# OIV-MA-AS323-09 Determination of natamycin in wines

**Type IV method**

## 1. Introduction

Different methods for the determination of natamycin are used based mainly on HPLC in combination with DAD or MS detection. Estimation of the performance limits - limit of detection and quantification - relies on the responsibility of the laboratories according to accreditation systems (e.g. ISO/EN 17025/2005) employing the recommendations of the OIV (OENO 7/2000, E-AS1-10-LIMDET) or other normative guidelines.

As there is lack of a reliable interlaboratory estimate of the critical level, a decision limit of 5 µg/l is temporarily adopted until a reliable interlaboratory estimate or other robust indicators of the critical level can be obtained.

## 2. Methods

### 2.1. Determination of natamycin (pimaricin) in wine by liquid chromatography coupled to high resolution mass spectrometry

#### 2.1.1. Scope

This method describes an analytical procedure for the determination of natamycin (pimaricin) in wine. The level of natamycin is expressed in micrograms per litre (µg/l) of wine. In-house validation has been carried out using solvent solutions, red wine and white wine over the concentration range 5 – 2600 µg/l.

#### 2.1.2. Principle

The level of natamycin in wine is determined by direct injection of the sample into a liquid chromatograph with a high-resolution mass-spectrometric detection system (LC-HR/MS). Quantification is achieved using the standard addition method. The sample is initially analysed to gain an estimated concentration of natamycin. The analysis is then repeated with standard addition calibration standards more suited to the concentration of natamycin in the sample.

#### 2.1.3. Reagents

##### 2.1.3.1. Analytes

2.1.3.1.1. Natamycin (Pimaricin) > 95%

2.1.3.2. Chemical

2.1.3.2.1. *Methanol, HPLC Fluorescence grade (CAS no. 67-56-1)*

2.1.3.2.2. *Purified water for laboratory use, for example of EN ISO 3696 grade (water for analytical laboratory use - specification and test methods [ISO 3696:1987])*

2.1.3.2.3. *Acetic acid, 100%, (CAS no. 64-19-7)*

2.1.3.3. Solutions

2.1.3.3.1. *Stock solution of natamycin (1000 µg/ml)*

Weigh to the nearest 0.1 mg approximately 10 mg of natamycin (2.1.3.1.1) in a 10 ml amber volumetric flask and make up to the mark with methanol:water:acetic acid (2.1.3.3.4). Cap and sonicate. Calculate the actual concentration in micrograms of natamycin per millilitre of solution.

2.1.3.3.2. *Working solution 1: natamycin (10 µg/ml)*

Pipette 100 µl of stock solution (2.1.3.3.1) into a amber 10 ml volumetric flask and make up to the mark with methanol:water:acetic acid (2.1.3.3.4)

2.1.3.3.3. *Working solution 2: natamycin (0.5 µg/ml)*

Pipette 500 µl of working solution one (2.1.3.3.2) into an amber 10 ml volumetric flask and make up to the mark with methanol:water:acetic acid (2.1.3.3.4)

*2.1.3.3.4. Solution of methanol:water:acetic acid (50:47:3, v/v)*

Using a measuring cylinder, measure 500 ml of methanol (3.2.1) into a 1 L volumetric flask. Add 470 ml water (2.1.3.2.2) and shake to mix. Add 30 ml acetic acid (2.1.3.2.3) and shake well.

*2.1.3.3.5. Methanol, 3% acetic acid*

Using a measuring cylinder add 30 ml of acetic acid (2.1.3.2.3) to a 1 L volumetric flask. Make up to the mark with methanol (2.1.3.2.1) and shake well.

***2.1.3.3.6. Water, 3% acetic acid***

Using a measuring cylinder add 30 ml of acetic acid (2.1.3.2.3) to a 1 L volumetric flask. Make up to the mark with water (2.1.3.2.2) and shake well.

**2.1.4. Apparatus**

NOTE: An instrument or item of apparatus is listed only where it is specialised or made to a particular specification, the usual laboratory glassware and equipment being assumed to be available.

2.1.4.1. Liquid Chromatograph (LC)

Equipped with an automatic injector, a 100 µl injection loop and a high resolution mass spectrometer.

*2.1.4.1.1. LC column*

Capable of obtaining reproducible natamycin peaks and capable of separating the natamycin peaks from interfering peaks originating from the sample matrix and/or the solvents used.

NOTE: Depending on the type of equipment used for the analysis, the appropriate operating conditions should be optimised.

*2.1.4.1.2. HPLC analysis*

The following column and parameters have been found to be suitable:

Column: Waters Sunfire C18, 150 x 2.1 mm, 3.5 µm

Column temperature: 30 °C

Flow rate:0.25 ml/min

Injection volume: 20 µl

Mobile phase A: Water:acetic acid, 97:3 (v/v) (2.1.3.3.6)

Mobile phase B: Methanol:acetic acid, 97:3 (v/v) (2.1.3.3.5)

Run time: 30 min

Autosampler tray 8 °C

Gradient:

| Time (min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|
| 0 | 90 | 10 |
| 25 | 10 | 90 |
| 27 | 10 | 90 |
| 27.1 | 90 | 10 |
| 30 | 90 | 10 |

Mass spectrometric detection (LC-HR/MS)

Ionisation mode: positive electrospray

Mass resolution: m/z/ m/z

AGC target: High dynamic range

Max Inj time: 50 ms

Scan range: m/z 480-670

Sweep gas: 60 L/min

Aux gas: 5 L/min

Spray voltage: 3.75 V

Natamycin: m/z 666.31069 $[M+H]^+$. confirmation ion m/z 503.22672

Retention time: 16.5 mins

2.1.5. Experimental procedure

Samples should be shaken to ensure homogeneity prior to sub-sampling.

