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ORIGINAL PAPER

A novel and rapid method for determination of natamycin in wines based on ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry: validation according to the 2002/657/EC European decision

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Abstract A novel, simple, and rapid reversed-phase liquid chromatography–tandem mass spectrometric methodology was developed for the analysis of natamycin in wine samples. Natamycin was protonated to form singly charged ions in an electrospray positive ion mode. Data acquisition under MS/MS was achieved by applying multiple reaction monitoring (MRM) of three fragment ion transitions (666.3→648.2, 666.3→503.3, and 666.3→485.2) to provide a high degree of sensitivity and specificity. Chromatographic separation was performed on a rapid resolution column using a mobile phase consisting of an acetonitrile/water mixture with a total run time of 5.0 min. After only filtration as pretreatment, the sample was injected into the chromatographic system. The proposed method was validated in terms of selectivity, trueness, precision, decision limit (CCα), and detection capability (CCβ) according to 2002/657/EC Commission decision. The values for trueness, reported as bias (%), agreed with those established by the aforementioned document. Repeatability (intraday variability) values were 12.37% at a concentration of 1.0 μg L^{-1} and 8.99–4.19% at concentrations between 2.5 and 10 μg L^{-1}. The overall within-laboratory (interday variability) reproducibility was 15.47% at a concentration of 1.0 μg L^{-1}, which was significantly lower than the indicative value reported in the EU decision. The results indicated that the proposed approach is a sensitive, fast, reproducible, and robust methodology suitable for the analysis of natamycin levels in wine samples.

Keywords Natamycin · Wines · LC-MS/MS · 2002/657/EC European decision

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Introduction

Natamycin (also known as pimaricin, E235) is a polyene macrolide antimycotic (Fig. 1) produced by *Streptomyces natalensis*. It is used as a fungicidal and antibiotic preservative in some branches of food industry [1]; however, if consumed in excess, natamycin can damage health by neutralizing antibodies to certain infections [2]. Natamycin is used in cheeses and sausages because of its ability to kill yeasts, fungi, and microbiological organisms which harm the maturation process. Therefore, according to the 95/2/EC directive, natamycin may be used for the surface treatment of semi-hard and semi-soft cheeses and dry, cured sausages at a maximum level of 1 mg dm^{-2} [3]. Natamycin has a limited, but important use in human medicine and, therefore, it is not acceptable as a food additive for general usage in foodstuffs. As a result, this macrolide cannot be added to wines because is not in the list of additives permitted by the Code of Enological Practices of the International Organization of Vine and Wine (OIV). The General Standards for Food Additives of the Codex Alimentarius define its uses without stipulating it in wine [4]. At same time, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) consider an acceptable daily intake (ADI) of natamycin to be 0.3 mg per kg body weight per day [5]. However, a clinical study in humans performed

