



Purity Evaluation Guideline: Patulin

BIPM PEG-04

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BIPM PEG-04 : Patulin

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1. Scope

This document provides technical guidance and reference data to assist with the assignment of the qualitative identity and quantitative characterization of a purified solid material containing patulin (PAT) as the primary component. In particular it is intended for the characterization of a Primary Reference Material (PRM)¹ intended for use to underpin the metrological traceability of routine testing procedures for the detection and quantification of contamination by PAT at trace levels in food, feedstuffs and primary produce.

2. Introduction

In collaboration with the National Institute of Metrology (NIM), China, and the National Metrology Institute of South Africa (NMISA), the BIPM initiated in 2016 a Capacity Building and Knowledge Transfer programme for Metrology for Safe Food and Feed in Developing Economies.² This project is designed to allow NMIs to work together to strengthen the world-wide mycotoxin metrology infrastructure, to provide knowledge transfer to scientists developing capabilities in this area and to enable NMIs in developing regions to produce calibrants, matrix reference materials and proficiency test samples to support testing and laboratory services for mycotoxin analysis within their countries.

As for all other areas of organic analysis PRMs consisting of well characterized, high purity compounds are required for each analyte subject to investigation. These materials are the ultimate source of higher-order metrological traceability for the assigned values of derived calibration solutions, matrix reference materials, proficiency test samples and ultimately the results of routine analysis. Access to pure organic compounds and calibration solutions prepared from these materials is an essential element in the measurement infrastructure supporting the delivery of reliable, comparable results. In the case of mycotoxins, purity analysis of source materials involves additional challenges linked to the limited amount of available material and its potential toxicity.

Patulin (PAT) is polyketide-derived heterocyclic lactone natural product, produced by moulds, particularly fungi of the genus *Aspergillus* and *Penicillium*. Often found in rotting apples and apple products, PAT can also occur in other mouldy fruits, grains and foods. Major human dietary sources of patulin are apples and apple juice made from affected fruit.³ The acute symptoms of exposure to patulin include liver, spleen and kidney damage and toxicity to the immune system.⁴ Nausea, gastrointestinal disturbances and vomiting have also been reported. PAT is potentially genotoxic however its carcinogenic potential has not been demonstrated yet.⁵ It is relatively stable when given a short-acting heat-treatment (pasteurization), especially in an acidic environment.⁶

A typical minimum allowed residue level is 50 µg/kg in food and juices.⁷ Levels of 25 µg/kg were established in the European Union with regard to solid apple products and of 10 µg/kg for apple-based baby food and juices.^{8,9}

The analytical difficulty and the importance of monitoring levels of PAT in baby food

and beverages have led to the need for suitable solution and matrix Certified Reference Materials. They are invaluable tools to ensure comparability and traceability in PAT measurements and are very useful for the implementation of written standards, legislation/regulations and laboratory accreditation.

A necessary requirement of the BIPM CBKT project was to obtain and characterize a primary reference material for PAT that could be used to anchor the metrological traceability¹⁰ of results linked through calibration to this material. This guideline summarizes the characterization and purity assignment studies undertaken to deliver a PRM for PAT required for the BIPM MNCBKT programme. It is intended to be of use to National Metrology Institutes and other Reference Measurement service providers needing to characterize their own primary material for PAT analysis. Reliance was placed on nuclear magnetic resonance spectroscopy (NMR) studies to confirm the qualitative identity of the main component of the material and to assign the mass fraction content of patulin it contained.

3. Nomenclature and Ring numbering

The conventional ring numbering and abbreviations established for the naming of PAT and related compounds are used. PAT is a furopyran lactide member of the polyketide natural products group. Its structure with the conventional numbering scheme is shown in Figure 1.