2.1.5.1. Screening

For each wine pipette 2 ml of sample into two 2 ml Eppendorf centrifuge vials. Add 0 µl, 20 µl and of natamycin working solution 2 at 0.5 µg/ml (2.1.3.3.3) to the vials respectively. This is equivalent to 0 µg/l and 5 µg/l natamycin added. Shake the vials for one minute and then centrifuge for 10 min at 14000 rpm. Filter an aliquot through 0.2 µm PTFE into an amber 2 ml vial. Analyse by LC-HR/MS (section 6) and estimate the concentration of natamycin in the sample (section 7).

If the estimated concentration of natamycin is less than 5 µg/l report the data as < 5 µg/l. If the estimated concentration of natamycin is greater than 5 µg/l follow section 5.2.

Quantitation

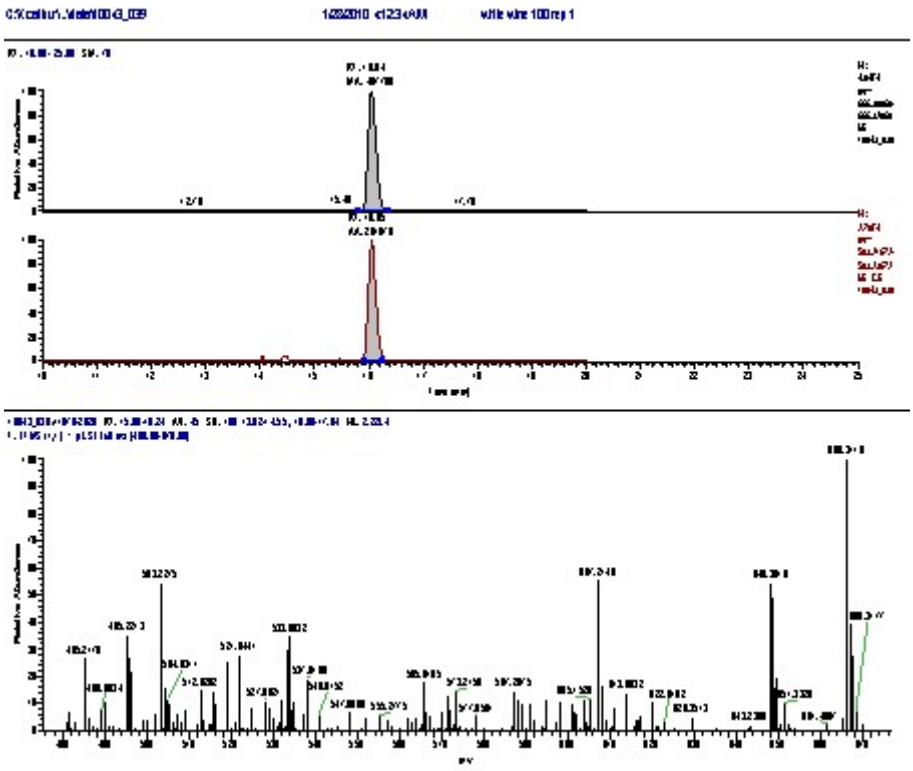
Natamycin determination for samples with an estimated concentration of greater than 5 µg/l. Pipette 2 ml of wine into five 2 ml Eppendorf centrifuge vials and add 0 l µ, 5 µl, 10 µl, 20 µl and 50 µl of natamycin working solution 1 (2.1.3.3.2) into the vials respectively. This is equivalent to 0 µg/l, 25 µg/l, 50 µg/l, 100 µg/l and 250 µg/l natamycin added. Shake the vials for one minute and then centrifuge for 10 min at 14000 rpm. Filter an aliquot through 0.2 µm PTFE into an amber 2 ml vial. Analyse by LC-HR/MS (section 6) and estimate the concentration of natamycin in the sample (section 7).

2.1.6. Analysis

Note: When starting measurements baseline stability and response linearity of the detector should be examined, together with verification of the detection limit. Maintain the same operating conditions throughout the measurement of all samples and calibration standards. Identify the natamycin peaks on the basis of the retention time and their accurate mass channel, and measure the peak areas. Inject each of the solutions as prepared onto the LC column. Measure the peak area of the natamycin peak in each of the quantification and confirmation channels. An example of a typical

chromatogram is given in Figure 1.

Figure 1. Typical LC-HR/MS chromatogram and mass spectrum for natamycin spiked into white wine at the equivalent of 50 µ g/l in the sample.



Plot the peak area for the main quantification channel against the concentration of natamycin added in micrograms per litre (µg/l). Determine the slope, intercept point and correlation coefficient of the regression line. The calibration curve shall be rectilinear and the correlation coefficient shall be 0.99 or better.

2.1.7. Expression of results

2.1.7.1. Calculation of analyte level

The natamycin concentration in the sample in micrograms per litre (µg/l) is calclulated using the following formula:

- C = b/a

where C = concentration of natamycin in the wine (µg/l), a = slope of the regression line, b = y-intercept point of the regression line

2.1.8. Confirmation

The presence of natamycin in the samples shall be confirmed by applying the following criteria:

The presence of a peak in both accurate mass channels *m/z* 666.31069 and *m/z* 503.22672 at the same retention time. Calculate the ratio of the peak area for the main quantification mass channel relative to the peak area of the confirmation channel. The criterion is that the ratios agree to ± 25% of those obtained from the standard addition calibration standards.

