# Certificate of Analysis

NRC·CNRC Certified Reference Material

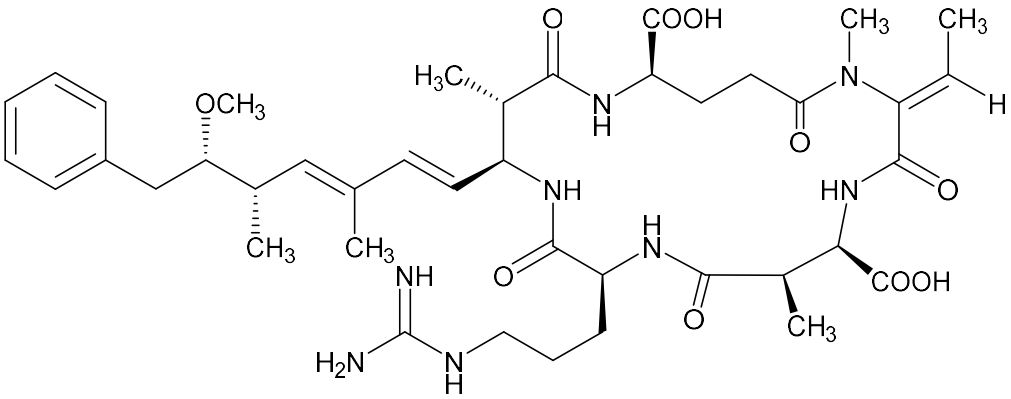
## CRM-NODR (Lot# 20070214)

Certified Calibration Solution for Nodularin-R

Nodularin-R (NODR) is a cyclic peptide toxin produced by freshwater cyanobacteria [1]. Nodularins have been associated with domestic and wild animal poisonings and pose a threat to human health through contamination of drinking water supplies. CRM-NODR is a certified instrument calibration solution prepared to aid the analyst in the determination of nodularin-R. Each ampoule contains approximately 0.5 mL of a solution of the toxin dissolved in aqueous methanol (1:1, v/v) at a concentration suitable for calibration of liquid chromatography experiments.

**Table 1:** Certified concentration values for nodularin-R in CRM-NODR.

| Compound | µmol/L (at +20 °C) | µg/mL (at +20 °C) | µg/g |
|---|---|---|---|
| Nodularin-R | 12.4 ± 0.5 | 10.3 ± 0.4 | 11.1 ± 0.4 |




**Nodularin-R**
CAS registry no: 118399-22-7
Molecular formula: $C_{41}H_{60}N_8O_{10}$
Molecular weight: 824.98 g/mol
$[M+H]^+$: *m/z* 825.4505

Expiry date: 1 year from date of sale.
Storage conditions: freezer (-12 °C or lower)

## Intended Use

CRM-NODR is a calibration solution CRM designed for analytical method development and accurate quantitation of nodularin-R. The concentration of toxin in this CRM is suitable for preparing a dilution series for calibration of instrumentation, such as liquid chromatography with detection by ultraviolet absorbance (LC-UVD) or mass spectrometry (LC-MS).

## Preparation of the CRM-NODR

Nodularin-R was obtained from Åbo Akademi University (Turku, Finland). The toxin was extracted from a culture of *Nodularia sp.*, purified by chromatography and dried *in vacuo*. A portion of the pure toxin was dissolved in 50% $CD_3OH/H_2O$ to give a stock solution that was used in the CRM preparation. The CRM-NODR solution was prepared in filtered (0.2 µm) and degassed methanol/water (1:1, v/v) and dispensed into amber ampoules pre-filled with argon, which were then immediately flame-sealed. Each ampoule contains approximately 0.5 mL of solution.

## Structural Confirmation and Purity Assessment

The molecular structure of the nodularin-R was confirmed by NMR spectroscopy, accurate mass measurement and tandem mass spectrometry (Figures 1-3). The purity of the nodularin-R was checked using the following techniques: 500 MHz proton NMR spectroscopy, LC-UVD, LC-MS, LC with chemiluminescence nitrogen detection (LC-CLND) [2], and capillary electrophoresis (CE-UV). Low levels of three impurities, the [D-Asp$^3$]- and [Dha$^7$]- analogues of NODR and a NODR methyl ester are present. The levels of impurities were estimated using LC-MS with CRM-NODR as the calibrant, assuming the same molar response (Table 2).

