41 |
| 37 | - | - | - | - | 5 | 1.67 | 5 | 1.83 | 10 | 0.33 | 10 | 0.33 |
| 38 | - | - | 5 | 13.39 | 10 | 1.01 | 10 | 0.88 | 10 | nd* | 10 | nd* |
| 39 | - | - | - | - | 5 | 1.82 | 5 | 1.78 | 10 | 0.30 | 10 | 0.30 |
| 40 | 5 | 35.20 | 5 | 54.10 | 10 | 13.70* | 10 | 6.70* | 10 | 2.40* | 10 | nd* |
| 41 | - | - | - | - | 10 | 1.43 | 10 | 1.23 | 10 | 0.16 | 10 | 0.50 |
| 43 | - | - | - | - | - | - | 10 | 2.00 | - | - | - | - |
| 44 | 5 | 12.44 | 5 | 5.73 | 10 | 0.55 | 10 | 0.73 | 10 | nd* | 10 | nd* |
| 45 | - | - | 10 | 6.50 | - | - | 10 | 0.75 | 10 | 0.17 | 10 | 0.10 |
| 47 | - | - | - | - | 5.6 | 1.19 | 5.6 | 1.29 | 5.6 | nd* | 5.6 | nd* |
| 48 | - | - | - | - | 5.5 | 1.34 | 5.5 | 1.18 | 5.5 | nd* | 5.5 | nd* |

nd = not detectable
- = missing
( )= method not carried out as described
* = outlier

Table 2.2 RESULTS, after concentration, HPLC (mg/kg)

| LAB | Level B | | | | Level C | | | | Level D | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | conc. | sample 2 | conc. | sample 8 | conc. | sample 3 | conc. | sample 6 | conc. | sample 5 | conc. | sample 7 |
| 1 | - | - | - | - | 10 | 1.26 | 10 | 1.15 | 10 | 0.35 | 10 | 0.26 |
| 4 | - | - | - | - | 10 | 1.10 | 10 | 1.30 | 10 | nd* | 10 | nd* |
| 5 | - | - | - | - | 10 | 0.92 | 5 | 1.46 | 10 | 0.24 | 10 | 0.32 |
| 6 | - | - | - | - | 10 | 0.92 | 10 | 1.37 | 10 | 0.22 | 10 | 0.23 |
| 9 | - | - | - | - | 5 | 1.00 | 5 | 1.08 | 10 | 0.31 | 10 | 0.31 |
| 12 | - | - | - | - | 5 | 1.31 | 5 | 1.48 | 10 | 0.32 | 10 | 0.23 |
| 16 | - | - | - | - | 5 | 0.79 | 10 | 0.82 | 10 | 0.27 | 10 | 0.24 |
| 17 | 5 | 7.42 | - | - | 5 | 1.13 | 10 | 2.12 | 10 | 0.36 | 10 | 0.17 |
| 18 | - | - | - | - | - | - | 5 | 1.50 | 5 | 0.50 | 5 | 0.40 |
| 20 | - | - | - | - | - | - | - | - | 10 | 0.30 | 10 | 0.50 |
| 21 | 5 | (9.97) | 5 | (5.59) | 10 | (0.63) | 10 | (0.29) | 10 | (0.12) | 10 | (0.17) |
| 22 | 10 | 11.60 | 10 | 11.70 | - | - | - | - | - | - | - | - |
| 23 | - | - | - | - | 10 | 1.10 | 10 | 1.30 | 10 | 0.40 | 5 | 0.40 |
| 24 | - | - | - | - | 5 | 1.46 | 5 | 1.77 | 10 | 0.31 | 10 | 0.38 |
| 26 | - | - | - | - | 10 | 1.10 | 10 | 0.22 | 10 | nd* | 10 | nd* |
| 27 | - | - | - | - | 10 | 1.26 | 10 | 0.73 | 10 | 0.29 | 10 | 0.18 |
| 30 | - | - | - | - | 10 | (2.80) | 10 | (2.00) | 10 | (0.50) | 10 | (nd) |
| 32 | - | - | - | - | 10 | 2.90 | 10 | 2.30 | 10 | 1.00* | 10 | 0.90* |
| 33 | - | - | - | - | 5 | 1.60 | 5 | 1.70 | 5 | 0.70 | 5 | 0.40 |
| 35 | - | - | - | - | 10 | 1.21 | 10 | 1.45 | 10 | 0.36 | 10 | 0.38 |
| 37 | - | - | - | - | 5 | 1.67 | 5 | 1.83 | 10 | 0.33 | 10 | 0.33 |
| 38 | - | - | - | - | 10 | 1.35 | 10 | 0.90 | 10 | 0.15 | 10 | nd |
| 39 | - | - | - | - | 5 | 2.15 | 5 | 2.12 | 10 | 0.42 | 10 | 0.43 |
| 41 | - | - | - | - | 10 | 0.94 | 10 | 1.28 | 10 | 0.18 | 10 | 0.43 |
| 43 | 13 | (11.00) | - | - | 17 | (2.00) | 4 | (2.00) | 17 | (0.50) | - | - |
| 45 | - | - | 10 | 8.27 | 10 | 0.88 | 10 | 2.02 | 10 | 1.45* | 10 | nd* |
| 47 | - | - | - | - | 5.5 | 2.10 | 5.5 | 2.00 | 5.5 | 0.70 | 5.5 | 0.40 |
| 48 | | | | | 5.5 | 2.00 | 5.5 | 2.30 | 5.5 | 0.40 | 5.5 | 0.50 |

nd = not detectable
- = missing
( )= method not carried out as described
* = outlier

