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Subject: Determination of the natamycin content of cheese rind and cheese. A collaborative study.

Annexes: 1

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REPORT 83.86 | Pr.no. 505 0290

Project: Harmonisation of methods of analysis for additives.

Subject: Determination of the natamycin content of cheese rind and cheese. A collaborative study.

Annex: 1

Summary

A collaborative test on the determination of natamycin in cheese rind was carried out.
The described method comprises
1. sampling
2. homogination
3. extraction
4. clean up
(5. concentration)
6. determination - spectroscopy
 - HPLC-UV.

For practical reasons the steps 3 to 6 only could be incorporated in this test.
Eight laboratories participated.
Three samples of decreasing levels were distributed.
The overall results were as follows:

Sample A	0.74 mg/dm^2
B	0.33 mg/dm^2
C	0.10 mg/dm^2

The quality of the results can be classified:

		A	B	C
spectroscopic	direct	good	reasonable	bad
	conc.	good	bad	reasonable/good
HPLC-UV	direct	reasonable	bad	bad
	conc.	-	not detectable	bad

The individual results are given in tables 1.1 to 1.4.

Comments from the participants on the method are reproduced and a new draft of the method is attached in the Annex.

Responsible: dr W.G. de Ruig
Collaborator/Rapporteur: J.J. van Oostrom, dr W.G. de Ruig
Projectleader: ir P.C. Hollman

The antimycoticum natamycin is widely used in the dairy industry to prevent mould formation on cheese. To this end the cheese rind is treated generally by means of a cheese coating that contains natamycin.

The legislation in various countries with respect to the use of natamycin differs.

To control its use, there has to be an internationally accepted method of analysis for the determination of natamycin in cheese rind.

There was a demand for such a method from EEC, IDF/ISO/AOAC and also from public analysts in the UK.

In the Netherlands a lot of work had already been done and a method was tested and improved by series of interlaboratory trials. Therefore in working groups of both EEC and IDF/ISO/AOAC as well as in the UK it was decided to test this method. The efforts were combined and it was concluded to start with a "pilot-collaborative study", with a limited number of participants.

Scope of the collaborative study

The proposed method describes the following steps

1. sampling
2. homogination
3. extraction
4. clean up

(5. concentration)

6. determination by either spectroscopic detection
   or HPLC-UV detection.

The concentration step has to be included as otherwise the signal is too low.

Description of the material

The samples consisted of lyophylized cheese rind, packed in brown bottles under nitrogen. Previously, stability tests had been carried out on such type of samples.

Three samples were distributed: A, B and C.

In sample A the natamycin content was so high that determination without a concentration step could be carried out.

The natamycin content of sample C was so low that concentration would be obligatory.
Sample B was in between A and C.
For calibration a reference sample was enclosed with an activity of 91,6%.
The method was to be applied on each of the three samples, in triplicate, with spectroscopic and with HPLC detection.
If the results by direct determination were poor, the concentration step as described should be included. This would probably be opportune for sample B and indispensable for sample C.
As the collaborative test did not include the sampling procedure, but started with an already freese-dried, homogenized product, the participants could not know the factors X and Y, mentioned in the method.
Therefore these factors were given: X = 15 g and Y = 25 cm^2.

Participants of the study

Nine institutes participants in this pilot study:

- Bundesgesundheitsamt, Max von Pettenkofer - Institut, Berlin, FRG (R. Tiebach).
- Chemische und Lebensmitteluntersuchungsanstalt, Hamburg, FRG (W. Frede).
- Gist-Brocades N.V., Delft, The Netherlands (C. Repelius).
- Laboratory of the Government Chemist, Londen, U.K. (D. Schuffam).
- Ministry of Agriculture, Fisheries and Food, Londen, U.K. (H.J. Judd).
- National Food Institute, Søborg, Denmark (M. Guldborg).
- State Institute for Quality Control of Agricultural Products, Wageningen, The Netherlands (W.G. de Ruig).
- Zuivelcontrole-Instituut, Leusden, The Netherlands (J. Leenheer).

Results

The results are reported in the tables.
In table 1 the results as reported by the participants are collected:
Table 1.1: Direct spectroscopic detection
1.2: Spectroscopic detection after concentration
1.3: Direct HPLC-UV detection
1.4: HPLC-UV detection after concentration.

For each of the four alternatives in table 2 the mean values per institute, and in table 3 the overall means and the repeatabilities and reproducibilities are collected.
In table 4 the correlations between the various alternatives are given:
Table 4.1: Spectroscopic-HPLC
4.2: Spectroscopic direct - after concentration
4.3: HPLC direct - after concentration.

Discussion

The overall mean values found for the 4 samples are:
A 0.74 mg/dm^2
B 0.33 mg/dm^2
C 0.10 mg/dm^2

To estimate the results, the following classification as to the reproducibility can be applied:

V_R	0-10%	good
V_R	11-20%	reasonable
V_R	21-40%	bad
V_R	> 40%	not detectable.