2.1.9. Method performance date

2.1.9.1. Linearity

The method is linear over the calibration range of 1 to 2640 / µgl in solvent, white wine and red wine matrices (figures 2, 3 and 4).

Figure 2. Ten point calibration graph of natamycin spiked into solvent in the range from 1 to 2600 µg/l.

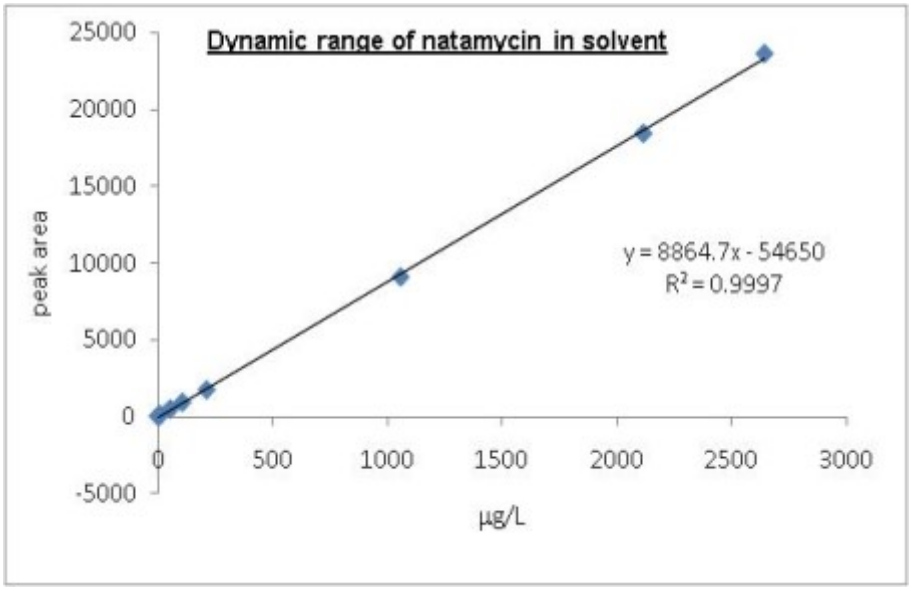



Table 1. Solvent calibration residuals.

| Natamycin (µg/l) | Predicted conc. (µg/l) | Residuals | Standard Residuals |
|---|---|---|---|

| | | | |
|---|---|---|---|
| 0 | 6.4 | -6.4 | -0.4 |
| 1.056 | 6.8 | -5.7 | -0.3 |
| 5.28 | 10.9 | -5.6 | -0.3 |
| 10.56 | 16.8 | -6.3 | -0.4 |
| 52.8 | 58.9 | -6.1 | -0.3 |
| 105.6 | 108.3 | -2.7 | -0.2 |
| 211.2 | 200.1 | 11.1 | 0.6 |
| 1056 | 1029.8 | 26.2 | 1.5 |
| 2112 | 2084.8 | 27.2 | 1.6 |
| 2640 | 2671.8 | -31.8 | -1.8 |

Figure 3. Ten point calibration graph of natamycin spiked into white wine in the range from 1 to 2600 µg/l.

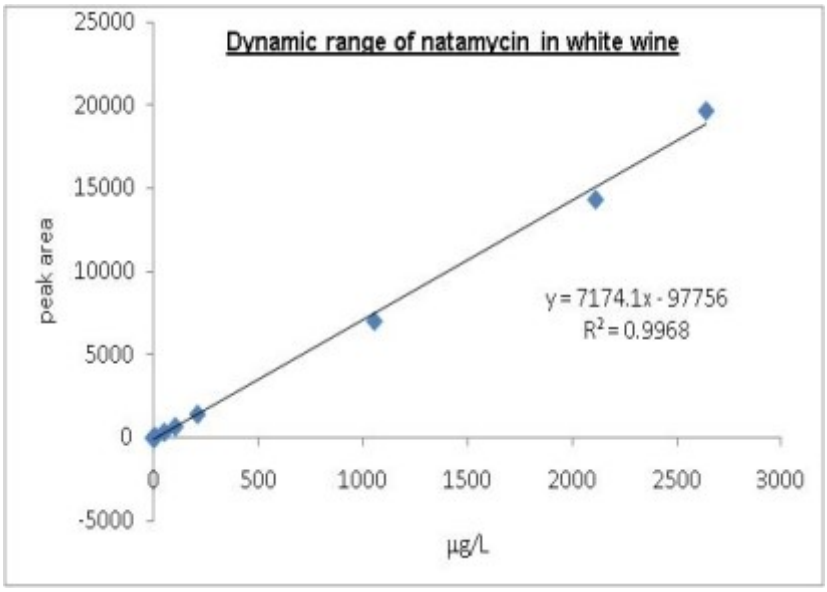



Table 2 White wine matrix calibration residuals.

| Natamycin (µg/l) | Predicted conc. (µg/l) | Residuals | Standard Residuals |
|---|---|---|---|
| 0 | 15.5 | -15.5 | -0.3 |
| 1.056 | 15.6 | -14.6 | -0.3 |
| 5.28 | 18.8 | -13.5 | -0.2 |
| 10.56 | 23.9 | -13.3 | -0.2 |
| 52.8 | 63.6 | -10.8 | -0.2 |
| 105.6 | 109.3 | -3.7 | -0.1 |
| 211.2 | 212.8 | -1.6 | 0.0 |
| 1056 | 989.0 | 67.0 | 1.2 |
| 2112 | 2003.2 | 108.8 | 2.0 |
| 2640 | 2742.7 | -102.7 | -1.8 |

Figure 4. Ten point Calibration graph of natamycin spiked into red wine in the range from 1 to 2600 µg/l.