Table 3.1 RECOVERY spectrometric

| LAB | added mg/kg | found mg/kg | found mg/kg | recovery % | recovery % | mean % | diff. % |
|---|---|---|---|---|---|---|---|
| 1 | 40.00 | 42.20 | 38.20 | 105.50 | 95.50 | 100.50 | 10.00 |
| 4 | - | - | - | - | - | - | - |
| 5 | 20.00 | 18.40 | - | 92.00 | - | 92.00 | - |
| 6 | 20.00 | 15.50 | 16.20 | 77.50 | 81.00 | 79.25 | 3.50 |
| 9 | 20.00 | 19.50 | 20.30 | 97.50 | 101.50 | 99.50 | 4.00 |
| 11 | 18.30 | 13.00 | - | 71.04 | - | 71.04 | - |
| 12 | 20.00 | 17.20 | 17.42 | 86.00 | 87.10 | 86.55 | 1.10 |
| 14 | 20.00 | 19.51 | 19.36 | 97.55 | 96.80 | 97.18 | 0.75 |
| 15 | 20.00 | 18.90 | 17.80 | 94.50 | 89.00 | 91.75 | 5.50 |
| 16 | - | - | - | - | - | - | - |
| 17 | 20.00 | 21.00 | 21.80 | 105.00 | 109.00 | 107.00 | 4.00 |
| 18 | 20.00 | 19.20 | 19.40 | 96.00 | 97.00 | 96.50 | 1.00 |
| 19 | 20.00 | 17.50 | 17.00 | 87.50 | 85.00 | 86.25 | 2.50 |
| 20 | 20.60 | 19.50 | - | 94.66 | - | 94.66 | - |
| 21 | 18.32 | 17.67 | 17.83 | 96.45 | 97.33 | 96.89 | 0.87 |
| 22 | 18.00 | 12.84 | 13.20 | 71.33 | 73.33 | 72.33 | 2.00 |
| 23 | 19.45 | 19.80 | 18.70 | 101.80 | 96.14 | 98.97 | 5.66 |
| 24 | 19.83 | 17.90 | 18.70 | 90.28 | 94.31 | 92.29 | 4.03 |
| 26 | 20.00 | 18.70 | 18.00 | 93.50 | 90.00 | 91.75 | 3.50 |
| 27 | 19.57 | 19.00 | 19.60 | 97.09 | 100.15 | 98.62 | 3.07 |
| 29 | 20.00 | 17.10 | - | 85.50 | - | 85.50 | - |
| 30 | 19.02 | 19.42 | - | 102.10 | - | 102.10 | - |
| 31 | 20.00 | 19.28 | 17.60 | 96.40 | 88.00 | 92.20 | 8.40 |
| 32 | 18.30 | 13.00 | - | 71.04 | - | 71.04 | - |
| 33 | 18.40 | 15.60 | - | 84.78 | - | 84.78 | - |
| 34 | 20.00 | 12.47 | 13.24 | 62.35 | 66.20 | 64.28 | 3.85 |
| 35 | 20.00 | 16.40 | 17.60 | 82.00 | 88.00 | 85.00 | 6.00 |
| 37 | 21.35 | 20.27 | 19.87 | 94.94 | 93.07 | 94.00 | 1.87 |
| 38 | 20.07 | 18.52 | - | 92.28 | - | 92.28 | - |
| 39 | 18.32 | 17.54 | 17.74 | 95.74 | 96.83 | 96.29 | 1.09 |
| 40 | 20.00 | 20.50 | 19.10 | 102.50 | 95.50 | 99.00 | 7.00 |
| 41 | 20.00 | 27.46 | 25.68 | 137.30 | 128.40 | 132.85 | 8.90 |
| 43 | 19.70 | 21.00 | - | 106.60 | - | 106.60 | - |
| 45 | 20.21 | 15.66 | 26.00 | 77.85 | 129.26 | 103.56 | 51.40 |
| 47 | 18.47 | 18.55 | - | 100.43 | - | 100.43 | - |
| 48 | 20.00 | 19.83 | - | 99.15 | - | 99.15 | - |

- = missing

Table 3.2 RECOVERY HPLC

| LAB | added mg/kg | found mg/kg | found mg/kg | recovery % | recovery % | mean % | diff. % |
|---|---|---|---|---|---|---|---|
| 1 | 40.00 | 39.41 | 30.42 | 98.53 | 76.05 | 87.29 | 22.48 |
| 4 | 50.00 | 51.00 | 45.70 | 102.00 | 91.40 | 96.70 | 10.60 |
| 5 | 20.00 | 18.30 | - | 91.50 | - | 91.50 | - |
| 6 | 20.00 | 19.10 | 19.10 | 95.50 | 95.50 | 95.50 | 0.00 |
| 9 | 20.00 | 20.80 | 20.40 | 104.00 | 102.00 | 103.00 | 2.00 |
| 11 | (18.30) | (19.50) | - | (106.56) | - | (106.56) | - |
| 12 | 20.00 | 18.55 | 18.76 | 92.75 | 93.80 | 93.28 | 1.05 |
| 14 | - | - | - | - | - | - | - |
| 15 | (20.00) | (16.60) | (17.20) | (83.00) | (86.00) | (84.50) | (3.00) |
| 16 | 20.00 | 19.09 | 19.31 | 95.45 | 96.55 | 96.00 | 1.10 |
| 17 | 20.00 | 22.00 | 17.55 | 110.00 | 87.75 | 98.88 | 22.25 |
| 18 | 20.00 | 20.50 | 20.70 | 102.50 | 103.50 | 103.00 | 1.00 |
| 19 | 20.00 | 20.87 | 19.31 | 104.35 | 96.55 | 100.45 | 7.80 |
| 20 | 20.60 | 23.00 | - | 111.65 | - | 111.65 | - |
| 21 | (18.32) | (14.21) | (15.69) | (77.57) | (85.64) | (81.60) | (8.08) |
| 22 | 18.00 | 15.20 | 14.40 | 84.40 | 80.00 | 82.22 | 4.44 |
| 23 | - | - | - | - | - | - | - |
| 24 | 19.83 | 15.90 | 20.10 | 80.19 | 101.37 | 90.78 | 21.18 |
| 26 | 20.00 | 20.30 | 9.40 | 101.50 | 47.00 | 74.25 | 54.50 |
| 27 | 19.57 | 14.97 | 16.88 | 76.49 | 86.25 | 81.37 | 9.76 |
| 29 | - | - | - | - | - | - | - |
| 30 | - | - | - | - | - | - | - |
| 31 | - | - | - | - | - | - | - |
| 32 | 18.30 | 17.40 | - | 95.08 | - | 95.08 | - |
| 33 | 18.40 | 16.00 | - | 86.96 | - | 86.96 | - |
| 34 | - | - | - | - | - | - | - |
| 35 | 20.00 | 14.60 | 12.40 | 73.00 | 62.00 | 67.50 | 11.00 |
| 37 | - | - | - | - | - | - | - |
| 38 | 20.07 | 17.84 | - | 88.89 | - | 88.89 | - |
| 39 | 18.32 | 19.05 | 19.02 | 103.99 | 103.82 | 103.90 | 0.16 |
| 40 | 20.00 | 24.00 | 20.00 | 120.00 | 100.00 | 110.00 | 20.00 |
| 41 | 20.00 | 16.30 | 15.80 | 81.50 | 79.00 | 80.25 | 2.50 |
| 43 | (19.70) | (24.00) | - | (121.83) | - | (121.83) | - |
| 45 | 20.12 | 14.00 | 26.50 | 69.60 | 131.74 | 100.67 | 62.14 |
| 47 | 18.47 | 16.40 | - | 88.79 | - | 88.79 | - |
| 48 | 20.00 | 18.90 | - | 94.50 | - | 94.50 | - |