Thus, the results obtained by this collaborative study are as follows:

		A level 0.6 mg/dm^2	B level 0.3 mg/dm^2	C level 0.1 mg/dm^2
Spectroscopic	direct	good	reasonable	bad
	conc.	good	bad	reasonable/good
HPLC	direct	reasonable	bad	bad
	conc.	-	not detectable	bad

In comparing the various detection alternatives no significant difference was found between the spectroscopic and the HPLC-UV detection. Comparing the measurements both without and with the concentration step a significant difference was found in case of the spectrophotometric determination for samples B ($\alpha < 0.05$) and C ($\alpha < 0.01$) and for the HPLC-UV determination for sample C ($\alpha < 0.05$).

A test on outliers or stragglers was carried out according to ISO 5725 using Cochran's maximum variance test to test the precision under repeatability conditions in the laboratories, and Dixon's outlier test to test the precision between laboratories.

Spectrophotometric, direct:
Cochran's test: no outliers or stragglers
Dixon's test : sample C: outlier lab 4
without lab 4: no outliers or stragglers.

Spectrophotometric, after concentration:
Cochran's test: straggler for sample A and sample B
straggler for sample A not note worthy;only two laboratories
Dixon's test : no outliers or stragglers.

HPLC-UV direct: no outliers or stragglers.

HPLC-UV, after concentration:
Cochran's test: sample C: outlier lab 6
Dixon's test : no outliers or stragglers.

Compared with each other, no significant difference is observed between spectroscopic and HPLC detection (table 4.1).
After concentration, the spectroscopic results are significant lower for samples B and C, and the HPLC results are significant lower for sample C. Here it has to be kept in mind that according to the concentrations, sample A was too high for determination with concentration and sample C too low for determination without concentration.

Having a general look at the results, the impression is made that "the simplest is the best", i.e. that a straightforward determination using the spectrophotometric detection without concentration, when applicable with respect to the content, will give the best results.

Comments

A general comment was that in 7.1.2.1 natamycin hardly dissolved in aqueous methanol (40 min in an ultrasonic bath!).

The dissolution succeeded when the natamycin was dissolved in a small volume pure methanol and then the composition of the solvent was adjusted by addition of an adequate amount of water. Alternatively, methanol only was used. As, according to the prescription, this solution is further diluted with aqueous methanol, using methanol in the first step practically will give the same overall result and may be preferred as the simplest way.

In 7.2.4 the standard series had to be diluted with methanol/water 2:1 in stead of the mobile phase because of decay of natamycin (about 0.1% per minute). Again, alternatively pure methanol was used.

As to the spectrophotometric detection one participant reported that sample C was not readable, because no cleary defined peak at 317 nm was detectable. Concentration showed no improvement because of turbidity.
Another participant reported that sample C after concentration with HPLC detection were not readable, because of broad, low, irregular bands.
On the other hand, another participant did not include the filtration step 8.8.2, because the solutions were clear already.

In the HPLC detection one participant reported the occurrence of two peaks closely before the natamycin peak (fig. 1). This peak occurred sometimes in the standard (fig. 1.2), sometimes not (fig. 1.1), but almost always in the samples (fig. 1.3-1.7). These peaks were suggested to be caused by decay products of natamycin. Natamycin dissolved in methanol never showed these peaks.

Other participants did not report these observations. In sofar as the chromatograms were sent with the results, they show distinct natamycin peaks only (fig. 2 and 3).

- 6 -

Further remarks from the participants

3.17 HPLC columns used:

3.18

- Analytical: 120 mm x 4,6 mm i.d.; guard: 100 mm x 3 mm i.d.
- Analytical: Li Chrosorb RP-8 10 µ 25 cm
  Li Chroprep RP-18 25-40 µm
- Guard: RP-18, 5 µ, 40 mm x 4.0 mm i.d.
- No guard column applied.

Calibration: - only 8, 12, 16 ng/20 µl
- sample B without concentration outside calibration curve, not reliable.

- Analytical column only will have a short lifetime. Peak broadening within 2 days from 4.7-8.5.

6.1

- Not exactly 10.0 g weighed
  Amount recognized in 7.1.3, 7.2.5, 8.9.1, 8.9.2.

6.2

- Use methanol in stead of water (comment: in our opinion disadvantage: less coagulation of fats and proteins).

6.4 Drop "... or so ....".

7.1.1 Use methanol as a blank.

7.1.2.1

- Dissolve in methanol, then add water.
- Dissolve and make up to volume with methanol.

7.2.4

- Concentrations of standard and samples low, therefore peak height instead of peak area; error relatively high.