**Table 2:** Information values for other compounds present in CRM-NODR at the time of packaging.

| Compound | $[M+H]^+$, *m/z* | Concentration (µmol/L) (at +20 °C) |
|---|---|---|
| [D-Asp$^3$]-Nodularin-R | *m/z* 811 | 0.09* |
| [Dha$^7$]-Nodularin-R | *m/z* 811 | 0.07* |
| Nodularin-R methyl ester | *m/z* 839 | 0.02* |

* These concentrations are not certified.

## Homogeneity

As this CRM is a true solution, it is expected to be homogenous. To confirm this, the concentration of nodularin-R in randomly selected ampoules representing 1.3% of those produced was measured by LC-UVD. Using an analysis of variance of the data, no heterogeneity could be detected.

## Stability Study

Extensive studies have been conducted to determine the stability of this toxin under various conditions. Nodularin-R is sensitive to oxygen and will also undergo gradual isomerization to the 6(Z)-Adda isomer [3] when exposed to light. A 15 month stability study was performed on CRM-NODR at various temperatures (-80, -16, +4, +20 and +37 °C). No loss of toxin was detectable at +4 °C during the course of that study. Less than 0.2% degradation was observed at +20 °C after 2 months, which

indicates good stability for shipment purposes. A 1% loss of material was observed at +37 °C after 2 months.

**Certified Value**

The certified value for CRM-NODR, 12.4 ± 0.5 µmol/L (at +20 °C) (Table 1), is based on results obtained at NRC using two independent analytical methods: quantitative nuclear magnetic resonance (qNMR) spectroscopy [4] and LC-CLND. Calibration of both of these techniques was performed using accurate caffeine solutions.

The results shown in this certificate are traceable to the SI standard through gravimetrically prepared standards of established purity. This product serves as a suitable reference material for laboratory quality assurance programs.

**Uncertainty**

The overall uncertainty estimate ($U_{CRM}$) for CRM-NODR includes uncertainties associated with batch characterization ($u_{char}$), between-bottle variation ($u_{hom}$) and instability associated with long-term storage ($u_{stab}$) [5-6]. These components can be combined as:

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{hom}^2 + u_{stab}^2}$$

where $k$ is the coverage factor (generally 2 or 3).

Quantitative measurements by CLND and QNMR contributed the most significant uncertainties. When combined they resulted in a relative uncertainty for batch characterization ($u_{char}$) of 0.017. As this CRM is a true solution, $u_{hom}$ was not significant, as solutions are inherently homogenous [7]. Nevertheless, tests on homogeneity were performed (see Section Homogeneity), and the between-bottle variance was determined to be no greater than the measurement variance, resulting in a relative value of 0.0031 for $u_{hom}$. A long-term stability study was performed on this CRM (see Section Stability Study) which showed no observable loss of material when stored under the recommended conditions. Nodularin-R exhibits reasonable stability at room temperature in the event that a delay in shipment occurs. The uncertainty due to stability ($u_{stab}$) [8] was not significant with a relative value of 0.0057. Applying a coverage factor of 2 resulted in a final relative standard uncertainty in the certified value of 0.036.

**Storage Instructions**

To ensure the stability of nodularin-R, the CRM and all dilutions thereof should be stored in the dark in a freezer (preferably at -12°C or lower).

**Expiry**

If stored unopened at the recommended storage conditions (see Section Storage Instructions), the certified concentration of the CRM is valid for 1 year from the date of sale.

**Instructions for Use**

Prior to opening, each ampoule should be allowed to warm to room temperature and the contents should be thoroughly mixed. The ampoule should be inverted several times, then held upright, tapped to ensure that most of the solution drains to the bottom, and opened at the pre-scored mark. Once an ampoule has been opened, accurate aliquots should be removed with calibrated volumetric equipment and transferred to volumetric flasks or vials. An increase in concentration due to evaporation of solvent will occur if the solution is left opened for more than a few minutes. It is recommended that the CRM

should not be evaporated to dryness because of the potential of losses on glass surfaces. Care must be taken to use apparatus with glass surfaces to transfer and store the solutions as losses of nodularins can occur on plastic [9]. Also, final solutions should contain at least 25% methanol to ensure complete solubility of the analyte.