- = missing
( )= method not carried out as described

Table 4 Mean values of results A and B direct, C and D after concentration

| LAB | A direct | | B direct | | C after concentration | | D after concentration | |
|---|---|---|---|---|---|---|---|---|
| | spec. | HPLC | spec. | HPLC | spec. | HPLC | spec. | HPLC |
| 1 | 61.30 | 56.09 | 14.60 | 13.42 | 1.22 | 1.21 | 0.16 | 0.31 |
| 4 | " | 61.60 | " | 15.35 | " | 1.20 | " | " |
| 5 | 58.94 | 51.21 | 13.80 | 14.15 | 1.11 | 1.19 | 0.13[b] | 0.28 |
| 6 | 59.08 | 56.33 | 13.92 | 15.07 | 1.01 | 1.15 | 0.23 | 0.23 |
| 9 | 61.50 | 62.05 | 16.20 | 15.23 | 1.44 | 1.04 | 0.31 | 0.31 |
| 11 | 59.80 | " | 15.95* | " | " | " | " | " |
| 12 | 64.89 | 65.90 | 14.66 | 17.38 | 0.91 | 1.40 | 0.23 | 0.28 |
| 14 | 65.50 | " | 11.12 | " | " | " | " | " |
| 15 | 66.07 | " | 18.15 | " | " | " | " | " |
| 16 | 51.96 | 42.71 | 14.32 | 13.34 | 0.97 | 0.81 | 0.31 | 0.26 |
| 17 | 52.27 | 45.00 | 13.32 | " | 1.14 | 1.63 | 0.24 | 0.27 |
| 18 | 67.90 | 68.20 | 16.35 | 17.10 | 1.30 | 1.50[a] | " | 0.45 |
| 19 | 60.19 | " | 18.28 | " | 1.82[a] | " | 0.45 | " |
| 20 | 71.50 | 81.30 | 17.10 | 18.90 | " | " | " | 0.40 |
| 21 | 58.33 | " | 14.62 | " | 0.31[b] | " | " | " |
| 22 | 61.75 | 37.00 | 13.40 | " | " | " | " | " |
| 23 | 76.45 | 63.70 | 16.30 | 14.40 | 1.35 | 1.20 | 0.30 | 0.40 |
| 24 | 66.87 | 64.99 | 14.89 | 14.38 | 1.42 | 1.62 | 0.22 | 0.35 |
| 26 | 63.80 | 57.15 | 13.90 | 11.35 | 0.45 | 0.66 | " | " |
| 27 | 72.15 | 57.64 | 15.25 | 13.95 | 0.92 | 1.00 | 0.05[b] | 0.24 |
| 29 | 59.65 | " | 15.10 | " | " | " | " | " |
| 30 | 58.61 | " | 14.93 | " | 1.10 | " | 0.16 | " |
| 31 | 52.70 | " | 11.42 | " | " | " | 0.64[a] | " |
| 32 | 44.00 | 51.75 | " | 14.75 | 1.46 | 2.60 | 0.14[b] | " |
| 33 | 68.70 | 75.65 | 15.70 | 17.85 | 1.60 | 1.65 | 0.60 | 0.55 |
| 34 | 65.84 | " | 17.56 | " | " | " | " | " |
| 35 | 73.30 | 65.25 | 17.00 | 9.77 | 1.45 | 1.33 | 0.40 | 0.37 |
| 37 | 63.67 | 63.33 | 14.58 | 12.34 | 1.75 | 1.75 | 0.33 | 0.33 |
| 38 | 63.15 | 53.34 | 15.45 | 13.44 | 0.95 | 1.13 | " | 0.08[b] |
| 39 | 66.74 | 65.36 | 15.37 | 15.32 | 1.80 | 2.14 | 0.30 | " |
| 40 | 63.30 | 89.50 | 16.60 | 24.00 | " | " | " | 0.43 |
| 41 | 61.65 | 67.67 | 14.79 | 8.68 | 1.33 | 1.11 | 0.33 | 0.31 |
| 43 | 62.75 | | 15.58 | | 2.00[a] | " | " | " |
| 44 | 63.65 | " | 15.29 | " | 0.64 | " | " | " |
| 45 | 43.59 | 42.50 | 10.09 | " | 0.75[a] | 1.45 | 0.14 | " |
| 47 | 66.47 | 67.05 | 14.71 | 23.30 | 1.24 | 2.05 | " | 0.55 |
| 48 | 66.12 | 68.60 | 15.49 | 22.75 | 1.26 | 2.15 | " | 0.45 |
| n | 36 | 26 | 35 | 23 | 27 | 23 | 20 | 20 |
| x | 62.50 | 60.80 | 15.19 | 15.49 | 1.21 | 1.43 | 0.28 | 0.34 |
| $s_x$ | 7.14 | 11.90 | 1.68 | 3.91 | 0.41 | 0.47 | 0.15 | 0.11 |
| $s_{\bar{x}}$ | 1.19 | 2.33 | 0.28 | 0.82 | 0.079 | 0.098 | 0.034 | 0.025 |
| | 1.70 | | -0.293 | | -0,222 | | -0,059 | |
| s | 2.62 | | 0.86 | | 0.125 | | 0.042 | |
| t | 0.65 | | -0.34 | | -1.77 | | -1.40 | |

a = duplicate omitted
b = duplicate not detectable

8547.25

Table 5 Results of natamycin determinations in collaborative study 1984

| | Level A direct | | | Level B direct | | | Level C after concentration | | | Level D after concentration | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | spectr. | HPLC | Sign. diff. | spectr. | HPLC | Sign. diff. | spectr. | HPLC | Sign. diff. | spectr. | HPLC | Sign. diff. |
| Number of participants | 36 | 27 | | 36 | 27 | | 29 | 23 | | 29 | 24 | |
| Number of outliers | 0 | 1 | | 1 | 4 | | 2 | 0 | | 9 | 4 | |
| % outliers | 0 | 4 | | 3 | 15 | | 7 | 0 | | 31 | 17 | |
| Results in mg/dm$^2$ | | | | | | | | | | | | |
| x 1) | 3.75 | 3.65 | no | 0.91 | 0.93 | no | 0.071 | 0.085 | no | 0.016 | 0.020 | no |
| r | 0.61 | 0.96 | ? | 0.16 | 0.19 | no | 0.034 | 0.057 | yes | 0.019 | 0.017 | no |
| 2.83 $s_{lab}$ | 1.13 | 1.90 | yes | 0.26 | 0.65 | yes | 0.062 | 0.070 | no | 0,019 | 0,016 | no |
| R | 1.29 | 2.13 | | 0.31 | 0.68 | | 0.070 | 0.089 | | 0.028 | 0,023 | |
| Results in mg/kg | | | | | | | | | | | | |
| x 1) | 62.5 | 60.8 | no | 15.2 | 15.5 | no | 1.19 | 1.43 | no | 0.27 | 0.34 | no |
| r | 10.4 | 16.0 | ? | 2.7 | 3.1 | no | 0.56 | 0.95 | yes | 0.32 | 0.28 | no |
| 2.83 $s_{lab}$ | 18.8 | 31.7 | yes | 4.4 | 10.8 | yes | 1.03 | 1.16 | no | 0.32 | 0.25 | no |
| R | 21.5 | 35.5 | | 5.1 | 11.3 | | 1.17 | 1.49 | | 0.46 | 0.38 | |
| Coefficients of variation | | | | | | | | | | | | |
| $CV_r$ % | 5.9 | 9.3 | | 6.2 | 7.1 | | 16.5 | 23.4 | | 42.5 | 29 | |
| $CV_1$ % | 10.6 | 18.4 | | 10.2 | 24.7 | | 31 | 29 | | 42.6 | 26 | |
| $CV_R$ % | 12.2 | 20.6 | | 11.9 | 25.7 | | 35 | 37 | | 60 | 39 | |

1) weight value

Remarks of participants

We remarked a rather rapid degradation of natamycin, when dissolved in methanol-water, as it is the case for sample extracts (without concentration step) and diluted standard solutions.