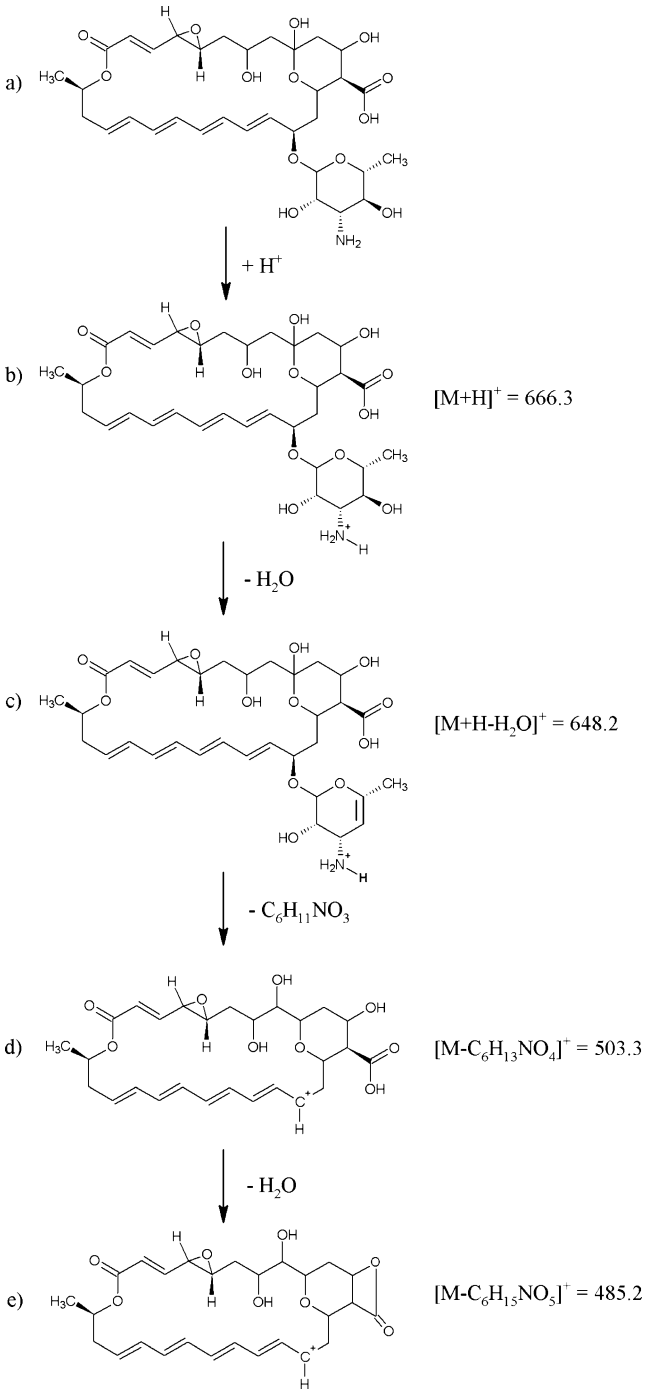

Fig. 1 Natamycin structure and possible fragmentation pathway (data acquisition under MS/MS was achieved by applying MRM of three fragment ion transitions). **a** Compound structure; **b** parent ion, *m/z* 666.3; **c** transition 1, 666.3→648.2; **d** transition 2, 666.3→503.3; and **e** transition 3, 666.3→485.2

in 1960 showed that natamycin, when used for systemic mycoses, induced nausea and diarrhea. Anorexia and other symptoms were also observed and daily natamycin ingest could affect the body's ability to heal of certain infections [6].

In late 2009 and early 2010, European disease control authorities detected natamycin in wines from some wine-producing countries and banned their sale and distribution. Currently, worldwide wineries and wine traders need to demonstrate that residues of natamycin are absent or in concentrations below 5.0 μg L^{-1}, using this value as a maximum residue limit (MRL) [7].

Considering the low concentrations and the need for routine analysis, it is critical to develop sensitive, robust, and rapid methodologies for qualitative and quantitative analysis of natamycin in wine samples. The physical and chemical properties of natamycin, such as low solubility in water, low volatility, and the presence of four conjugated double bonds, allow its analysis by liquid chromatography (LC) with UV–visible and diode array detection (DAD) without derivatization [8]. The JECFA therefore reported an assay for the determination of natamycin in sausages and cheeses using high-performance liquid chromatography (HPLC) with UV detection [5]. Also, numerous methodologies have been developed for determination of natamycin in cheese-derived products using molecular absorption spectrophotometry and HPLC [9–11]. On the other hand, some reviews [12, 13] and several liquid chromatography–tandem mass spectrometry-based methods have been proposed for analyzing antifungal and antibiotic macrolides in various foodstuffs [14–27]. Currently, there is only one literature report regarding the determination of natamycin in wines [7]. In this work, a chromatographic approach coupled to electrospray ionization–high resolution mass spectrometry (LC-ESI-HRMS) was employed. Thus, natamycin was chromatographically resolved in 30 min with a conventional C_{18} column; detection and quantification limits of 3.0 and 5.0 μg L^{-1}, respectively, were reported. Considering these figures of merit and the MRL established for natamycin in wines, it is noticeable that the detection capability of this previously published method is insufficient to avoid false positives or negatives. In comparison, the methodology proposed herein offers better chromatographic conditions and the analytical figures of merit have been highly improved. In addition, validation has been thoroughly performed using the concepts of CCα and CCβ according to the 2002/657/EC European decision [28]. Thus, this proposed methodology is suitable for the routine analysis of natamycin in wine samples.

In this work, we evaluated the potential of reversed-phase liquid chromatography using a column with particle size below 2 μm (1.7 μm) coupled to tandem mass spectrometry for the separation/determination and quantification of natamycin in white and red wines. The specificity of tandem mass spectrometry allowed the analysis of the compound using calibration curves prepared in the sample matrix without needing prior sample cleanup. Minimum sample preparation, short chromatographic runs, and low

detection and quantification limits are some of the advantages of the proposed method.