A useful procedure that ensures accurate dilutions involves using a balance to determine weights of the dispensed aliquot and the final diluted solution, assuming that methanol/water (1:1, v/v) is used as the diluent (the density of the CRM solution is 0.925 g/mL at +23 °C). *Note:* The volume of the solution is not certified. Only the concentration is certified.

**Safety Instructions**

Nodularins are known to be hepatotoxic and may also be genotoxic. Only qualified personnel should handle the solution and appropriate disposal methods should be used. Heavy gloves and eye protection should be used when opening the ampoule in the event the glass shatters. A material safety data sheet (MSDS) is available for CRM-NODR.

**References**

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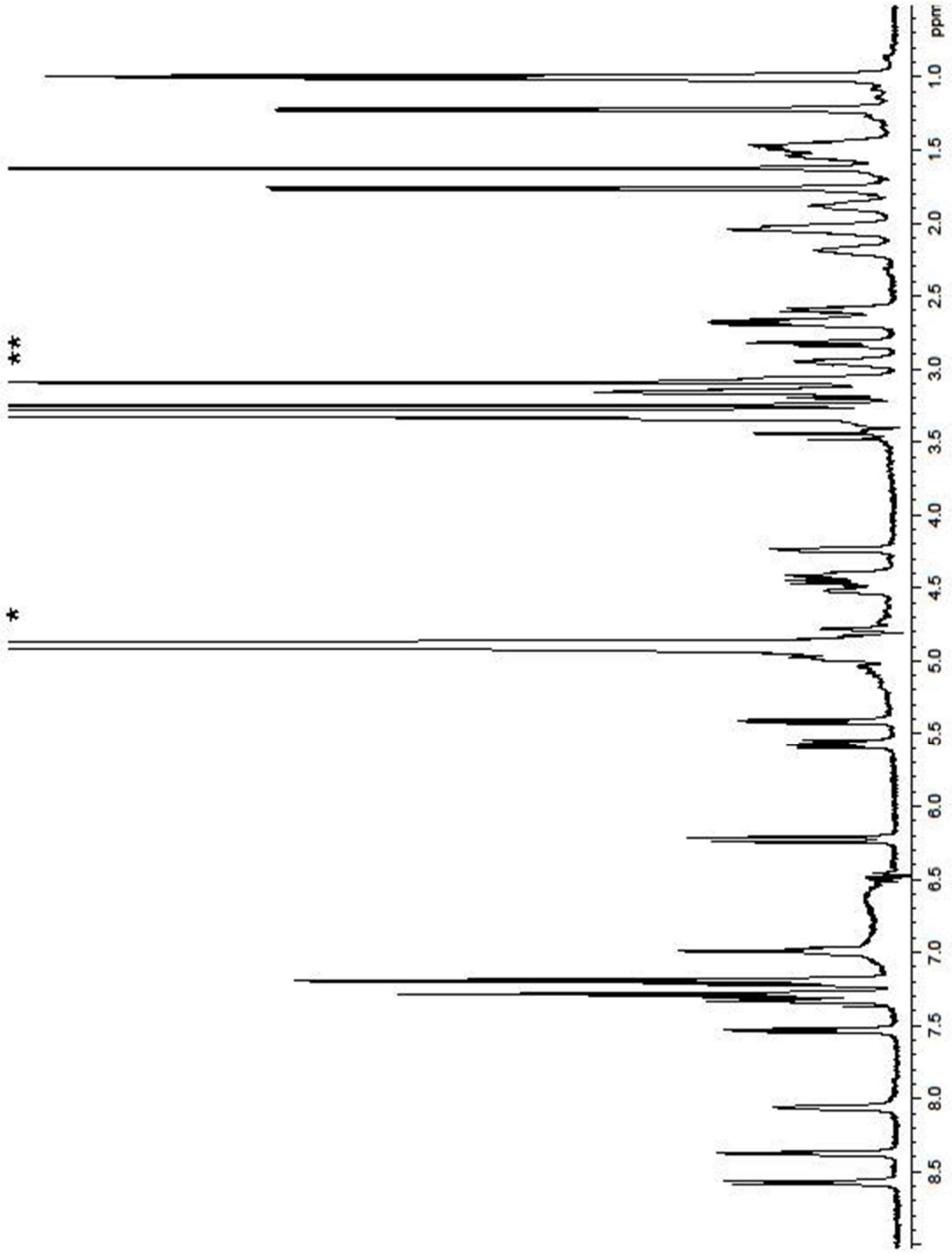


**Figure 1:** Proton NMR spectrum of nodularin-R in water/$CD_3OH$. The truncated peaks represent the protons from water (*) and methanol (**).