When performing the recovery experiments with local untreated cheese, we noted decreasing peak heights for added natamycin in the course of the day, while simultaneously a peak of a degradation product emerged (retention time of natamycin : 10,2 min, of degradation product : 8,2 min)

We therefore tried to inject sample extracts as rapidly as possible after preparation.

For samples, which had to be concentrated before final determination, you proposed to fill up the eluate from the Sepak cartridge to 5 ml with methanol. We would prefer to fill up with water and to filter the solution, before injecting into the HPLC, in order to reduce the amount of substances which decrease column life.

1. Notice should be given at the beginning of the determination procedure that all the process should be carried out as much as possible light-shaded since natamycin is particularly unstable against light.

2. Natamycin concentration in HPLC had better be expressed not as ng/20ml but as µg/ml (ppm). In this case, the calculation formula of natamycin concentration in cheese (mg/kg) should be changed from C = 0.75 x B to C (mg/kg) = 15 x B' (µg/ml).

4. Recovery tests should be carried out at lower spikage levels, too (e.g. 5.0 and 1.0 mg/kg).

5. Explanatory notes had better be given for calculation formulae.

1. As in the first study our columns have other dimensions, analytical column: 120 mm x 4,6 mm i.d., and guard column: 100 mm x 3 mm i.d.

2. The results for the spectrophotometric procedure are based on drawing of the line from the absorption at 311 nm to the absorption at 329 nm.

3. Generally we use two injections in the HPLC, when the calculation is based on an external standard, so in this study we have also used two injections pro sample.

4. The final solutions of sample no. 1 and sample no. 4 are diluted 10x to keep the attenuation of the recorder fixed at 0,005, and to be within the range of the standard curve.

5. One of the samples, no. 3 is measured at the spectrofotometer after both 5x and 10x concentration. In the evaluation of the study You have to use the result after 10x concentration because generally the high concentration is necessary. We only give You the figures for 5x concentration to show You that the result is higher.

7. The calculation of the natamycin content according to the formulas 13), 16), and the formulas on the pages 8, 9, and 10 are given in reduced form. It is more informative first to give the full formula, and You have to give all formulas number.

Eine weitere Verzögerung ergab sich, weil die von Ihnen angegebenen Bedingungen für die HPLC (Fließmittelzusammensetzung) bei uns kein vernünftiges Chromatogramm ergaben. Bei der geforderten Verwendung von Methanol/Wasser/Essigsäure = 60/40/5 (V:V:V) als Fließmittel erhielten wir einen zu breiten Natamycinpeak nach einer zu langen Retention von ca. 2228 sec. (37 min) (siehe beiliegendes Chromatogramm). Wir haben daraufhin die Zusammensetzung des angegebenen Fließmittels vielfach verändert, jedoch mit wenig Erfolg für vernünftige Peaks und kürzere Retentionszeiten. Deshalb haben wir uns entschlossen, das von uns in der 'Milchwissenschaft' 38 (3), 145-147 (1983) angegebene Fließmittel Methanol/ Phosphatpuffer = 70/30 (V:V; 3,026 g $KH_2PO_4$ in 1 Liter $H_2O$ bidest.) zu verwenden. Säulenmaterial und Fließgeschwindigkeit entsprechen ansonsten Ihrer Vorschrift.

Weiterhin konnten wir nach Ihrer Vorschrift keine Konzentrierung der Proben 3, 5, 6 und 7 erreichen (s. Chromatogramme: Versuch 1, 3, 5, 6 und 7 zehnfach konzentriert), was insbesondere für die spektralphotometrische Bestimmung erforderlich gewesen wäre. Auch Probe 1 mit hohem Natamycingehalt ließ sich nicht konzentrieren. Die Gründe hierfür sind uns nicht bekannt. In Abänderung Ihrer Vorschrift verwendeten wir lediglich die C 18 Kartuschen von Chrompack anstelle der von Waters.

1) Die Proben und der Standard wurden nach Erhalt bis zum Untersuchungsbeginn bei -18°C gelagert. Die Untersuchungen wurden vom 20.8. - 25.8.84 vorgenommen.

4) Bei den Proben 1 und 4 sind deutliche Unterschiede in den Werten, die nach der HPLC- und der UV-Methode ermittelt wurden, festzustellen.

Wenn die dazugehörigen HPLC-Chromatogramme betrachtet werden, kann man vor dem eigentlichen Natamycin-Peak zwei mehr oder weniger deutliche Peaks mit Retentionszeiten von ca. 16-17 Minuten erkennen. Solche Vorpeaks treten auch bei reinen alten Standards auf, man kann sie wohl als Signale für Natamycin Abbauprodukte ansehen. Diese Abbauprodukte wurden aber bei der UV-Bestimmung als "Gesamt-Natamycin" miterfaßt. An einem Beispiel sei dies demonstriert:

| | | |
|---|---|---|
| Probe 1 | Natamycin-Peak, Höhenwert ..... | 59461 |
| | daraus berechneter Gehalt, mg/kg..... | 59.36 |
| | Natamycin-Peak und Vorpeaks | |
| | Summe der Höhenwerte ..... | 63.26 |
| | "Gesamt-Natamycin" Gehalt, UV, mg/kg | 64.90 |

Wir wollen dem Problem der Bildung der Abbauprodukte weiter nachgehen.

5) Ist es nicht möglich zur Vereinfachung des Untersuchungsablaufes, die spektralphotometrische Detektion in Lösung 8.8.1 durchzuführen und auf 8.7.1 bis 8.7.3 zu verzichten?

We regret we have experienced severe difficulties with the H.P.L.C. aspects of the above trial. In general we are experiencing prolonged retention times and in view of this poor sensitivity due to reduced and diffuse peaks.

1. In our institute the relative unusual column (150 x 4,6 mm id) as described in 3.17 was not available. Therefore we employed a Radial-Pak-C18-cartridge (Waters) with the dimensions 100 x 8 mm, equipped with a C18-Guard-PAK precolumn.

2. The retention times of natamycin were less stable as usual in HPLC but fluctuated although flow and pressure of the system seemed to be constant. Perhaps some proteins remain in solution during the preparation of the samples, and later influence the retention times on the HPLC-column.

3. The diluted solutions described in 7.2.3 with natamycin contents in the range of 0,1-0,8 ppm could not be analysed successful by our HPLC-system injecting only 20 microliters. Therefore we injected 200 µl to test the linearity of the calibration curve. However in the case of samples with low natamycin contents this proceeding caused poor results, for the natamycin peaks shifted forwards sometimes from 9-10 to 3-4 minutes. These shifts may also be caused by remaining proteins in the sample solutions, as suggested before.

4. The procedure of concentration with sep-pak cartridges seems to be not allways reproducible, and the rates of recovery are varying. The dilution step in 8.2 causes precipitates, which perhaps bind some natamycin.