Experimental

Chemical and standards

Natamycin VETRANAL® (32417) analytical standard was obtained from Fluka (Steinheim, Germany). Acetonitrile, methanol, and water Optima® LC-MS grade were purchased from Fisher Scientific (Fair Lawn, New Jersey). Formic acid was obtained from Fisher Scientific (Loughborough, UK). Methanolic working standard solutions were prepared by stepwise dilution from a 1.0 mg L^{-1} natamycin stock standard solution immediately before use. Intermediate 100 μg L^{-1} spikedsamples of red and white wines were prepared, the natamycin contents of which were previously determined and resulted to be absent or below the detection capability of the proposed method. Quantification was achieved by preparing spiked red and white wines samples with appropriate amounts of the analyte. The solutions were maintained at 4 °C, protected from light, and kept in flasks. For the solid-phase extraction step, 3 mL and 60 mg Oasis HLB™ cartridges (Waters, Milford, USA) were used.

HPLC instrumentation and conditions

An Acquity™ Ultra High Performance LC system (Waters, Milford) equipped with autosampler injection and pump systems (Waters, Milford) was used. The autosampler vial tray was maintained at 15 °C. The needle was washed with proper mixtures of acetonitrile and water. The separation was performed by injecting a 10-μL sample onto an ACQUITY UPLC® BEH C_{18} (Waters, Milford, USA) analytical column with 2.1-mm internal diameter, 50-mm length, and 1.7-μm particle size. The binary mobile phases consisted of water with 0.1% (v/v) of formic acid (A) and acetonitrile with 0.1% (v/v) of formic acid (B) delivered at 0.45 mL min^{-1}. The C_{18} gradient was started at an initial composition of 70% A and 30% B, then 3-min linear gradient to 0% A, held for 0.5 min. A return to the initial conditions was accomplished by a 0.2-min gradient to 70% A, where it was held for 1.3 min. Thus, the total chromatographic run time was 5.0 min. The column was held at a temperature of 30 °C. Under these conditions, no sample contamination or sample to sample carryover was observed.

Mass spectrometry instrumentation and MS/MS conditions

Mass spectrometry analyses were performed on a Quattro Premier™ XE Micromass MS Technologies triple quadrupole mass spectrometer with a ZSpray™ electrospray ionization source (Waters, Milford, USA). The source was operated in the positive (ES+) mode at 350 °C with N_2 as the nebulizer and the source temperature was kept at 150 °C. The capillary voltage was maintained at 3.0 kV and the extractor voltage was set at 5.0 kV. Ultrapure nitrogen was used as desolvation gas with a flow of 800 L h^{-1}. Argon was used as the collision gas at a flow of 0.18 mL min^{-1}. Detection was performed in multiple reaction monitoring (MRM) mode of selected ions at the first (Q_1) and third quadrupole (Q_3). To choose the fragmentation patterns of m/z (Q_1)→m/z (Q_3) for the analytes in MRM mode, direct infusion (via syringe pump) into the MS of natamycin standard solution in methanol was performed and the product ion scan mass spectra were recorded. The data were acquired using MassLinx Mass Spectrometry Software (Waters, Milford, USA).

Sample preparation

Red and white Argentinean wine varieties (Malbec, Cabernet Sauvignon, Syrah, Tempranillo, Merlot, Petit Verdot, Pasionado, Bonarda, Chardonnay, Sauvignon Blanc, Pinot Noir, Torrontes, Viognier, among others) from 2005 to 2010 vintages were used for validation and application studies. The samples were supplied by local wineries. A 1-mL aliquot of wine was filtered through a 0.2-μm polytetrafluoroethylene (PTFE) syringe filter into a 1.5-mL amber glass vial prior to analysis by LC-MS/MS. On the other hand, to assess the matrix effect, a cleanup through a solid-phase extraction (SPE) procedure was performed. The SPE was developed and optimized using varying volumes of conditioning/elution solvents and sample. One-milliliter sample aliquots were applied to Oasis HLB cartridges equilibrated with 1 mL of methanol and 1 mL of water, and eluted with 1 mL of 20, 40, 60, 80, or 100% of acetonitrile or methanol. Natamycin recovery was above 98% in the 100% methanolic fraction.