7.2.6 Samples C after concentration very low, broad, irregular peaks; not detectable.

8. Include concentration step in all cases (comment: when signal to noise ratio is high enough, the concentration step in a needless complication. It depends on the sensitivity of the apparatus as well).

8.3 Describe activation of Sep-pak in detail.

8.5 Dry Sep-pak after rinsing with 10 ml H_2O (comment: risk of decay).

8.6

- Elute in 5 ml.
- Elute in 5 ml and drop 8.7.1, then in 8.9.1. 0.30 instead of 0.27 and 0.15 instead of 0.135.

8.8.1 Filtration through 0.2 µm filter stopped (comment: 0.45 µm was prescribed).

8.8.2 Not applied, because the solutes were clear already.

Table 1.1 Results of the spectrophotometric method, without concentration (mg/dm^2)

Sample	Institute no. 1	2	3	4	5	6	7	8
A	0.76 0.86 0.76	0.81 0.82 0.79	0.776 0.824 0.747	0.73 0.78 0.75 0.69	0.77 0.84 0.86	0.81 0.75 0.73	0.83 0.75 0.83	0.834 0.779 0.680
B	0.41 0.36 0.35	0.34 0.39 0.40	0.383 0.367 0.373	0.32 0.30 0.28 0.29	0.39 0.44 0.46	0.36 0.36 0.33	0.39 0.43 0.43	0.418 0.459 0.397
C	not readable	0.17 0.15 0.12	0.151 0.167 0.174	0.08 0.08 0.07 0.08	very turbid not readable	0.14 0.16 0.16	0.17 0.17 0.15	0.163 0.152 0.130

Table 1.2 Results of the spectrophotometric method, after concentration (5x or 10x) (mg/dm^2)

Sample	Institute no. 1	2	3	4	5	6	7	8
A	- - -	- - -	0.672 0.775 0.687	- - -	0.65 0.66 0.65	- - -	- - -	- - -
B	0.38 0.43 0.34	0.23 0.22 0.22	0.332 0.336 0.300	- - -	0.15 0.31 0.26	0.30 - 0.25	0.34 0.37 0.39	0.271 0.288 0.255
C	- - -	0.11 0.10 0.13	0.115 0.110 0.124	- - -	0.10 0.13 0.12	0.087 - 0.076	0.10 0.11 0.11	0.087 0.091 0.104

Table 1.3 Results of the HPLC detection, without concentration (mg/dm^2)

	Institute no.							
Sample	1	2	3	4	5	6	7	8
A	0.64 0.70 0.70	0.62 0.60 0.62	0.720 0.729 0.698	0.74 0.71 0.74 0.70	0.57 0.54 0.55	0.90 0.91 0.93	0.76 0.71 0.81	0.728 0.679 0.806
B	0.27 0.30 0.25	0.32 0.30 0.30	0.315 0.324 0.286	0.35 0.31 0.40 0.43	0.25 0.37 0.29	0.42 0.42 0.41	- - -	0.142 0.146 0.150
C	0.05 0.06 0.08	0.09 0.09 0.09	0.081 0.068 0.070	- - -	0.16 0.14 0.15	0.11 0.12 0.11	- - -	not detec- table

Table 1.4 Results of the HPLC detection, after concentration (mg/dm^2)

	Institute no.							
Sample	1	2	3	4	5	6	7	8
A	- - -	- - -	(4 µl)* 0.596 0.688 0.554	- - -	- - -	- - -	- - -	- - -
B	- - -	0.19*** 0.30*** 0.30***	(8 µl)* 0.261 0.272 0.242	- - -	- - -	0.59*** - 0.49***	(5x)** 0.44 0.46 0.44	(5x)** 0.102 0.168 0.145
C	0.04 0.04 0.04	0.05 0.05 0.06	(20 µl)* 0.059 0.048 0.056	0.065 0.060 0.078 0.053	- - -	0.10 - 0.07	(10x)** 0.07 0.07 0.06	(5x)** 0.025 0.020 0.028

* = injected volume
** = concentration
*** = peak surface far beyond calibration curve, results not reliable

Table 2. Mean values per institute per method of detection (mg/dm^2)

Sample	Method of detection		Institute no. 1	2	3	4	5	6	7	8
A	spectr.	direct	0.79	0.81	0.78	0.74	0.82	0.76	0.80	0.76
		conc.	-	-	0.71	-	0.65	-	-	-
	HPLC	direct	0.68	0.61	0.72	0.72	0.55	0.91	0.76	0.74
		conc.	-	-	0.61	-	-	-	-	-
B	spectr.	direct	0.37	0.38	0.37	0.30	0.43	0.35	0.42	0.42
		conc.	0.38	0.22	0.32	-	0.24	0.28	0.37	0.27
	HPLC	direct	0.27	0.31	0.31	0.37	0.30	0.42	-	0.15
		conc.	-	0.26	0.26	-	-	0.54	0.45	0.14
C	spectr.	direct	-	0.15	0.16	0.08	-	0.15	0.16	0.15
		conc.	-	0.11	0.12	-	0.12	0.08	0.11	0.09
	HPLC	direct	0.06	0.09	0.07	-	0.15	0.11	-	-
		conc.	0.04	0.05	0.05	0.06	-	0.08	0.07	0.02