**Ayant utilisé pour le dosage par HPLC une colonne RP8 dont les performances se sont révelées très médiocres, nous avons dû refaire une colonne dont les caractéristiques sont différentes:**

**L : 150 mm**

**ID : 4,6 mm**

**Phase: Nucléosil C 18 dp : 5 µm**

**Une seconde extraction a dû être réalisée avec le reste des échantillons, en ajoutant un volume de solvant proportionnel à la quantité prélevée (ce que nous avons indiqué dans une colonne "Solvent").**

Joint ISO/IDF/AOAC group of experts E 43 - Selected Food Additives

Members

Carl Dr M. (FRG)

Crow Mr J. (Australia)

Cvak Dr Z. (Czechoslovakia)

Gray Mr I.K. (New Zealand)

Hollman ir P.C.H. (the Netherlands)

Iwaida Dr Masahiro (Japan)

Kosikowski Dr F. (USA)

Linaza Dra Maria Teresa (Spain)

Mair-Waldburg Dr H. (FRG)

Overström Dr H. (Sweden)

Stijve Dr M.T. (Switzerland)

Weik Dr R.W. (AOAC)

EEC Working Group of Experts responsible for the method of analysis for natamycin.

| | |
|---|---|
| Belgium | : M.J.M. de Groodt |
| | M.D. Dewulf |
| Denmark | : Mrs M. Guldberg |
| | B. Christensen |
| Federal Republic of Germany | : R. Tiebach |
| France | : Mrs C. Soulès |
| Greece | : E. Tsigoridas |
| Italy | : M. Aureli |
| Netherlands | : W.G. de Ruig |
| United Kingdom | : R. Wood |
| Commission | : Mrs O. Demine |

List of collaborators

J. Crow, Dairy Technical Services, Melbourne, (Australia)

V. Eckelmans, Ch.R. Winné, Centraal Laboratorium Ministerie van Economische Zaken, Brussel (Belgium)

Schneider, Milchwirtschaftliche Untersuchungsanstalt München der Landesvereinigung der Bayerischen Milchwirtschaft e.V., Munchen (B.R.D.)

G. Engel, Institut für Mikrobiologie Bundesanstalt für Milchforschung, Kiel (B.R.D.)

W. Frede, Chemische und Lebensmitteluntersuchungsanstalt im Hygienischen Institut, Hamburg (B.R.D.)

R. Tiebach, Max von Pettenkofer-Institut des Bundesgesundheitsamtes, Berlin (B.R.D.)

Führling, Landesanstalt für Lebensmittel, Arzneimittel und gerichtliche Chemie, Berlin (B.R.D.)

Ch. Gertz, Chemische Untersuchungsanstalt, Hagen (B.R.D.)

H. Köbler, Chemische Landesuntersuchungsanstalt Stuttgart, Stuttgart (B.R.D.)

B. Hoffmann, Chemisches Landesuntersuchungsamt Nordrhein-Westfalen, Munster (B.R.D.)

Mrs S. Littman, c/o Chem. Untersuchungsamt, Hamm (B.R.D.)

Z. Cvak, Vyzkumny ustav mlékarensky, Praha (Czechoslovakia)

Mrs M. Guldborg, National Food Institute, The Central Laboratory, Div. A: Nutrients and Food Additives, Søborg (Denmark)

Mrs A. Dupont, Laboratoire Central de Recherche et d'Analyse, Massy (Essonne) (France)

E. Tsigaridas, General Chemical State Laboratory, Athens (Greece)

Masahiro Iwaida, Nestlé K.K., Kobe Port (Japan)

J. Daenen, Food Inspection Service, Maastricht (Netherlands)

J.P. Geerts, Cheese Control Station "Friesland", Leeuwarden (Netherlands)

W.G. de Ruig, State Institute for Quality Control of Agricultural Products, Wageningen, The Netherlands

C. Repelius, Gist-Brocades NV, Delft (Netherlands)

J. Leenheer, Netherlands Inspection Institute for Milk and Milk Products, Leusden (Netherlands)

J.M. Nuijens, Food Inspection Service, Haarlem (Netherlands)

S. v.d. Reek, DMV-Campina B.V., Veghel (Netherlands)

Mrs T. Linazo Iglesias, Laboratorio Agrario del Estado en Madrid, Madrid (Spain)

Mrs I. Ibanez Rico, Laboratorio Arbitral Central de Frandes, Madrid (Spain)

H. Overström, SMR:s Central Laboratorium, Molmö (Sweden)

M.T.Stijve, Société d'Assistance Technique pour Produits Nestlé S.A., La Tour de Peilz (Switzerland)

M.J. Scotter, Ministry of Agriculture, Fisheries and Food, London (UK)

D. Scuffam, Laboratory of the Government Chemist, London (UK)

M. Barnett, Dr Augustus Voelcker and Sons Ltd, London (UK)

J.A. Palgrave, Moir and Palgrave, London (UK)

E.C. Conchie, Ruddock and Sherratt Public Analyst's Laboratory, Chester (UK)

V. Husbands, Department of Regional Chemist, Strathclyde Regional Council, Glasgow (UK)

T.E. Johnson, Herbert J Evans and Partners (UK)

C.N. Grange, Bostock Hill and Rigby, Birmingham (UK)

R. Fawcett, Kent County Analytical Laboratory, Kent (UK)

J.H. Shelton, Leo Taylor and Lucke, Bedford House, London (UK)

A.J. Harrison, County of Avon Scientific Services, Bristol (UK)

Annex 6

1985-04-01

# DETERMINATION OF THE NATAMYCIN CONTENT OF CHEESE RIND AND CHEESE

Content

# DETERMINATION OF THE NATAMYCIN CONTENT OF CHEESE RIND AND CHEESE

## 1 SCOPE AND FIELD OF APPLICATION

This Standard describes a method for determining the natamycin content of cheese rind and cheese.

The limit of detection in the interior of the cheese is 2 mg/kg.

The limit of determination in the cheese rind is 0,5 mg/dm$^2$ *).

### 1.1 Reference

ISO 707 Milk and milk products - Methods of sampling.

### 1.2 Definition

'Natamycin content' means the amount of this substance, as determined by the method described below, expressed in mg/dm$^2$.

### 1.3 Principle

A weighed quantity of sample is extracted with methanol. The extract is diluted with water to precipitate most of the fat and is then cooled.

After filtration and clean-up the natamycin content is determined by a spectrometric or an HPLC method.

For low concentrations a concentration step is included before measurement.

## 2 REAGENTS AND REFERENCE SUBSTANCES

Note: Brand names are mentioned for identification only, and do not exclude other brands, which may satisfy as well.

2.1 Methanol, chemically pure.

2.2 Aqueous methanol, prepared by mixing two volumes of methanol with one volume of water.

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*) These limits were fixed by the Joint IDF/ISO/AOAC Group of Experts E 43 "Selected Food Additives" in its meeting of 7 May 1985, based upon the results of a collaborative study carried out in 1984.

2.3 Natamycin preparation, with a known natamycin content = P %.

Note: When using this preparation as a standard take the natamycin content into account (see 7.1.1.1).

2.4 Acetic acid, glacial p.a.

3 APPARATUS, GLASSWARE AND AUXILIARY EQUIPMENT

3.1 Balance, capable of weighing to 1 mg.

3.2 Slicing machinery

3.2.1 For the analysis of cheese rinds:
a Slicer, or similar apparatus that will enable portions of cheese rind 5 mm thick and about 3 cm wide to be obtained (see figure 1).