Results and discussion

Optimization of MS parameters and MRM transitions

Preliminary experiments were conducted to find the best instrumental conditions that would allow identification of natamycin in wine samples at trace levels. Natamycin standard solution (1 mg L^{-1}) in methanol was introduced into the MS system at a flow rate of 20 μL min^{-1} via a syringe pump. Identification of the parent ion was performed in the full-scan mode by recording mass spectra from m/z 100 to 800 in positive mode. As established by the 2002/657/EC Commission decision in the item 2.3.3.2

for quantitative mass spectrometric detection, a minimum of three identification points are required to meet the identification performance criteria [29]. In this work, monitoring of one precursor ion and three daughter ions 'earned' 5.5 identification points (1 for the parent ion and 1.5 for each daughter ion) and, therefore, fulfilled the mentioned criteria giving the necessary specificity to identify a substance correctly. To evaluate the transitions in the MRM mode, the precursor and the product ions of natamycin were selected according to the analyte's fragmentation pattern (Fig. 1). The MRM conditions were further optimized for the analytes to obtain maximum sensitivity (Table 1). Quantitative results including ion ratios were calculated using QuanLynx software bundled with MassLynx v4.1. As a result, the most sensitive transition (666.3→503.3) was selected for quantification.

Optimization of chromatographic conditions

To improve natamycin chromatographic retention, a reversed-phase column (ACQUITY UPLC® BEH C_{18} of 2.1×50 mm, 1.7 μm dimensions) and several mobile phases such as methanol, acetonitrile, and water were investigated. The results indicated that a mixture of water/acetonitrile as gradient solvents was optimal; this gradient was compatible with the necessary conditions of high organic content in the mobile phase to allow formation of a fine spray of small droplets in the ESI interface. To enhance the signal response, mobile phase modifiers such as acetic acid and formic acid were also studied. The type (acetic or formic acid) and concentration (from 0.05 to 4.0% (v/v)) of buffer were evaluated. The use of formic acid led to improved peak shape and retention time compared with acetic acid. The use of either buffer gave improved retention time, peak shape, and sensitivity compared with no buffer. As a result, a 0.1% (v/v) formic acid concentration provided the maximum response for the generation of the protonated $[M+H]^+$ natamycin ion, which was used for further MRM experiments. The effect of the mobile phase flow rate on the separation/retention of natamycin was evaluated using van Deemter plots. Ten microliters of the standard sample was injected onto the reversed-phase system at varying flow rates, from 0.1 to 1.0 mL/min with isocratic separation. A flow rate of 0.45 mL min^{-1} gave the best results in terms of chromatographic conditions and ESI efficiency. In addition, the effect of column temperature on the retention of natamycin was studied. The Van't Hoff plot for natamycin on the C_{18} column over the temperature interval from 20 to 60 °C was evaluated. Natamycin retention's behavior decreased when increasing the column temperature. The optimal retention conditions were obtained when the temperature was fixed at 30 °C. This temperature was selected for further experiments. Under the optimal mentioned conditions, the analyte was eluted at 2.52 min from the column within a 5.0-min total run cycle; these values are considerably shorter than those recently reported by Roberts et al. [7].

Table 1 Optimized MRM detection parameters for ion transitions of natamycin

Transition (*m/z*)	Dwell time (s)	Cone voltage (V)	Collision voltage (V)
666.3→648.2	0.08	15	13
666.3→503.3[a]	0.08	15	11
666.3→485.2	0.08	15	11

[a] Transition used for quantification

Method validation

According to the 2002/657/EC document [28], both the decision limit (CCα) and the detection capability (CCβ) are important parameters to validate a method. The CCα was defined as "the limit at and above which it can be concluded with an error probability of α (α=0.05), that a sample is non-compliant", and CCβ as "the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β (β=0.05)". In a first approach, the CCα and CCβ values were calculated using the protocols provided by the 2002/657/EC decision along with the guidelines and equations proposed by Antignac et al. [30] about blank samples' analyses. However, after experimental evaluation, this calculation approach was discarded because of the lack of consideration of homoscedastic or heteroscedastic behavior, matrix effect, and the variability at each studied concentration. Therefore, a second approach proposed by the International Standard ISO 11843–2 [31] and tailored by Van Loco et al. [32], which measures variances associated with the concentrations levels of the calibration curve, was used to calculate CCα and CCβ. Thus, the impact of homoscedasticity and heteroscedasticity over the calculation of CCα, CCβ, and precision was considered. These last aspects were of great importance for the present work because the experimental results for red wine samples showed a heteroscedastic behavior, whereas those corresponding to white wines followed a homoscedastic trend. Thus, the analytical performance, repeatability, within-laboratory reproducibility, and CCα and CCβ values were evaluated and calculated considering these tendencies. Hence, under the assumption of linearity and homoscedasticity CCα and CCβ were given by [32]:

$$CC\alpha = t_{df,1-\alpha}\frac{Sy}{b}\sqrt{1+\frac{1}{n}+\frac{\bar{x}^2}{j\sum_{i=0}^{I}(\bar{x}_i-\bar{x}^2)}} \quad (1)$$

$$CC\beta = \delta_{df,\alpha,\beta}\frac{Sy}{b}\sqrt{1+\frac{1}{Ij}+\frac{\bar{x}^2}{j\sum_{i=0}^{I}(\bar{x}_i-\bar{x}^2)}} \quad (2)$$