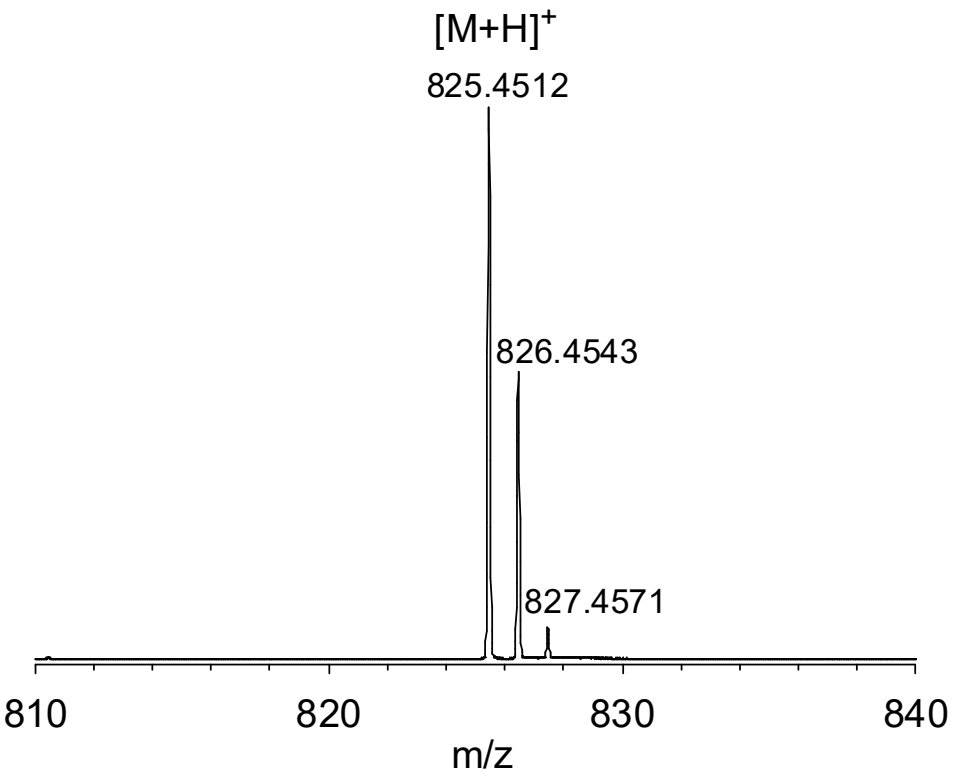


**Figure 2:** High resolution mass spectrum of nodularin-R. CRM-NODR was diluted in 50% $CH_3CN/H_2O$ with 0.1% HCOOH and infused into a Waters QToF Premier mass spectrometer at 1 µL/min. Leucine enkephalin (556.2771 Da) was used as the lock-mass ion to correct for any short-term variability in calibration, and was infused into the mass spectrometer via a separate channel at 1 µL/min. The observed mass of the $[M+H]^+$ ion was 825.4512, which is in good agreement with the theoretical mass of 825.4505.