3.2.2 For the analysis of cheese:
Fine-slicer, for cutting slices of cheese 0.7 mm thick. See figure 2.
Note: A David planning-machine is suitable.

3.3 Grinder or blender
Note: A Moulinex 'Moulinette' is suitable.

3.4 A sharp knife, for cutting slices of cheese into small pieces.

3.5 Magnetic stirrer or shaking-machine.

3.6 Measuring cylinders, 100 and 50 ml.

3.7 Conical flasks, 200 ml, of coloured glass with ground-glass stoppers.

3.8 Volumetric flasks, 100 and 50 ml.

3.9 Pipette, 5 ml.

3.10 Disposable syringes, 10 ml.

3.11 Microfilters, 0,45 µm pore size
0,20 µm pore size
(resistent to attack from alcoholic solutions).

3.12 Folded filters, ϕ 15 cm.

3.13 Funnel, about 7 cm in diameter.

3.14 Spectrometer, suitable for measurements at wavelengths of about 310 nm, about 317 nm and also 329 nm, equipped with cells having an optical path of 1 cm.

3.15 Freezer, operating in the temperature range -15 to -20°C.

3.16 Liquid Chromatograph with U.V. detector and recorder and/or integrator.

3.17 Analytical column stainless steel: 150 mm x 4.6 mm id, packed with Lichrosorb RP 8, particle size 5 µm.

3.18 Guard column stainless steel: 100 mm x 2.1 mm id, packed with Perisorb RP 8, particle size 30-40 µm.

3.19 Sep-pak C18 cartridges, Waters no. 51910.

## 4 SAMPLING

See ISO 707.
A whole cheese, or a segment of a cheese representative of the whole, shall be presented to the laboratory.

## 5 PREPARATION OF THE SAMPLE

5.1 Cheese rind laboratory sample.

5.1.2 If necessary, cut the sector or portion sample into smaller sectors or portions so that the width of the cheese rind is not more than about 3 cm.

5.1.3 Cut the whole rind to a thickness of 5 mm from the sectors or portion thus obtained.

5.1.4 Cut from the rind obtained a rectangular piece and measure the surface in $cm^2$ (about 20-40 $cm^2$), weigh the piece in g. Note the surface and mass.

5.1.5 Grate carefully and mix the whole cheese rind, including the weighed and measured piece. Transfer immediately to a sample jar a quantity of the sample thus prepared.

5.1.6 Clean, after each sample, all tools which have been in contact with the cheese or cheese rind, first with hot water followed by methanol and dry thoroughly for instance with a stream of compressed air.

5.2 Cheese laboratory sample.

5.2.1 After removing the rind as described in paragraph 5.1.3, slice with the fine-slicer (3.2.2) the whole of the outer section of the cheese as prepared in paragraph 5.1.2.

5.2.2 Cut from the slices of cheese a rectangular piece and measure the surface in $cm^2$ (about 20-40 $cm^2$), weigh the piece in g. Note the surface and mass.

5.2.3 Cut all the slices of cheese - including the weighed and measured piece of cheese - into small pieces of 1 to 2 mm and mix carefully. Transfer immediately to a sample jar a quantity of the sample thus prepared.

5.2.4 Clear, after each sample, all tools which have been in contact with the cheese first with hot water followed by methanol and dry thoroughly for instance with a stream of compressed air.

## 6 DETERMINATION

6.1 In the case of cheese rind, accurately weigh, to the nearest 10 mg, about 10 g of the test sample for analysis into a 200 ml conical flask and add 100 ml of methanol (2.1).
In the case of cheese, accurately weigh, to the nearest 10 mg, about 5 g of the test sample for analysis into 100 ml conical flask and add 50 ml of methanol (2.1).
Stir the contents of the conical flask for 90 min with a magnetic stirrer or shake for 90 min in a shaking-machine.

6.2 If cheese rind, add 50 ml of water.
If cheese, add 25 ml of water.

6.3 Place the conical flask in the freezer immediately and allow to stand for about 60 min.

6.4 Filter the cold extract through a folded filter, discarding the first 5 ml of filtrate.
Bring the filtrate to room temperature.
Note: The filtration has to be carried out when the suspension is still cold. When warmed up, there is a risk of turbid filtrates.

6.5 Put a part of the filtrate in a syringe (3.10) and filter through a microfilter of 0,45 µm pore size and then through a microfilter of 0.20 µm pore size (5.11).

## 7 DETECTION

### 7.1 Spectrometric detection

7.1.1 Measuring standard solution.

7.1.1.1 Immediately before use, dissolve 50 mg of 100% natamycin (calculated from 50 x $\frac{100}{P}$ mg of natamycin standard) (2.3) in 100 ml of methanol (2.1).

Take 5 ml of this solution and dilute to 50 ml with aqueous methanol (2.2), then dilute 5 ml of the diluted solution again with aqueous methanol (2.2) to 50 ml.
The natamycin concentration of the final solution in 5 µg/ml. Use this solution, or another solution with appropriate concentration close to that of the sample solution, measured in 7.1.2, for calibration of the apparatus.

7.1.1.2 Record the spectrum of the standard solution in the range 300~340 nm. Measure the absorption at the maximum at about 317 nm, the minimum of about 311 nm and at 329 nm exactly. Use aqueous methanol (2.2) as a blank.
Note: As natamycin is instable in aqueous methanol, measure as rapidly as possible.

7.1.1.3 Calculate a constant A from the equation

$$C_N = A \times E_{natam} \qquad (1)$$

where

$C_N$ = concentration of the natamycin standard solution in µg/ml.
$E_{natam}$ = net absorption of this natamycin solution.

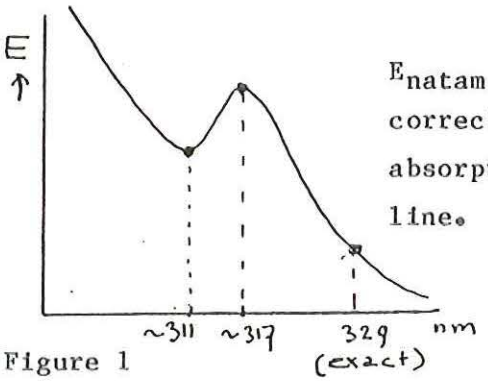

Figure 1

$E_{natam}$ is the absorption at about 317 nm corrected using the straight line between the absorption at about 311 and 329 nm as a base line.

$E_{natam}$ can be taken from the UV spectrum (see figure 1), or can be calculated with the formula

$$E_{natam} = (E_1)_N - \frac{2}{3}(E_2)_N - \frac{1}{3}(E_{329})_N \qquad (2)$$

where

$(E_1)_N$ = the maximum absorption at about 317 nm.
$(E_2)_N$ = the minimum absorption at about 311 nm.
$(E_{329})_N$ = the absorption at 329 nm.

Note: The exact position of the maximum at 317 and the minimum at 311 nm can be slightly shifted, due to variations in apparatus calibration. Use always the actual maximum and minimum values.