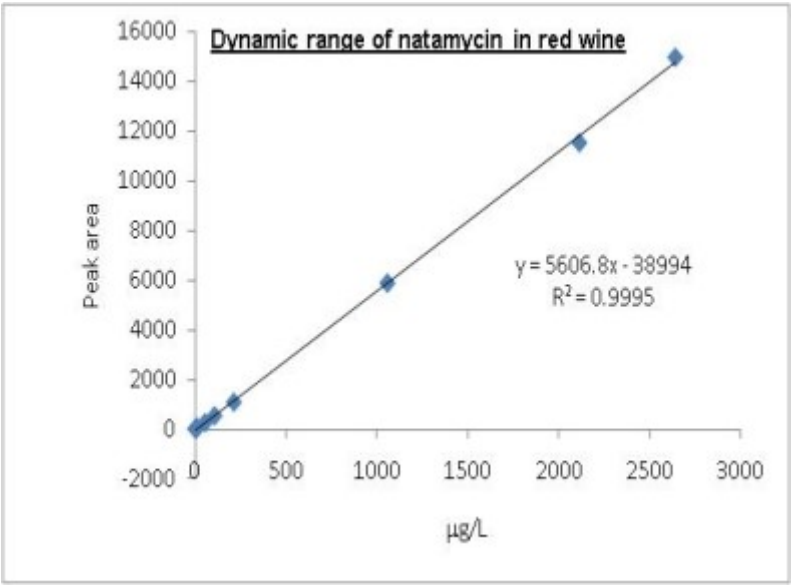



Table 3. Red wine matrix calibration residuals.

| Natamycin (µg/l) | Predicted conc. (µg/l) | Residuals | Standard Residuals |
|---|---|---|---|
| 0 | 7.2 | -7.2 | -0.3 |
| 1.056 | 8.2 | -7.1 | -0.3 |
| 5.28 | 10.9 | -5.7 | -0.3 |
| 10.56 | 16.8 | -6.2 | -0.3 |
| 52.8 | 52.1 | 0.7 | 0.0 |
| 105.6 | 102.1 | 3.5 | 0.2 |
| 211.2 | 199.8 | 11.4 | 0.5 |
| 1056 | 1055.2 | 0.8 | 0.0 |
| 2112 | 2063.7 | 48.3 | 2.3 |

| | | | |
|---|---|---|---|
| 2640 | 2678.4 | -38.4 | -1.8 |

2.1.9.2. Accuracy and Precision

The method was assessed for repeatability at the interventiont limit of 5 µg/l and at 200 µg/l in solvent, white wine and red wine matrices (tables 4, 5 and 6). The accuracy was assessed by spiking a known amount at two different levels into white and red wine. The analysis was then performed by a second analyst without the knowledge of the spiked natamycin concentration. The results are shown in table 7.

Table 4. Repeatability of natamycin spiked into solvent (methanol:water:acetic acid, 50:47:3 v/v) at two concentrations; 5 and 200 µg/l.

| | **Conc. Natamycin µg/l** | **Recovery (%)** |
|---|---|---|
| **Solvent std at 5 ng/ml rep 1** | 5.3 | 99.7 |
| **Solvent std at 5 ng/ml rep 2** | 5.4 | 101.8 |
| **Solvent std at 5 ng/ml rep 3** | 5.8 | 108.6 |
| **Solvent std at 5 ng/ml rep 4** | 5.7 | 108.2 |
| **Solvent std at 5 ng/ml rep 5** | 5.8 | 109.0 |
| **Solvent std at 5 ng/ml rep 6** | 5.9 | 112.2 |
| **Solvent std at 5 ng/ml rep 7** | 5.7 | 108.4 |
| **Solvent std at 5 ng/ml rep 8** | 6.4 | 120.2 |
| | | |
| **Average** | 5.8 | 108.5 |
| **Std deviation** | 0.3 | 6.2 |
| **RSD (%)** | 5.7 | 5.7 |

| | | |
|---|---|---|
| **Solvent std at 200 ng/ml rep 1** | 238.3 | 112.9 |
| **Solvent std at 200 ng/ml rep 2** | 237.1 | 112.4 |
| **Solvent std at 200 ng/ml rep 3** | 231.5 | 109.7 |
| **Solvent std at 200 ng/ml rep 4** | 228.0 | 108.1 |
| **Solvent std at 200 ng/ml rep 5** | 244.0 | 115.7 |
| **Solvent std at 200 ng/ml rep 6** | 220.7 | 104.6 |
| **Solvent std at 200 ng/ml rep 7** | 229.4 | 108.7 |
| **Solvent std at 200 ng/ml rep 8** | 251.7 | 119.3 |
| | | |
| **Average** | 235.1 | 111.4 |
| **Std deviation** | 9.8 | 4.7 |
| **RSD (%)** | 4.2 | 4.2 |

Table 5. Repeatability of natamycin spiked into white wine at two concentrations; 5 and 200 µg/l.