Table 3. Mean values, repeatabilities and reproducibilities

Sample	Method of detection		n	x	s(x)	range	V(x)	s_r	s_R	V_r	V_R
A	spectr.	direct	8	0.78	0.028	0.74-0.82	3.5	0.048	0.049	6.1	6.3
		conc.	2	0.68	0.042	0.65-0.71	6.2	0.040	0.052	5.8	7.6
	HPLC	direct	8	0.71	0.107	0.95-0.91	15.0	0.033	0.108	4.6	15.2
		conc.	1	0.61	-	-		0.069		11.2	
B	spectr.	direct	8	0.38	0.043	0.30-0.43	11.4	0.026	0.051	6.9	13.6
		conc.	7	0.30	0.062	0.22-0.38	20.8	0.041	0.071	13.6	23.8
	HPLC	direct	7	0.30	0.084	0.15-0.42	27.7	0.035	0.089	11.4	29.1
		conc.	5	0.33	0.162	0.14-0.54	49.0	0.042	0.158	13.4	50.8
C	spectr.	direct	6	0.14	0.031	0.08-0.16	21.6	0.014	0.037	10.4	26.9
	ibid. without lab. 4		5	0.16	0.008	0.15-0.16	5.3	0.016	0.016	10.5	10.5
		conc.	6	0.10	0.016	0.08-0.12	15.6	0.011	0.016	10.3	15.0
	HPLC	direct	5	0.10	0.036	0.06-0.15	37.3	0.009	0.036	9.3	36.3
		conc.	7	0.05	0.020	0.02-0.08	37.4	0.009	0.020	15.7	36.1

Table 4.1 Spectroscopic versus HPLC detection

Sample	A			B			C		
Detection	Spect.	HPLC	Diff. Spect-HPLC	Spect.	HPLC	Diff. Spect-HPLC	Spect.	HPLC	Diff. Spect-HPLC
Lab 1	0.79	0.68	+0.11	0.37	0.27	+0.10	-	0.06	
2	0.81	0.61	+0.20	0.38	0.31	+0.07	0.15	0.09	+0.06
3	0.78	0.72	+0.06	0.37	0.31	+0.06	0.16	0.07	+0.09
4	0.74	0.72	+0.02	0.30	0.37	-0.07	0.08	-	
5	0.82	0.55	+0.27	0.43	0.30	+0.13	-	0.15	
6	0.76	0.91	-0.15	0.35	0.42	-0.07	0.15	0.11	+0.04
7	0.80	0.76	+0.04	0.42	-	-	0.16	-	
8	0.76	0.74	+0.02	0.42	0.15	+0.27	0.15	-	
Mean	0.782	0.711	+0.071[1)]	0.380	0.304	+0.070[1)]	0.142	0.096	+0.063
s	0.028	0.107	0.127	0.043	0.084	0.118	0.031	0.036	0.025
C.V.	3.5	15.0		11.4	27.2		21.6	37.3	
s(mean)			0.045			0.045			0.015
t			1.59			1.57			4.35
	1) not significant			1) not significant					