And in the case of heteroscedasticity:

$$CC\alpha = \frac{tdf,1-\alpha}{b}\sqrt{S^2_{CC\alpha}+\left[\left(\frac{1}{j\sum_{i=1}^{I}w_i}\right)+\frac{\bar{x}_w^2}{j\sum_{i=1}^{I}w_i(\bar{x}_i-\bar{x})^2}\right]S_y^2} \quad (3)$$

$$CC\beta = \frac{\delta_{df,\alpha,\beta}}{b}\sqrt{S^2_{CC\beta}+\left[\left(\frac{1}{j\sum_{i=1}^{I}w_i}\right)+\frac{\bar{x}_w^2}{j\sum_{i=1}^{I}w_i(\bar{x}_i-\bar{x})^2}\right]S_y^2} \quad (4)$$

where b is the slope of the regression curve, $\bar{x}$ the mean concentration, t the associated t values, δ the non-centrality parameter, S_y the standard error of the estimate, J the number of the replicates per concentration level of the spiked samples, and I the number of concentration levels for the spiked samples: $i=1, 2\ldots I$. The non-centrality parameter can be approximated by $\delta_{\alpha,\beta}=t_{df,1-\alpha}+t_{df,1-\beta}$; and $\bar{x}_w=\sum_{i=1}^{I}w_ix_i/\sum_{i=1}^{I}w_i$. The weighing factors are $w_i=1/S_i^2$, and S_i^2 is obtained by regressing the standard deviation at each level (S_i) versus the concentration following the variance equation: $S^2=(a'+b'X)^2$. The variance at $CC\alpha(S^2_{CC\alpha})$, and $CC\beta(S^2_{CC\beta})$ were determined by $S^2_{CC\alpha}=(a'+b'CC\alpha)^2$ and $S^2_{CC\alpha}=(a'+b'CC\beta)^2$, respectively. These standard deviations were imput into Eqs. 3 and 4 and they were solved by iteration.

CCα and CCβ calculation

An approach based on spiked samples was developed. Thus, five white and red wine varieties (Chardonnay, Sauvignon Blanc, Pinot Noir, Torrontes, and Viognier; Malbec, Cabernet Sauvignon, Syrah, Tempranillo, and Merlot, respectively) were evaluated. A total of 35 samples composed by five blank samples from each variety of white and red wines, 10 replicates at the MRL (5.0 μg L^{-1}), 5 replicates at 0.5MRL (2.5 μg L^{-1}), 1.5MRL (7.5 μg L^{-1}), 0.2MRL (1.0 μg L^{-1}), and 2MRL (10.0 μg L^{-1}) were used. A coefficient of determination (R^2) greater than 0.7 from the standard deviation regression curve at each concentration level showed the presence of a heteroscedastic trend in the measurements of all the red wines. In the case of the white wines, R^2 less than 0.7 demonstrated the presence of homoscedasticity in the measurements. As a result, CCα and CCβ values were calculated according to Eqs. 1 and 2 for white wines, whereas Eqs. 3 and 4 were employed for red wines. Experimentally, to calculate CCα and CCβ from the spiked samples mentioned, the responses obtained from total ion chromatograms (TICs) and the extracted ion chromatograms (EICs) for two of the most specific natamycin ion transitions, 666.3→503.3 and 666.3→485.2 respectively, were used. The values are shown in Table 2 for red and white wine spiked samples. As seen, the values for CCα and CCβ from the TICs in the case of red wine were higher than those calculated from the EICs. The standard error of the estimate S_y exerts a strong influence in the calculation of these parameters when heteroscedasticity and homoscedasticity occur. The variability associated with the signal (S_y) in the TICs is a product of adding the individual variances of the fragments generating it; thus, the limit of decision and detection capability values are higher. Therefore, if S_y increases, the CCα and CCβ increase as well [33]. On the other hand, the CCα and CCβ values reported for each transition were comparable. As a result, the most sensitive and least variable transition—666.3→503.3—was used to calculate the CCα and CCβ values in red and white wines. In the case of white wine samples, TICs and EICs' values were comparable, which can be explained by the homoscedastic behavior. Additionally, limits of detection (LoD) and limits of quantification (LoQ) values were evaluated as $3.3\times S_y/b$ and $10\times S_y/b$, respectively, and they are also reported in Table 2.