| | **Conc. Natamycin µg/l** | **Recovery (%)** |
|---|---|---|
| **White wine 5.3 ng/ml rep 1** | 5.3 | 99.1 |
| **White wine 5.3 ng/ml rep 2** | 4.4 | 82.8 |
| **White wine 5.3 ng/ml rep 3** | 5.1 | 96.0 |

| | | |
|---|---|---|
| White wine 5.3 ng/ml rep 4 | 4.9 | 92.5 |
| White wine 5.3 ng/ml rep 5 | 4.6 | 86.4 |
| White wine 5.3 ng/ml rep 6 | 5.1 | 96.4 |
| White wine 5.3 ng/ml rep 7 | 4.8 | 90.9 |
| White wine 5.3 ng/ml rep 8 | 4.9 | 92.2 |
| Average | 4.9 | 92.0 |
| Std deviation | 0.3 | 5.4 |
| RSD (%) | 5.9 | 5.9 |
| White wine 211 ng/ml rep 1 | 217.6 | 103.1 |
| White wine 211 ng/ml rep 2 | 223.3 | 105.8 |
| White wine 211 ng/ml rep 3 | 213.0 | 101.0 |
| White wine 211 ng/ml rep 4 | 216.8 | 102.7 |
| White wine 211 ng/ml rep 5 | 211.4 | 100.2 |
| White wine 211 ng/ml rep 6 | 208.6 | 98.9 |
| White wine 211 ng/ml rep 7 | 204.2 | 96.8 |
| White wine 211 ng/ml rep 8 | 214.4 | 101.6 |
| | | |
| Average | 213.7 | 101.3 |
| Std deviation | 5.8 | 2.8 |
| RSD (%) | 2.7 | 2.7 |

Table 6. Repeatability of natamycin spiked into red wine at two concentrations; 5 and 200 µg/l.

| | **Conc. Natamycin ug/l** | **Recovery (%)** |
|---|---|---|
| **Red wine 5.3 ng/ml rep 1** | 5.3 | 99.7 |
| **Red wine 5.3 ng/ml rep 2** | 5.0 | 93.8 |
| **Red wine 5.3 ng/ml rep 3** | 3.8 | 72.5 |
| **Red wine 5.3 ng/ml rep 4** | 5.1 | 96.5 |
| **Red wine 5.3 ng/ml rep 5** | 5.0 | 95.0 |
| **Red wine 5.3 ng/ml rep 6** | 5.5 | 103.5 |
| **Red wine 5.3 ng/ml rep 7** | 4.3 | 80.9 |
| **Red wine 5.3 ng/ml rep 8** | 4.8 | 90.7 |
| | | |
| **Average** | 4.9 | 91.6 |
| **Std deviation** | 0.5 | 10.2 |
| **RSD (%)** | 11.1 | 11.1 |
| | | |
| **Red wine 211 ng/ml rep 1** | 183.9 | 87.1 |
| **Red wine 211 ng/ml rep 2** | 178.4 | 84.5 |
| **Red wine 211 ng/ml rep 3** | 181.1 | 85.8 |

| | | |
|---|---|---|
| **Red wine 211 ng/ml rep 4** | 197.5 | 93.6 |
| **Red wine 211 ng/ml rep 5** | 178.2 | 84.5 |
| **Red wine 211 ng/ml rep 6** | 184.2 | 87.3 |
| **Red wine 211 ng/ml rep 7** | 181.2 | 85.9 |
| **Red wine 211 ng/ml rep 8** | 171.3 | 81.2 |
| | | |
| **Average** | 182.0 | 86.2 |
| **Std deviation** | 7.5 | 3.6 |
| **RSD (%)** | 4.1 | 4.1 |

Table 7. Accuracy of natamycin spiked into white and red wine at two concentrations; 125 and 220 µg/l.

| | **Theoretical concentration (µg/l)** | **Obtained concentration (µg/l)** | **Accuracy (%)** | **Z Score** |
|---|---|---|---|---|
| White wine A rep 1 | 125 | 135 | 108 | 0.50 |
| White wine A rep 2 | 125 | 142 | 114 | 0.85 |
| White wine A rep 3 | 125 | 138 | 110 | 0.65 |
| White wine B rep 1 | 220 | 230 | 105 | 0.28 |
| White wine B rep 2 | 220 | 230 | 105 | 0.28 |

| | | | | |
|---|---|---|---|---|
| White wine B rep 3 | 220 | 239 | 109 | 0.54 |
| Red wine A rep 1 | 220 | 213 | 97 | -0.20 |
| Red wine A rep 2 | 220 | 234 | 106 | 0.40 |
| Red wine A rep 3 | 220 | 223 | 101 | 0.09 |
| Red wine B rep 1 | 125 | 129 | 103 | 0.20 |
| Red wine B rep 2 | 125 | 129 | 103 | 0.20 |
| Red wine B rep 3 | 125 | 120 | 96 | -0.25 |

Calculations

Z scores calculated as: $= \frac{(obtained\ concentration - theoretical\ concentration}{target\ standard\ deviation}$

Where:

Target standard deviation = 0.16 x spiked concentration

i.e according to Horwitz

2.2. Détermination of natamycin (pimaricin) in wine by HPLC/DAD

2.2.1. Scope

This method describes an analytical procedure for the determination of natamycin (pimaricin) in wine by HPLC. The level of natamycin is expressed in micrograms per litre (µg/l) of wine.

The described method has been laboratory validated taking into account the influence of the matrix wine (e.g. white wine or red wine).

*2.2.2. Principle*

Non-sparkling wine samples are directly injected into the HPLC system. Sparkling wine samples are degased first by filtration or by using an ultrasonic bath. The analyte is separated from the matrix on a C8-column. The fraction window with the analyte is

automatically transferred to a C18-column for further separation. Natamycin is detected at 304 nm and 319 nm. Additionally the DAD spectrum is used for identification. Quantification is done with reference to external standards.