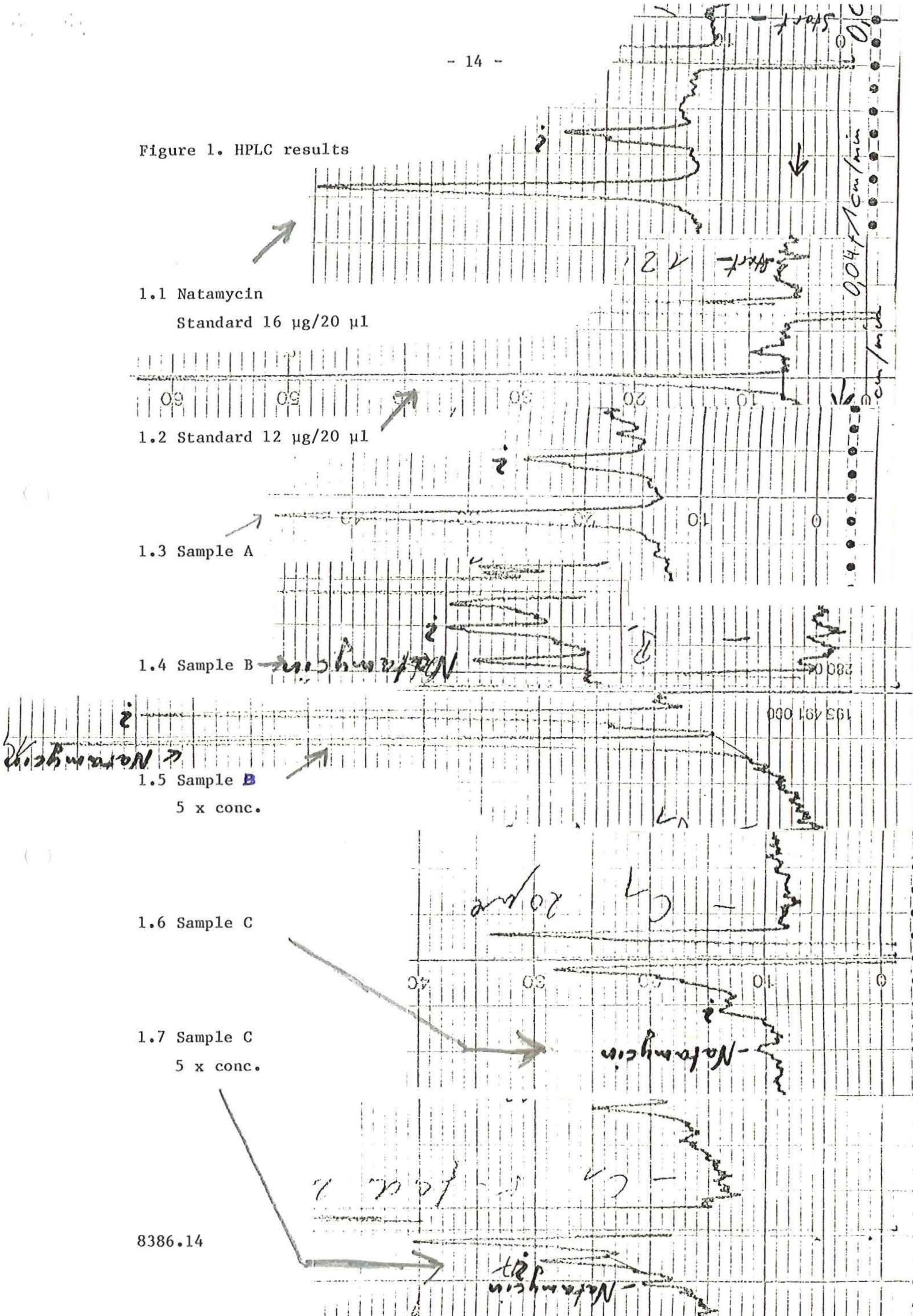
Table 4.2 Spectroscopic direct versus spectroscopic conc.

Sample	A			B			C		
Detection	Direct	Conc.	Diff. Dir.-Conc.	Direct	Conc.	Diff. Dir.-Conc.	Direct	Conc.	Diff. Dir.-Conc.
Lab 1	0.79	-		0.37	0.38	-0.01	-	-	
2	0.81	-		0.38	0.22	+0.16	0.15	0.11	+0.04
3	0.78	0.71	+0.07	0.37	0.32	+0.05	0.16	0.12	+0.04
4	0.74	-		0.30	-	-	0.08	-	
5	0.82	0.65	+0.17	0.43	0.24	+0.19	-	0.12	
6	0.76	-		0.35	0.28	+0.07	0.15	0.08	+0.07
7	0.80	-		0.42	0.37	+0.05	0.16	0.11	+0.05
8	0.76	-		0.42	0.27	+0.15	0.15	0.09	+0.06
Mean	0,782	0,680	+0.12 1)	0.380	0.297	+0.094*	0.142	0.105	+0.052**
s	0.028	0.042	0.071	0.043	0.062	0.073	0.031	0.016	0.013
C.V.	3.5	6.2		11.4	20.8		21.6	15.6	
s(mean)			0.050			0.028			0.006
t			2.4			3.42			8.9
	1) not significant			* significant $\alpha < 0.05$			** significant $\alpha < 0.01$		

Table 4.3 HPLC-UV direct versus HPLC-UV conc.

Sample	A			B			C		
Detection	Direct	Conc.	Diff. Dir.-Conc.	Direct	Conc.	Diff. Dir.-Conc.	Direct	Conc.	Diff. Dir.-Conc.
Lab 1	0.68	-		0.27	-		0.06	0.04	+0.02
2	0.61	-		0.31	-		0.09	0.05	+0.04
3	0.72	0.61	+0.11	0.31	0.26	+0.05	0.07	0.05	+0.02
4	0.72	-		0.37	0.26	+0.05	-	0.06	
5	0.55	-		0.30	-		0.15	-	
6	0.91			0.42	0.54	-0.12	0.11	0.08	+0.05
7	0.76			-	0.45		-	0.07	
8	0.74			0.15	0.14	+0.01	-	0.02	
Mean	0.711	0.61	+0.11[1)]	0.304	0.330	-0.002	0.096	0.053	+0.032*
s	0.107	-		0.084	0.162	0.080	0.036	0.020	0.015
C.V.	15.0	-		27.7	49.0		37.3	37.4	
s(mean)						0.040			0.008
t						0.06			4.33
	1) not significant			1) not significant			* significant α < 0.05		