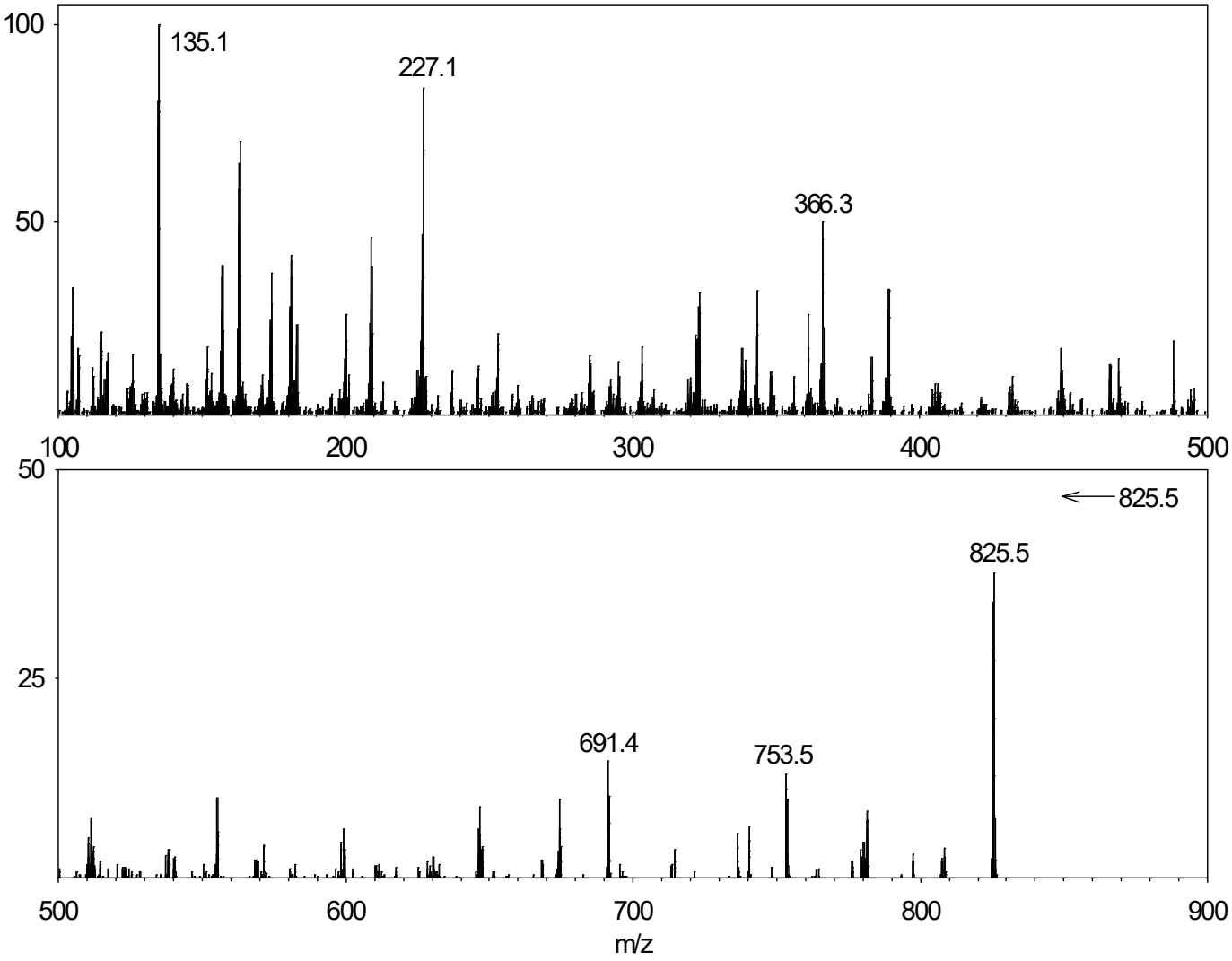


**Figure 3:** Product ion mass spectrum of the $[M+H]^+$ ion (*m/z* 825.5) of nodularin-R generated from an LC-MS/MS analysis. Conditions: Agilent 1200 LC and AB-Sciex API4000 QTRAP MS with electrospray ionization; 75 V declustering potential; 65 V collision energy; Zorbax SB-C18, 1.8 µm, 50 mm × 2 mm i.d. column at +40 °C; 0.2 mL/min flow; gradient elution with 30% to 65% B over 5 min, where A = water, B = 95% acetonitrile, both with 2 mM ammonium formate and 50 mM formic acid.