*2.2.3. Reagents and Material*

*2.2.3.1.* Reagents

*2.2.3.1.1.* Water, deionised

*2.2.3.1.2.* Methanol, HPLC grade *(CAS no. 67-56-1).*

*2.2.3.1.3.* Formic acid, p. a. *(CAS no. 64-18-6).*

*2.2.3.1.4.* Acetic acid, p. a. *(CAS no. 64-19-7).*

*2.2.3.1.5.* Hydrochloric acid, p. a., 0,1 N *(CAS no. 7647-01-0).*

*2.2.3.1.6.* Matrix wine, natamycin not detectable

*2.2.3.1.7.* Natamycin, > 95 % *(CAS no. 7681-93-8).*

The purity is verified by photometric measurement at 291 nm, 304 nm and 319 nm of a natamycin solution in hydrochloric acid, 0,1 N against a blank of hydrochloric acid, 0,1 N:

| Reference data according to the literature | 291 nm | 304 nm | 319 nm |
|---|---|---|---|
| Extinction (1 Gew.% Natamycin, 1 cm cell) | 758 | 1173 | 1070 |

Alternative:

After dilution (e. g. dilution factor 20) the stock solution (2.1.3.3.1.) can also be used for the photometric measurement, e. g. pipette 1,0 ml stock solution into a 20 ml volumetric flask and fill up to the mark with hydrochloric acid, 0,1 N. Measure against a blank with the same composition of solvents as the diluted stock solution.

2.2.3.2. Preparation of the mobile phase

2.2.3.2.1. Solutions for the mobile phase:

2.2.3.2.1.1. 5 ml acetic acid added to 2 l methanol

2.2.3.2.1.2. 5 ml acetic acid added to 2 l deionised water

2.2.3.2.2. Eluent 1: methanol-acetic acid / deionised water-acetic acid (65 / 35)

2.2.3.2.3. Eluent 2: methanol-acetic acid / deionised water-acetic acid (80 / 20)

2.2.3.3. Preparation of the stock and standard solutions

All solutions have a limited stability and have to be stored dark and cold in a

refrigerator. The stock solution (2.1.3.3.1.1) has a shelf life up to several weeks but the concentration has to be checked shortly before usage (e.g. see alternative method, 2.2.3.1.7.). Dilution I ( 2.2.3.3.1.2) and II (2.2.3.3.1.3) and the standard solutions (2.2.3.3.2) have to be prepared daily.

2.2.3.3.1. Preparation of the stock solution and dilutions

2.2.3.3.1.1. Stock solution (approximately 100 mg/l)

Weight in about 5 mg natamycin (3.1.7) and transfer with methanol into a 50 ml volumetric flask. Add 0,5 ml formic acid, make sure that all the natamycin is dissolved, temperate at 20 °C and make up to the mark with methanol.

2.2.3.3.1.2. Dilution I (approximately 5 mg/l)

Pipette 2,5 ml of the stock solution (2.1.3.3.1.1) into a 50 ml volumetric flask and make up to the mark with deionised water.

2.2.3.3.1.3. Dilution II (approximately 1 mg/l)

Pipette 4 ml of Dilution I (2.2.3.3.1.2) into a 20 ml volumetric flask and make up to the mark with the matrix wine (2.2.3.1.6).

2.2.3.3.2. Preparation of the standard solutions

For the standard solutions dilute Dilution II (2.2.3.3.1.3) to the desired concentrations with the matrix wine (2.2.3.1.6), e. g. 50 µl into a 10 ml volumetric flask equals 5 µg/l:

| Volumetric flask | 10 ml | 10 ml | 10 ml | 10 ml | 10 ml | 10 ml | 10 ml |
|---|---|---|---|---|---|---|---|
| Volume of Dilution II (µl) | 50 | 100 | 200 | 400 | 500 | 1000 | 3700 |
| Amount of natamycin (µg/l) | 5 | 10 | 20 | 40 | 50 | 100 | 370 |

2.2.4. Apparatus

Usual laboratory equipment, in particular the following:

2.2.4.1. HPLC-DAD apparatus with a 6 port HPLC valve and two isocratic pumps or a gradient pump to enable fractionation

2.2.4.2. HPLC-column RP-8

2.2.4.3. HPLC-column RP-18

2.2.4.4. Photometer

2.2.5. Sampling

Non-sparkling wine samples are directly injected into the HPLC system. Sparkling wine samples are first degased by filtration or by using an ultrasonic bath. If samples need to be stored the storage conditions should be cold and dark.

2.2.6. *Procedure*

2.2.6.1. Operating conditions of HPLC

The following columns and parameters have been found to be suitable:

Column 1: C 8-column (e.g. Select B 125*4mm/5 µm endcapped, Merck)

Mobile phase: Eluent 1 (2.2.3.2.2) at room temperature

Flow rate: 1 ml/min

Column 2: C 18-column (e.g. Lichrospher 125*4mm/5µm, Merck)

Mobile phase: Eluent 2 (2.2.3.2.3) at 30°C

Flow rate: 1 ml/min

Injection volume: 500 µl

UV-detection: 304 nm and 319 nm

Fraction window: The position of the fraction window has to be checked prior to the following analysis (fig. 1). The range of the fraction window has to be set at 0,5 min. before and after the desired peak elutes from the C 8-column.