Figure 1. HPLC results



1.1 Natamycin
Standard 16 µg/20 µl

1.2 Standard 12 µg/20 µl

1.3 Sample A

1.4 Sample B

1.5 Sample B
5 x conc.

1.6 Sample C

1.7 Sample C
5 x conc.

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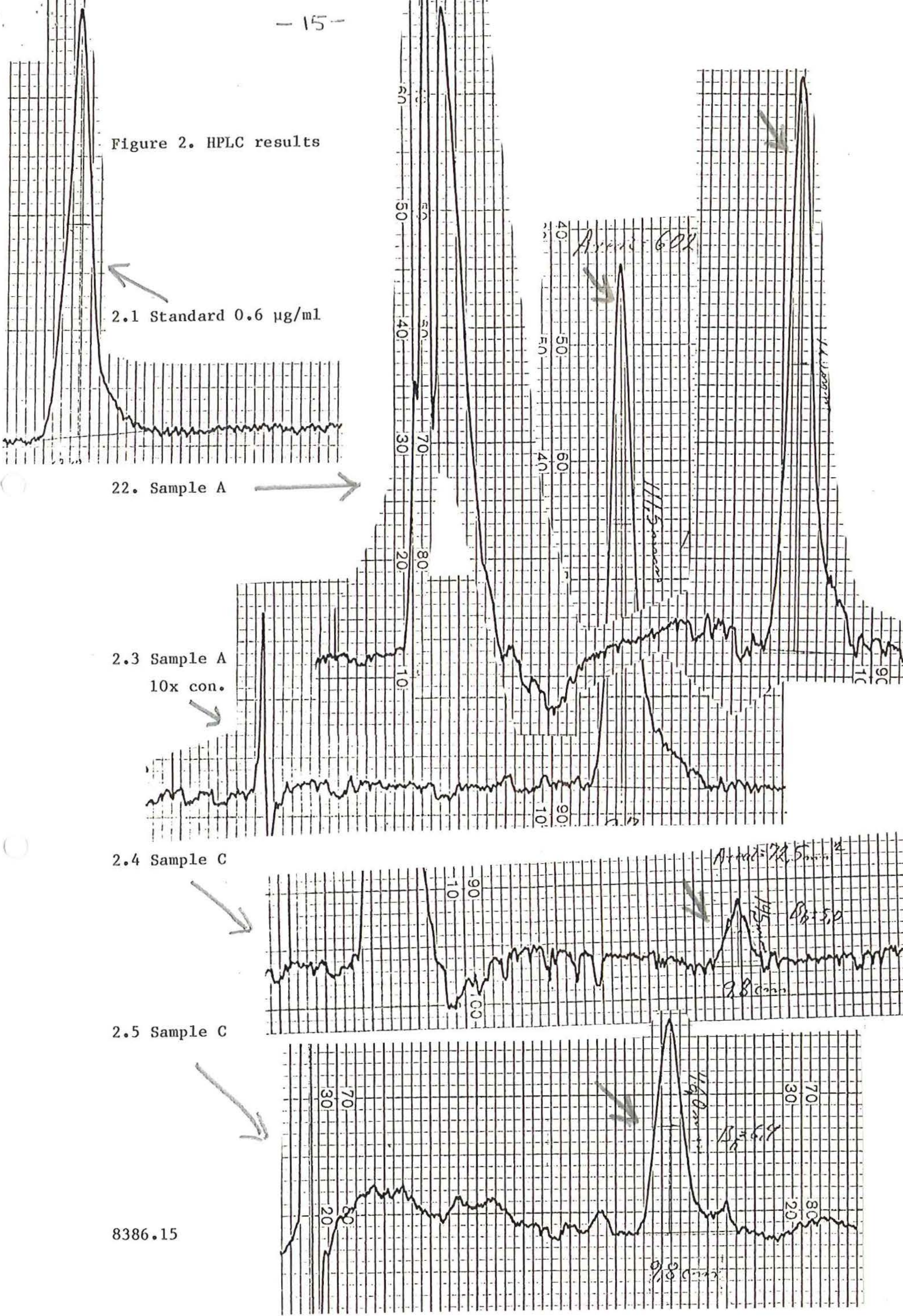
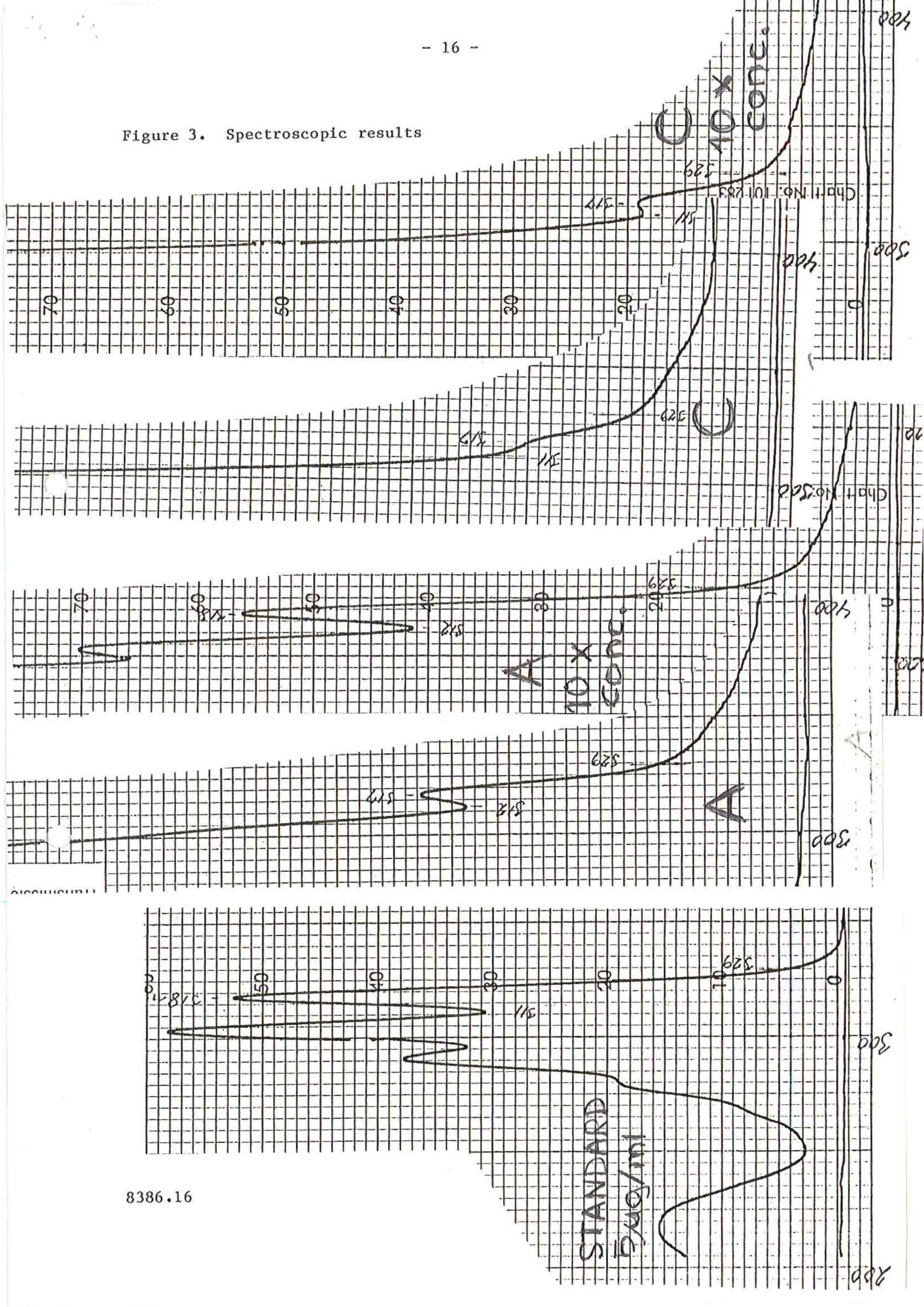