7.1.2 Measuring sample solution

7.1.2.1 Record the spectrum of the sample solution in the range 300-340 nm. Measure the absorption obtained in 6.5 at the maximum at about 317 nm = $(E_1)_S$, the minimum at about 311 nm = $(E_2)_S$ and at 329 nm exactly = $(E_{329})_S$. Use the aqueous methanol (2.2) as a blank.

7.1.2.2 Calculate the natamycin concentration of the sample in mg/kg using the equation

$$C_s = 15\ A \times E_{natam} \qquad (3)$$

where

$C_s$ = natamycin concentration of the sample in mg/kg.

A = constant, as determined in 7.1.1.3.

$E_{natam}$ = absorption of the sample solution, baseline corrected according to fig. 1, or calculated from the equation

$$E_{natam} = (E_1)_S - \frac{2}{3}(E_2)_S - \frac{1}{3}(E_{329})_S \qquad (4)$$

The value $C_s$ will be used for the determination of the natamycin concentration in the cheese, below the cheese rind, to detect migration of natamycin into the cheese.

7.1.2.3 Calculate the amount of natamycin on the surface of the cheese rind in mg/dm$^2$ using the equation

$$C_s' = 0.1\ C_s \times \frac{X}{Y} \qquad (5)$$

$$= 1.5 \times A \times \frac{X}{Y}\ E_{natam} \qquad (6)$$

$$= 1.5 \times A \times \frac{X}{Y}\ (E_1)_S - \frac{2}{3}(E_2)_S - \frac{1}{3}(E_{329})_S \qquad (7)$$

where

$C_s'$ = concentration at the surface of the cheese rind, in mg/dm$^2$

X = mass of the piece of cheese rind in gram

Y = surface of the piece of cheese rind in cm$^2$

A = constant found in paragraph 7.1.1.3.

The value $C_s'$ will be used for the determination of the natamycin concentration on the surface of the cheese rind.

7.1.3 If the natamycin concentration of the sample is so low that detection is impossible or almost impossible (signal/noise ratio < 3) and you still want to know the quantity, concentrate the filtrate (6.5) as described in paragraph 8.

7.2 Detection with HPLC

7.2.1 Adjustment of the liquid chromatograph

The following chromatographic conditions are recommended.

Mobile fase : Methanol-water-acetic acid 60 + 40 + 5.

Flow : 1 ml/min.

Detector set: 303 nm, 0,005 AUFS.

Recorder : 10 mV.

Theorecital (typical) plate count: minimal 1500.

Typical retetion time.

Note: When another type of column is applied, the methanol:water ratio may have to be adapted. The relative amount of acetic acid, however, is essential to keep the absorption maximum at 303 nm.

7.2.2 Before each series of samples a standard with a known quantity of natamycin must be injected to appoint the retention time and to check the calibration curve.

7.2.3 Preparation of the calibration curve

Pipette 1-2-4-6 and 8 ml of standard solution (7.1.1.1) into a series of 50 ml volumetric flasks and make up to volume with aqueous methanol (2.2).

These solutions contain 0.1, 0.2, 0.4, 0.6 and 0.8 µg/ml respectively. Inject 20 µl of these solutions. Measure the surface or height of the peaks and plot the found values on the y-axis against the injected quantities in ng on the x-axis.

7.2.4 Inject 20 µl of the clear filtrate obtained in paragraph 6.5. Measure the surface or the height of the peak with the same retention time as the natamycin standard solutions.
Measure as rapidly as possible.

7.2.5 Calculation
The quantity of natamycin in the injected aliquot can be found by interpolation on the standard curve.

7.2.5.1 Calculate from the found concentration of natamycin in the filtrate the natamycin concentration in the cheese (rind) in mg/kg with the formula

$$C = 15 \times B \qquad (8)$$

where
B = the quantity of natamycin in µg/ml
C = the concentration in the cheese (rind) in mg/kg.

7.2.5.2 Calculate the amount of natamycin on the cheese rind surface in $mg/dm^2$ with the formula

$$C' = 0.075\ B \times \frac{X}{Y} \qquad (9)$$

$$= 0.1\ .\ C \times \frac{X}{Y} \qquad (10)$$

where
C' = the amount of natamycin in the cheese rind surface in $mg/dm^2$.
X = the mass of the piece of cheese rind in gram.
Y = the surface of the piece of cheese rind in $cm^2$.

7.2.6 If the peak surface or peak height of the sample, found in paragraph 7.2.4 is so low that interpolation on the standard curve is impossible or almost impossible and you still want to know the quantity, concentrate the filtrate (6.5) as described in paragraph 8.