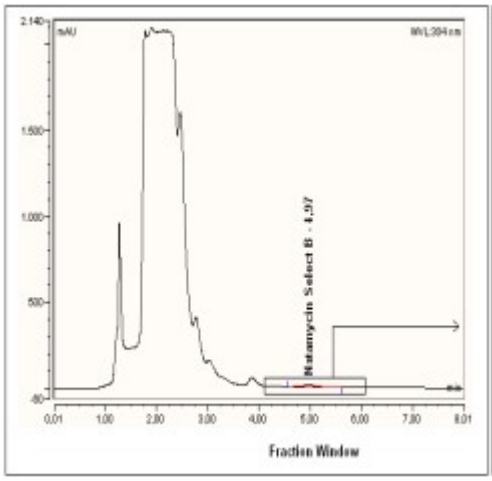



Fig. 1 Column 1 Fraction window

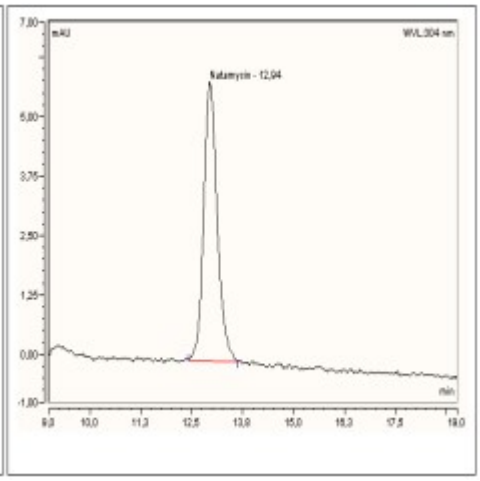



Fig. 2 Column 2
White wine spiked with natamycin (50 µg/l)

2.2.6.2. Identification/ Confirmation

Identification of peaks is done by the comparison of retention times between standards and samples for both measured wavelengths 304 nm and 319 nm. Using the chromatographic system and parameters of 2.2.6.1 the retention time for natamycin is approximately 12,9 min (fig. 2).

The DAD spectrum is used for further confirmation of positive findings (fig. 3 and fig.

4).

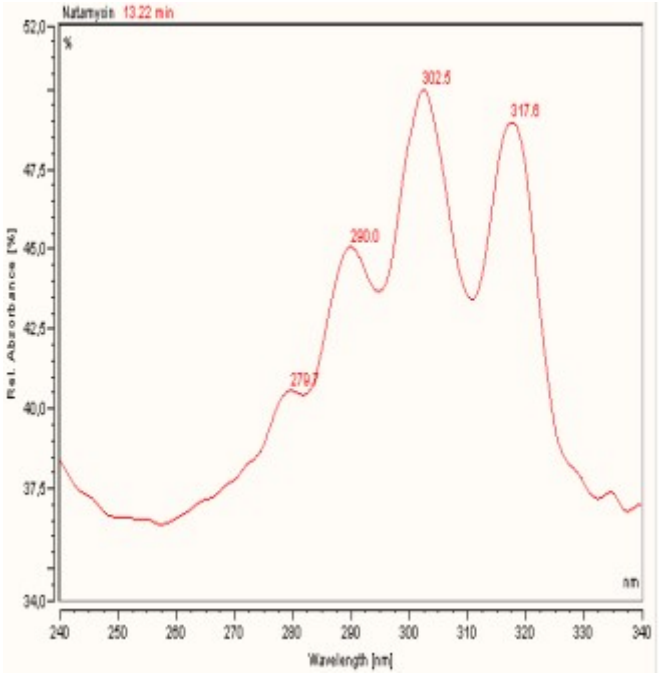



Fig. 4 DAD spectra of natamycin

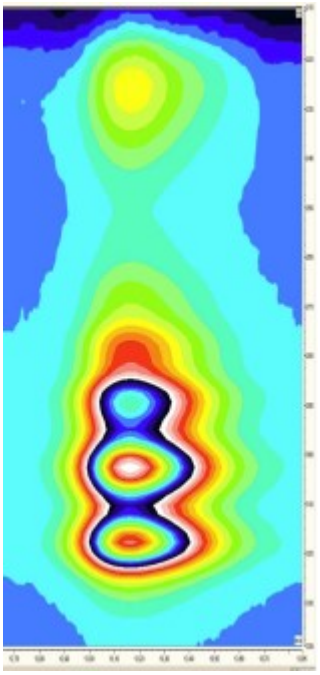


Fig. 3 3D-DAD spectra of natamycin

2.2.7. *Calculation and expression of results*

A calibration curve of the standard solutions (2.2.3.3.2) is prepared using the chromatograms measured at 304 nm. The quantification of natamycin is performed following the external calibration method. A linear calibration curve is generated by comparison of the peak areas and the relevant concentrations. The correlation coefficient should be at least 0,99.

The expression of the results is µg/l.

*2.2.8. Method performance data*

Detection limit, Quantification limit

The detection limit and quantification limit were determined according to DIN 32645 (direct determination: multiple measurement of a blank matrix sample, n=10, and a calibration curve that covers the total working range).

Detection limit: 2,5 µg/l

Quantification limit: 8,5 µg/l

Linearity

The linearity in a wine matrix is confirmed in the calibration range of 5 µg/l to 100

µg/l (fig. 5).