Figure 2. HPLC results

Figure 3. Spectroscopic results



8386.16

STATE INSTITUTE FOR QUALITY CONTROL
OF AGRICULTURAL PRODUCTS
WAGENINGEN

ANNEX

DETERMINATION OF THE NATAMYCIN CONTENT OF CHEESE RIND AND CHEESE

2nd draft, 1983-11-01

1 SCOPE AND FIELD OF APPLICATION

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

1.1 Definition

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

1.2 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 spectrophotometrical or a HPLC method.

2 REAGENTS AND REFERENCE SUBSTANCES

2.1 Methanol, chemically pure.

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

2.3 Natamycin preparation, with a known natamycin content.

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:
Slicer, for cutting off a cheese rind 5 mm thick and about 3 cm wide.

3.2.2 For the analysis of cheese:
Fine-slicer, for cutting slices of cheese 0,7 mm thick.
N.B. A David planing-machine is suitable.

3.3 Grater.
N.B. 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 Disposible syringes, 10 ml (e.g. Terumo SS 10 S).

3.11 Microfilters, 0,45 µm pore size (e.g. Gelman Acrodisc 4184).

3.12 Folded filters, ∅ 15 cm.

3.13 Funnel, about 7 cm in diamter.

3.14 Electric photometer, suitable for measurements at wavelengths of about 310 nm, about 317 nm and also 329 nm, equipped with cuvettes 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.