Matrix effect

As is known, one downside of ESI-MS or ESI-MS/MS ionization/detection is that the ionization process is susceptible to matrix signal suppression or enhancement. The liquid chromatography–mass spectrometry response obtained from a standard can differ significantly from matrix samples. The origin and mechanism of matrix effects are not understood fully and there are many possible

Table 2 CCα, CCβ, LoD, and LoQ values (μg L^{-1}) for red and white wines

Response	Red wines				White wines			
	CCα	CCβ	LoD	LoQ	CCα	CCβ	LoD	LoQ
TIC	1.20	3.13	NA	NA	0.67	1.61	NA	NA
666.3→503.3[a]	0.27	0.69	0.68	2.07	0.59	1.42	1.13	3.42
666.3→485.2	0.23	0.58	NA	NA	0.68	1.63	NA	NA

NA not applicable

[a]Transition used for quantification

Table 3 Repeatability (intraday variability) and within-laboratory (interday) reproducibility

Concentration (μg L^{-1})	Repeatability		Within-laboratory reproducibility	
	SD	RSD (%)	Bias (%)[a]	RSD (%)
1.0	0.17	12.37	14.0	15.47
2.5	0.23	8.99	−1.0	11.24
5.0	0.25	5.50	−3.0	8.40
7.5	0.32	4.36	0.5	6.82
10	0.43	4.19	0.5	5.29

SD standard deviation, *RSD* relative standard deviation

[a]Bias (%)=[(measured content−spiked level/spiked level)×100]

sources of ion suppression [34–38]. Published approaches to minimize the ion suppression effects include using a more selective extraction procedure for matrix cleanup, providing more chromatographic retention of analytes, changing buffer and its concentration, flow-splitting or nanospray, post-column addition, and two-dimensional chromatography [39]. Consequently, the effect of sample matrix was assessed by comparing the signal of the analyte in matrices of both high-quality and table wines (spiked samples) to the signal in pure solvent (methanol). Thus, calibration curves from spiked matrix and spiked pure solvent samples were created. The percentage of the quotient of the slopes (b) in the spiked and solvent samples was used as an indicator of the extent of the ion suppression or signal enhancement, which was calculated as $100-(b_{spiked}/b_{solvent}\times 100)$. No signal enhancement, but response reductions of approximately 30% and 5% were observed as a result of matrix interference for red wine and red table wine, respectively. On the other hand, the ion enhancement/suppression in white wine was also evaluated following the same procedure and a negligible change of the signal—approximately 2%—was observed. Accordingly, in order to improve the signal-to-noise ratio and reduce the effect of ion suppression in red wine, a procedure using SPE was developed. The assay conditions were mentioned above. The results derived from SPE applied to red wine and solvent-spiked samples showed no substantial improvement of sensitivity. Thus, the samples were only filtered before introducing them into the LC system.

Trueness, repeatability, and within-laboratory reproducibility

A Certified Reference Material of wine with an informed value for natamycin does not exist. However, it is acceptable to assess the trueness—expressed as bias (%)—of the measurements through recovery of known added amounts of the analyte to a blank matrix [28]. Following the 2002/657/EC document and with the aim of estimating the trueness, intraday repeatability, and interday reproducibility, a total of 35 spiked samples were analyzed: 5 blank samples, 10 replicates at the MRL (5.0 μg L^{-1}), 5 replicates

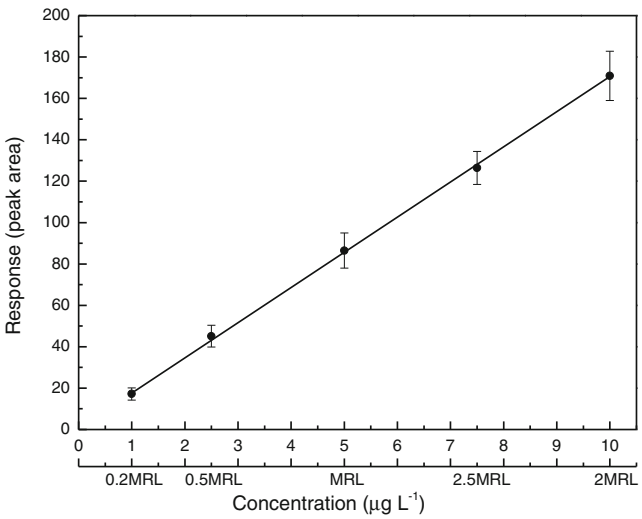

Fig. 2 Linearity and behavior (as variance) of red wine spiked samples. The *error bars* were calculated considering the standard deviations of the measurements at each concentration