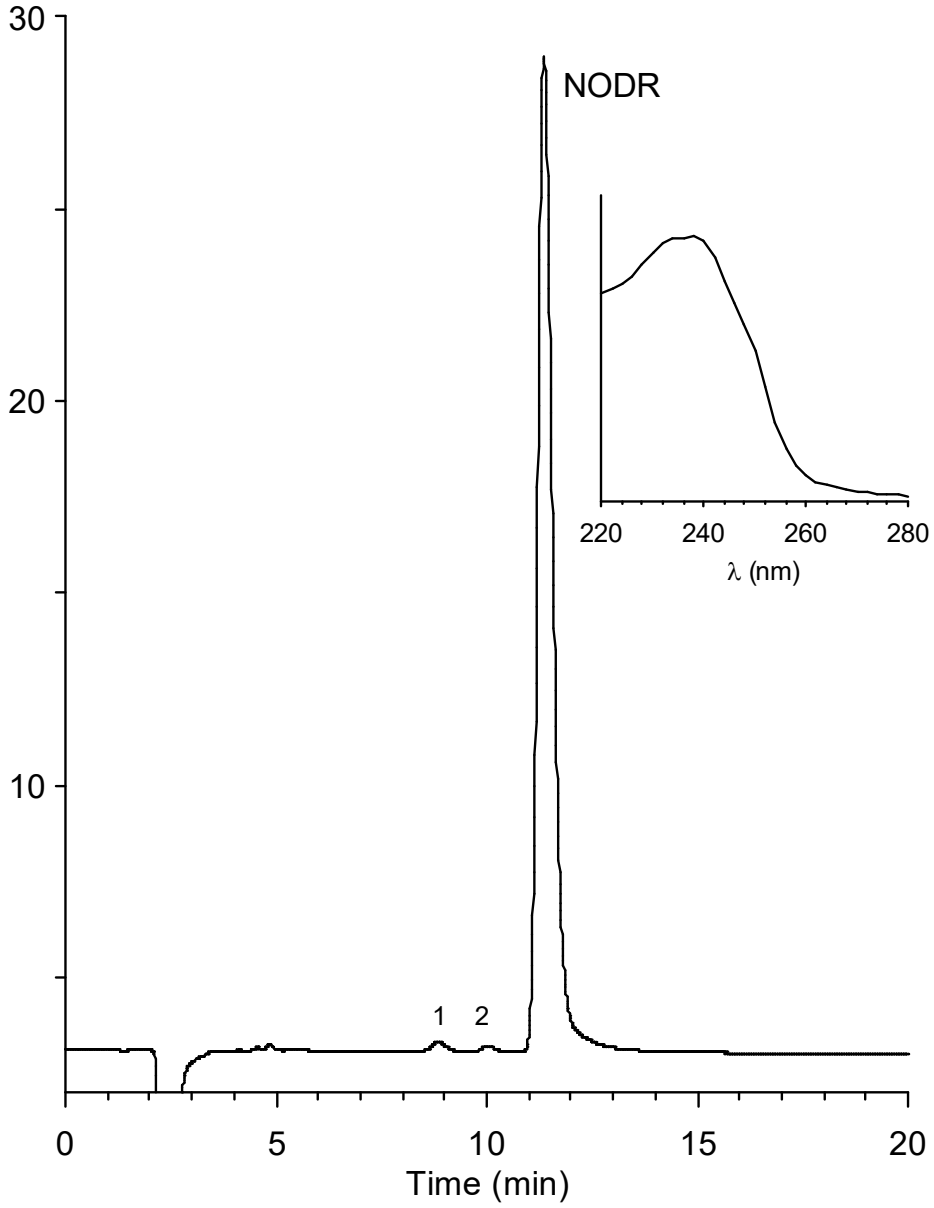


**Figure 4:** Analysis of CRM-NODR by liquid chromatography with ultraviolet detection (LC-UVD). Conditions: Zorbax SB-C8, 2.1 × 150 mm column at +40 °C; isocratic analysis with $CH_3OH/H_2O$ (1:1) with 0.2% HCOOH; 0.2 mL/min; 10 µL injection; UV detection at 238 nm. The UV spectrum was acquired using a diode array detector in a separate run. Peaks labeled 1 and 2 are [D-Asp$^3$] and [Dha$^7$] analogues of NODR (respectively).