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 DIS 707 Milk and milk products - sampling.

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 obtained rind 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 pre-treated.

5.1.6 Clean, after each sample, all tools which have been contacted 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 contacted 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, weigh 10.0 g of the test sample for analysis into a 200 ml conical flask and add 100 ml of methanol.
In the case of cheese, weigh 5.0 g of the test sample for analysis into 100 ml conical flask and add 50 ml of methanol.
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 flaks in the freezer and allow to stand for about 60 min.

6.4 Filter the cooled extract through a folded filter, discarding the first 5 ml of filtrate.
Bring the filtrate to room temperature.

6.5 Put a part of the filtrate in a syringe (3.10) and filter through a microfilter (3.11).

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7 DETECTION

7.1 Spectrophotometrical detection.

7.1.1 Measure the absorption of the solution obtained in paragraph 6.5 by the maximum at about 317 nm, the minimum at about 311 nm and at 329 nm exactly. Use the aqueous methanol (2.2) as a blank.

7.1.2 Determination of the constant A.

7.1.2.1 Immediately before use, dissolve 50 mg of natamycin in 100 ml of methanol (2.1).
Dilute 5 ml of this solution with aqueous methanol (2.2) to 50 ml, then dilute 5 ml of the diluted solution again with aqueous methanol (2.2) to 50 ml.
The natamycin concentration of the end solution is 5 µg/ml.

7.1.2.2 Determine the maximum and minimum absorption at, respectively, about 317 and 311 nm, and record the absorptions:
- the maximum absorption at about 317 nm is E_1:
- the minimum absorption at about 311 nm is E_2, and
- the absorption at 329 nm against aqueous methanol (2.2) is E_{329}.

7.1.2.3 Calculate the constant A from the equation:

$$A = \frac{C}{E_1 - 1/2(E_2 + E_{329})}$$

where
C is the natamycin concentration, in µg/ml, of the solution measured.

7.1.3 Calculation
Calculate the natamycin concentration, in mg/dm^2, of the cheese-rind or cheese sample with the formula:

$$1.5 \times A \times \frac{X}{Y}\left\{E_1 - 1/2(E_2 + E_{329})\right\}$$

where:

A is the constant found in paragraph 7.1.2.3

X is the mass of the piece of cheese rind or cheese in gram

Y is the surface of the piece of cheese rind or cheese in cm^2

E_1, E_2 and E_{329} are the absorptions of the sample extract measured at the wavelengths laid down in paragraph 7.1.2.2.

7.1.4 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

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

Flow : 1 ml/min.

Detector set: 303 nm, 0,005 AUFS.

Recorder : 10 mV.

Chart speed : 1 cm/min.

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 Inject 20 µl of the clear filtrate obtained in paragraph 6.5.

7.2.4 Preparation of the calibration curve

Dilute, from the obtained standard solution (7.1.2.1), 1-2-4-6 and 8 ml in 50 ml methonol/water (2:1).

These solutions contain respectively 2-4-8-12 and 16 ng/20 µl. Inject 20 µl of these solutions. Measure the surface or the height of the peaks and plot the found values on the y-axis against the injected quantities in ng on the x-axis.

7.2.5 Calculation

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

Calculate from the found number of ng the natamycin content in mg/dm^2 with the following formula:

$$0.075 \times C \times \frac{X}{Y}$$

where:

C is quantity of natamycin in ng in 20 µl.

X is the mass of the piece of cheese(rind) in gram.

Y is the surface of the piece of cheese(rind) in cm^2.

7.2.6 If the peak height of the sample, found in paragraph 7.2.2 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.

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 Spectrophotometrical 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 in 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 Put the solution in a syringe and filter through a microfilter.

8.8.3 Inject 20 µl of the clear filtrate obtained in paragraph 8.8.2. Measure the surface (in mm^2) of the peak.

8.9 Calculation after concentration.

8.9.1 For spectrophotometrical detection.
Calculate the natamycin content, in mg/dm^2, with the formula:

for about 5 times concentration:

$$A \times 0{,}27 \times \frac{X}{Y} \times \left\{ E_1 - 0.5\ (E_2 + E_{329}) \right\}$$

for 10 times concentration:

$$A \times 0{,}135 \times \frac{X}{Y} \times \left\{ E_1 - 0.5\ (E_2 + E_{329}) \right\}$$

where A, X, Y, E_1, E_2 and E_{329} as in paragraph 7.1.3.

8.9.2 For HPLC detection.
Calculate the natamycin content, in mg/dm^2, with the formula:

for 5 times concentration

$$0.015 \times C \times \frac{X}{Y}$$

for 10 times concentration

$$0.0075 \times C \times \frac{X}{Y}$$

where C, X and Y as in paragraph 7.2.5.