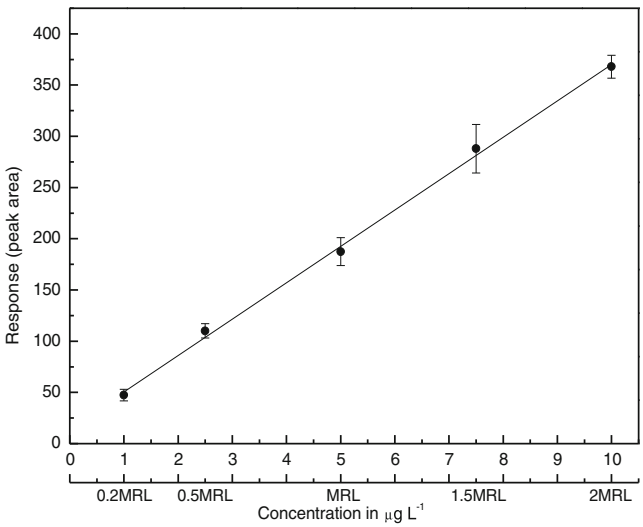

Fig. 3 Linearity and behavior (as variance) of white wine spiked samples. The *error bars* were calculated considering the standard deviations of the measurements at each concentration

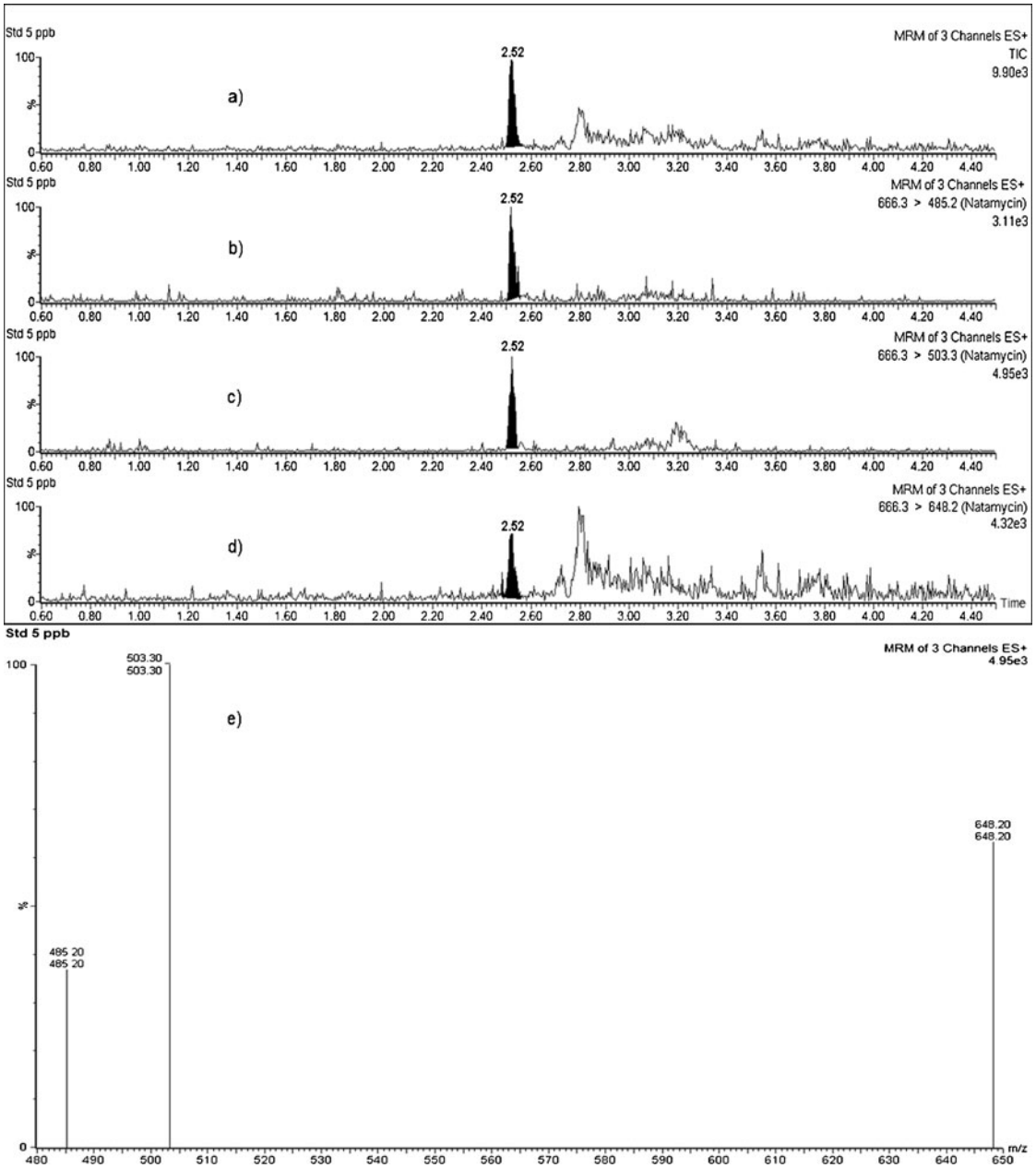

Fig. 4 TIC and EICs resulting from analysis of a wine sample spiked with 5.0 μg L^{-1} of natamycin. **a** TIC, **b** EIC for transition 666.3→485.2, **c** EIC transition 666.3→503.3, **d** EIC for transition 666.3→648.2, and **e** MRM spectrum obtained using the conditions listed in Table 1

at 0.5MRL (2.5 μg L^{-1}), 1.5MRL (7.5 μg L^{-1}), 0.2MRL (1.0 μg L^{-1}), and 2MRL (10 μg L^{-1}); the same experiment was repeated on two other independent occasions with at least a 1-week interval. All the experimental results were in agreement with the bias (%) values established by the European decision: concentration range no greater than 1 μg L^{-1}, acceptable bias between −50% and +20%, and concentration range from greater than 1 to 10 μg L^{-1}, acceptable bias between −30% and +10% (Table 3). Repeatability as intraday variability was determined by calculating the relative standard deviation (RSD (%)) for the replicated measurements. The obtained values were 12.37% at a concentration of 0.2MRL (1.0 μg L^{-1}), and 8.99–4.19% for the concentrations between 0.5MRL (2.5 μg L^{-1}) and 2MRL (10.0 μg L^{-1}) (Table 3). The overall within-laboratory reproducibility (Table 3) was 15.47% at a concentration of 0.2MRL (1.0 μg L^{-1}), which was significantly lower than the indicative value (less than 23% for concentrations lower than 100.0 μg L^{-1}) reported in the EU decision. In summary and taking into account the matrix complexity, the reported values for the method assessment parameters could be considered highly satisfactory.

Linearity

Linearity was evaluated from values closer to the CCα up to approximately 200 μg L^{-1} for both type of wines. The linearity of the calibration curves for red and white wine spiked samples was satisfactory with determination coefficients (R^2) of 0.9996 and 0.9987, respectively. As mentioned earlier, heteroscedasticity was observed for red wine, whereas white wines samples data exhibited a homoscedastic behavior (Figs. 2 and 3 illustrate both types of behavior from 1.0 to 10.0 μg L^{-1}).