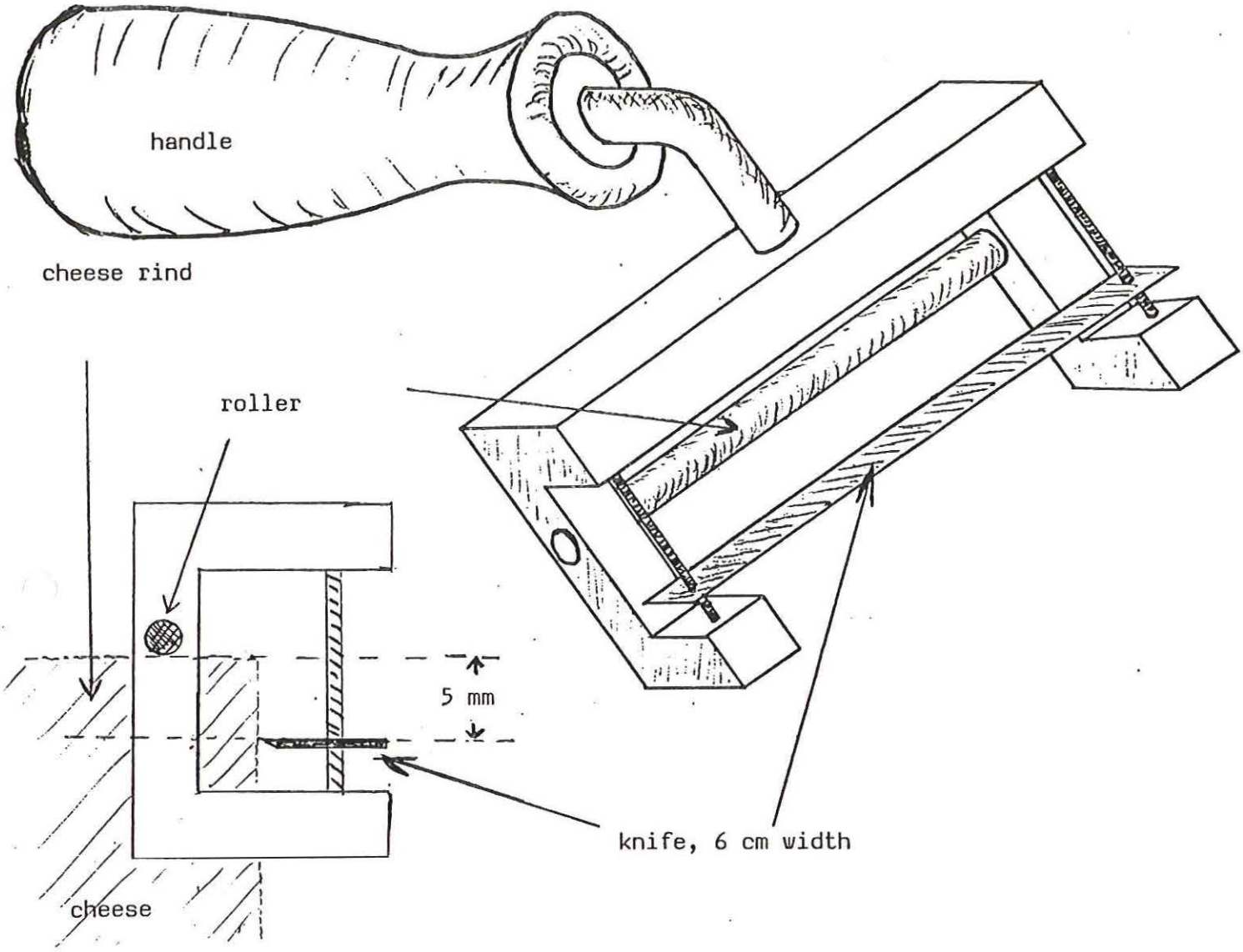


Figure 1 Slicer, that will enable porties of cheese rind 5 mm thick (3.2.1)

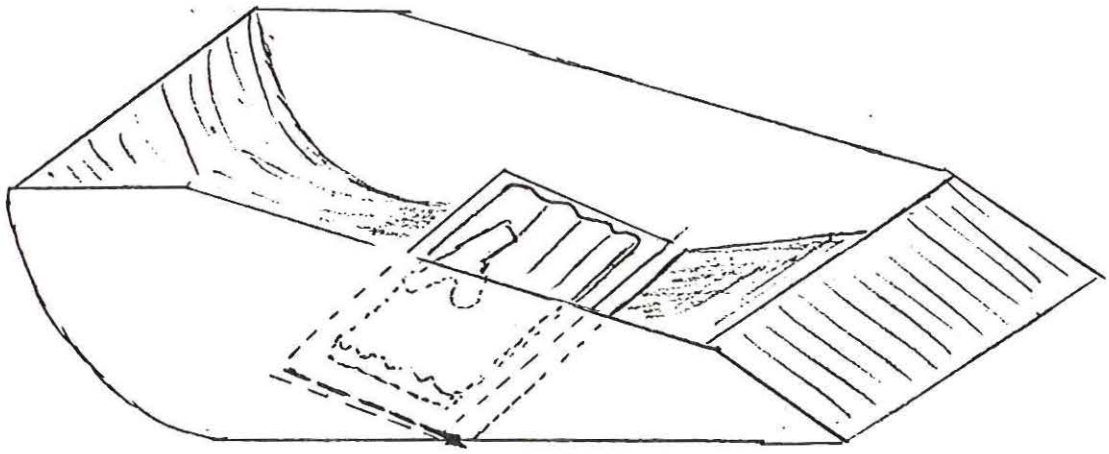

Figure 2 Fine-slicer, for cutting slices of cheese 0.7 mm thick (3.2.2)

## 8 CONCENTRATION OF THE FILTRATE

8.1 Decide if a concentration of about 5 or about 10 times is desired. Base this decision on the data found in paragraph 7.1.1 or 7.2.3 and the required detection limit.

8.2 Pipette 25 or 50 ml (resp. 5 and 10 times concentration) of the filtrate (6.5) in a beaker. Add 50 or 100 ml water and mix.

8.3 Activate a sep-pak C18 cartridge using 3-5 ml of methanol, then wash with 10 ml of water.

8.4 Pass the solution (8.2) through the cartridge with a speed of ± 25 ml/min with the aid of a syringe.

8.5 Rinse the cartridge with 10 ml water.

8.6 Elute the natamycin with 3 ml methanol.

8.7 Spectrometric detection.

8.7.1 Add 1,5 ml water and mix.

8.7.2 Put the solution in a syringe and filter through a microfilter of 0,45 µm pore size and then through a microfilter of 0,20 µm pore size, into a cuvette.

8.7.3 Measure the absorption as described in paragraph 7.1.1.

8.8 Detection with HPLC.

8.8.1 Fill up the solution (8.6) to 5 ml with methanol.

8.8.2 Inject 20 µl of the clear filtrate obtained in paragraph 8.8.1. Measure the surface or height of the peak with the same retention time as the natamycin standard solutions. Measure as rapidly as possible.

8.9 Calculation after concentration.

8.9.1 For spectrophotometrical detection.

8.9.1.1 Calculate the natamycin concentration of the sample in mg/kg with the formula:
for about 5 times (5.6 x) concentration:

$$C_s = 2.7\ A \times E_{natam} = 2.7\ A \times \quad (E_1)_N - \frac{2}{3}(E_2)_N - \frac{1}{3}(E_{329})_N \qquad (11)$$

for about 10 times (11.1 x) concentration

$$C_s = 1.35\ A \times E_{natam} = 1.35\ A \quad (E_1)_N - \frac{2}{3}(E_2)_N - \frac{1}{3}(E_{329})_N \qquad (12)$$

where A, $E_1$, $E_2$ and $E_{329}$ are as in paragraph 7.1.2.2.

8.9.1.2 Calculate the amount of natamycin on the surface of the cheese rind in mg/dm$^2$ for about 5 times (5.6 x) and about 10 times (11.1 x) concentration with the formula:

$$C_s' = 0.1\ C_s \times \frac{X}{Y} \qquad (13)$$

where X and Y are as in paragraph 7.1.2.3.

8.9.2 For HPLC detection.

The quantity of natamycin in the injected aliquot can be found by interpolation on the standard curve.

8.9.2.1 Calculate the natamycin concentration of the surface in mg/kg with the formula:
for 5 times concentration

$$C = 3\ B \qquad (14)$$

for 10 times concentration

$$C = 1.5\ B \qquad (15)$$

where B is the same as in paragraph 7.2.5.1.

8.9.2.2 Calculate the amount of natamycin on the cheese rind surface in mg/dm$^2$ with the formula

$$C' = 0.1\ C \times \frac{X}{Y} \qquad (16)$$

where X and Y are as in paragraph 7.2.5.2.

## 9 REPEATIBILITY AND REPRODUCIBILITY

The repeatibility and reproducibility of the method according to a collaborative study carried out in 1984 with 36 laboratories on 8 samples proved to be as follows.

| Level | | Spectrometric | | HPLC | | |
|---|---|---|---|---|---|---|
| mg/dm2 | mg/kg | $CV_r$ % | $CV_R$ % | $CV_r$ % | $CV_R$ % | |
| 4 | 60 | 5.9 | 12.2 | 9.3 | 20.6 | direct determination |
| 1 | 15 | 6.2 | 11.9 | 7.1 | 25.7 | |
| 0.08 | 1.3 | 16.5 | 35 | 23.4 | 37 | concentration 10x |
| 0.02 | 0.3 | 42.5 | 60 | 29 | 39 | |

CV = Coefficient of variation.
Relative repeatibility $r_{rel} = 2.83 \times CV_r$.
Relative reproducibility $R_{rel} = 2.83 \times CV_R$.