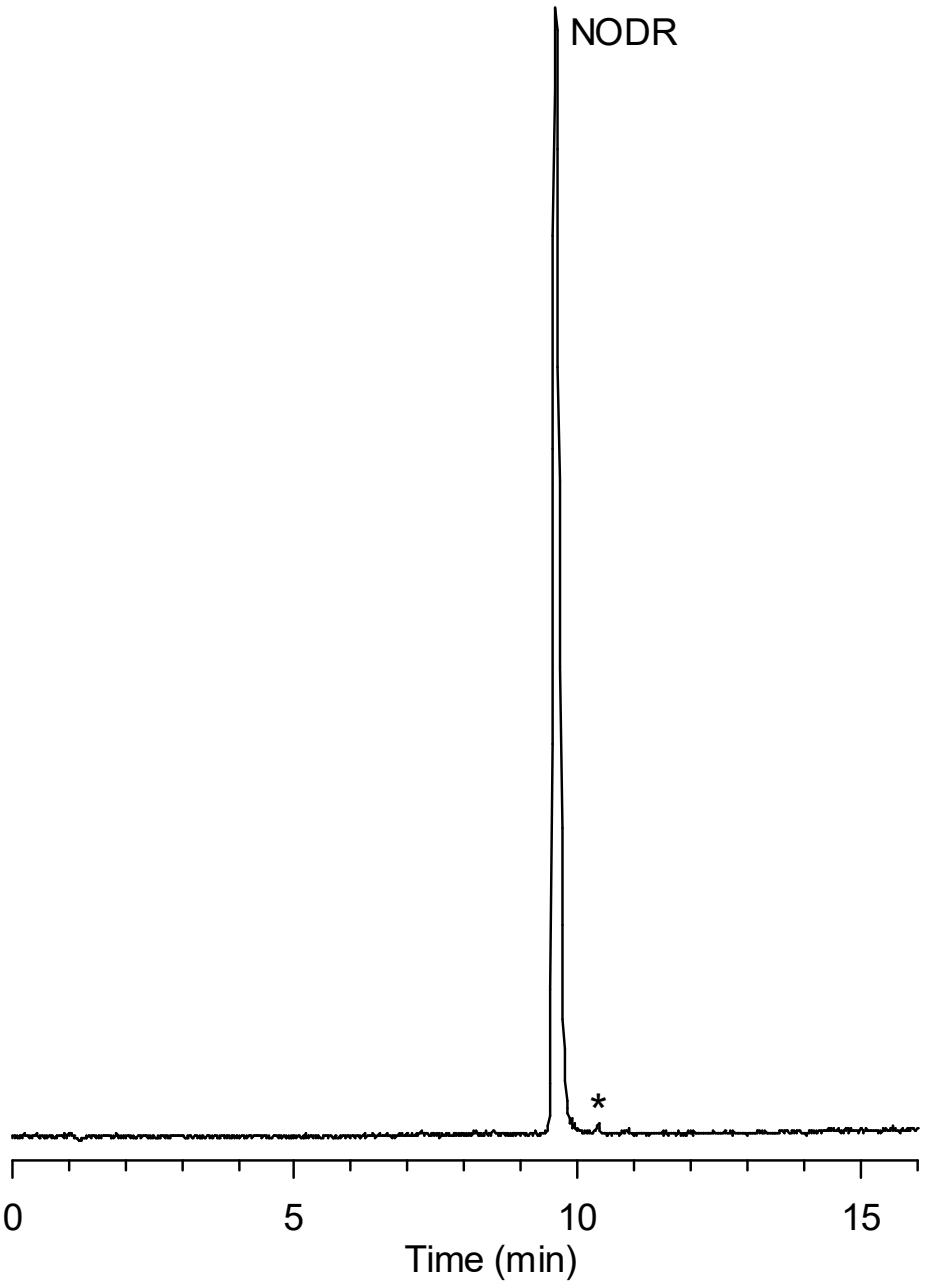


**Figure 5:** Analysis of a dilution of the CRM-NODR stock solution by liquid chromatography with mass spectrometry detection (LC-MS). Conditions: Keystone BDS Hypersil C8, 2.1 × 50 mm column at +40 °C; gradient analysis with 20% to 80% B over 10 min (hold to 20 min) where A = $H_2O$; B = 95% $CH_3CN/H_2O$ both with 50 mM HCOOH and 2 mM $NH_4COOH$; 0.2 mL/min flow. Full scan analysis using an AB-Sciex API165 single quadrupole mass spectrometer. The * indicates the NODR methyl ester.

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***Signed*** **:** 

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