Ruggedness and specificity

Analyses of six blank red and white samples from various varieties were performed to check the ruggedness of the method. Analyses of these samples indicated that no interferences for each of the three ion transitions selected for natamycin were present at the analyte's retention time. Over the time period employed for developing this method, variation of the retention time never exceeded 2.0%. This meets the requirements reported in Section 2.3.3.1 of the 2002/657/EC decision. In addition, the ratio between the two most specific ion transitions (666.3→503.3 and 666.3→485.2) was 1.77±0.08. Over a 3-week period, the tolerance of the relative ion abundances varied no more than 5%, in compliance with the maximum permitted tolerance for relative ion transitions (Section 2.3.3.2. of the 2002/657/EC decision). On the other hand, analysis of real samples indicated that no effect on the natamycin determination was observed due to provenance or manufacturing practices; Fig. 4 shows the TIC and EICs (a–d) and the MRM spectrum (e) for a red wine spiked sample at a concentration of 5.0 μg L^{-1}.

Application to real samples

The optimized methodology was applied to the control of sub-trace levels of natamycin in wines. Thus, red and white wine samples (approximately 3,000) from different varieties and between 2005 and 2010 vintage were analyzed. Natamycin either was absent or complied with the MRL in 99.0% of the samples.

Conclusions

Direct injection of wine samples in the LC-MS/MS analysis of natamycin was optimized to obtain the best performance. The method was sensitive, rapid, simple, and offered low solvent consumption. The effect of ion suppression of the matrix was also evaluated. The red wine samples showed greater ion suppression of the signal than white wine samples. The methodology was validated according to the 2002/657/EC European decision and was applied to the routine monitoring and control of natamycin in Argentinean wines and could be extended to the analysis of natamycin in wines from different countries.

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