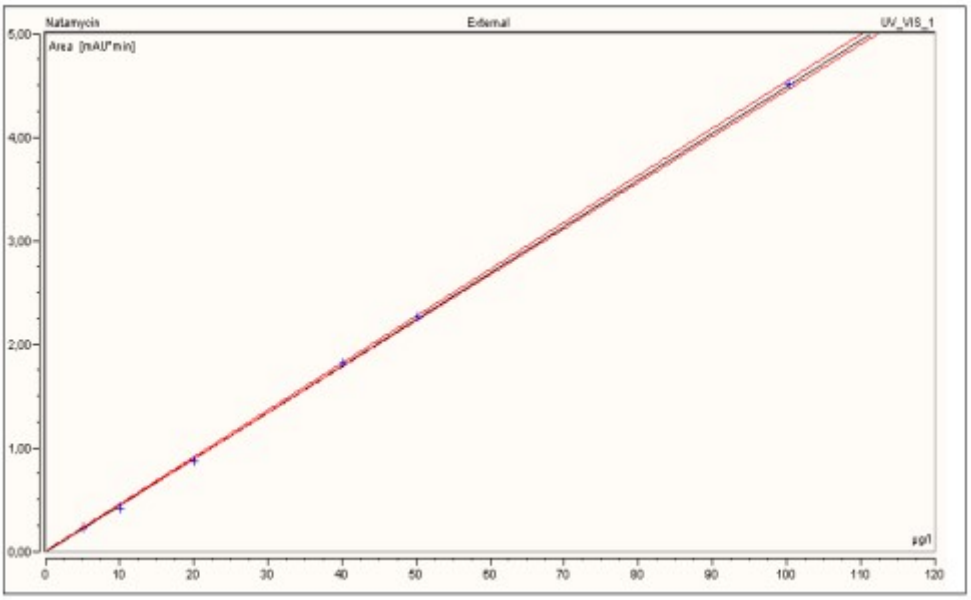



Fig. 5 : Six point calibration graph of natamycin spiked into white wine matrix in the range from 5 to 100 µg/l, R2=0,9999

2.2.9. Trueness and Precision

Trueness and repeatability were assessed by spiking a known amount of natamycin into white, rosé and red wine and measuring each of these samples five times. The results are shown in table 1.

| Matrix | Natamycin content in matrix (µg/l) | Spiked natamycin content (µg/l) | Measured natamycin content (µg/l) | Recovery rate (%) | Z-Score |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| White wine | n. d. | 5.02 | 5.04 | 100.4 | 0.0 |
| | | | 4.70 | 93.6 | -0.2 |
| | | | 5.12 | 102.0 | 0.1 |
| | | | 5.29 | 105.4 | 0.2 |
| | | | 4.97 | 99.0 | 0.0 |
| | | **Average** | **5.02** | **100.1** | |
| | | **Std dev.** | **0.22** | | |
| | | **RSD (%)** | **4.3** | | |
| | | **Repeatability r** | **0.85** | | |
| Rosé wine | n. d. | 5.02 | 4.79 | 95.4 | -0.1 |
| | | | 4.83 | 96.2 | -0.1 |
| | | | 4.76 | 94.8 | -0.1 |
| | | | 4.79 | 95.4 | -0.1 |
| | | | 4.73 | 94.2 | -0.2 |
| | | **Average** | **4.78** | **95.2** | |
| | | **Std dev.** | **0.04** | | |
| | | **RSD (%)** | **0.78** | | |
| | | **Repeatability r** | **0.15** | | |

| | | | | | |
|---|---|---|---|---|---|
| Red wine | n. d. | 5.02 | 4.61 | 91.8 | -0.2 |
| | | | 4.65 | 92.6 | -0.2 |
| | | | 4.89 | 97.4 | -0.1 |
| | | | 4.67 | 93.0 | -0.2 |
| | | | 4.34 | 86.5 | -0.4 |
| | | **Average** | **4.63** | **92.3** | |
| | | **Std dev.** | **0.20** | | |
| | | **RSD (%)** | **4.2** | | |
| | | **Repeatability r** | **0.77** | | |
| Red wine | n. d. | 21.2 | 19.73 | 93.1 | -0.2 |
| | | | 20.66 | 97.5 | -0.1 |
| | | | 21.16 | 99.8 | 0.0 |
| | | | 19.73 | 93.1 | -0.2 |
| | | | 19.58 | 92.4 | -0.3 |

| | | | | | |
|---|---|---|---|---|---|
| | | **Average** | **20.17** | **95.2** | |
| | | **Std dev.** | **0.70** | | |
| | | **RSD (%)** | **3.5** | | |
| | | **Repeatability r** | **2.7** | | |

| | | | | | |
|---|---|---|---|---|---|
| Red wine | n.d. | 53.2 | 51.84 | 97.4 | -0.1 |
| | | | 51.91 | 97.6 | -0.1 |
| | | | 51.42 | 96.7 | -0.1 |
| | | | 50.12 | 94.2 | -0.2 |
| | | | 50.62 | 95.2 | -0.2 |
| | | **Average** | **51.18** | **96.2** | |
| | | **Std dev.** | **0.78** | | |
| | | **RSD (%)** | **1.5** | | |
| | | **Repeatability r** | **3.1** | | |

Table 1 Accuracy of natamycin spiked into white. rosé and red wine; n.d. “not detected”. detection limit 2.5 µg/l

Calculations (Table 1):

Repeatability r = Std dev. * $t_{4;0.95}$ * $2^{1/2}$

Z score = (measured amount-spiked amount)/ target standard deviation *

* according to Horwitz

target standard deviation = 1/100 * spiked amount * $2^{(1 - 0.5 \log \text{spiked amount})}$

**References**


- DIN 32645:2008-11
- UV- und IR-Spektren wichtiger pharmazeutischer Wirkstoffe. Editio Cantor Aulendorf. 1978. Herausgeber/ Editior Hans-Werner Dibbern in Zusammenarbeit mit E. Wirbitzki
- Macarthur R. Feinberg M. Bertheau Y. 2010. Construction of measurement uncertainty profiles for quantitative analysis of genetically modified organisms based on interlaboratory validation data. Journal of the Association of Official Analytical Chemists. 93(3). 1046 – 1056.
- FV 1351. Dominic Roberts and Adrian Charlton. Determination of natamycin in

wine by liquid chromatography coupled to high resolution mass spectrometry: standard operating procedure and method performance data. OIV SCMA March 2010.

- FV 1355. Tomasz Brzezina. Natamycin in Wein. OIV SCMA March 2010.