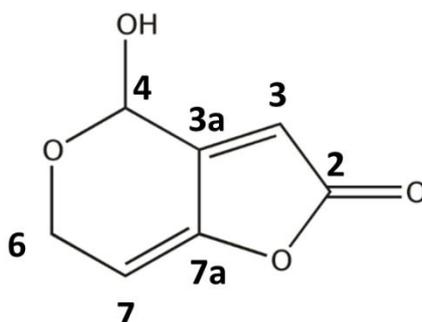


Figure 1: PAT structure with numbering scheme.¹¹

4. Properties of Patulin

4.1 Hazard Identification

Patulin poses risks for human health if handled inappropriately. Although conclusive evidence has not been reported for natural occurrences of poisoning it has been shown to be toxic at high concentrations in the laboratory.¹² It causes irritation when applied dermally to humans; Oral administration in humans causes stomach irritation, nausea, and vomiting.

Under EC regulation 1272/2008 risks arising from exposure to patulin are classified as:

- Acute toxicity, oral (Category 2) H300
- Skin irritation (Category 2) H315

DISCLAIMER: The recommendations in this section are based on the review of literature reports but have not been verified by the BIPM.

4.1.1 Protective measures

Avoid inhalation of dust, vapours, mist or gas. Wear full-face particulate filtering respirator type N100 (US) or type P3 (EN 143) respirator cartridges when working with the solid material. Wear protective gloves, goggles and clothing. Take care to avoid skin exposure if handling solutions and work in ventilated areas. Wash hands thoroughly after handling.

4.1.2 Emergency procedures

General advice: Immediately call a POISON CENTRE or doctor/physician. Show this safety information to the doctor in attendance. Move out of the dangerous area.

If inhaled: Move into fresh air. If not breathing give artificial respiration. Consult a physician.

In case of skin contact: Wash off with soap and plenty of water. Consult a physician.

In case of eye contact: Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

If swallowed: Immediately call a POISON CENTRE or doctor/physician. Never give anything by mouth to an unconscious person. Rinse mouth with water.

4.1.3 Spillage

Contain spillage and then collect by wet-brushing and place in container for disposal. Keep in suitable, closed containers for disposal according to local regulations.

4.2 Physical and Chemical Properties

| | |
|----------------------|--|
| Common Name: | Patulin |
| IUPAC and CAS Names: | 4-Hydroxy-4,6-dihydrofuro[3,2-c]pyran-2-one 4-Hydroxy-4H-furo[3,2-c]pyran-2(6H)-one |
| Synonyms: | PAT, Clavicin, Clavatin, Expansin |
| CAS Registry Number: | 149-29-1 |
| Molecular Formula: | C ₇ H ₆ O ₄ |
| Molar Mass: | 154.12 g/mol |
| Monoisotopic mass: | 154.0266 |
| Melting point: | 105-108 °C (after drying for 1 hr at 60 °C) ¹³ 111 °C ¹⁴ |
| Appearance: | Colorless or white odorless solid |
| Solubility | Highly soluble in water and polar solvents (MeOH, acetone, ACN) |
| UV maxima | 276 nm in MeOH ($\epsilon = 1436 \pm 4$) ¹³ 277 nm in EtOH ¹⁵ |

4.3 Qualitative identification

4.3.1 NMR Materials and methods

Chemicals:

- Patulin (PAT); BIPM Reference OGO.180a

NMR Solvents:

- Deuterium oxide (D₂O); BIPM Reference OGS.025d
- Deuterated methanol (CD₃OD); BIPM Reference OGS.028d
- Acetone-*d*₆; BIPM Reference OGS.029b

Deuterated NMR solvents were purchased commercially and used without further treatment.

4.3.2 Sample preparation

For qualitative NMR analyses sample sizes typically in the range (5 – 9) mg of PAT were weighed accurately and made up in 1 mL of deuterated solvent in a glass vial. The sample solution was mixed in a vortex shaker and transferred using a disposable glass pasteur pipette into NMR tubes (HG-Type: high grade class, 5 mm o.d., with polyethylene caps). Each sample mixture was transferred to the NMR tube immediately after dissolution in the deuterated solvent and these tubes were protected from exposure to light by wrapping with foil until they were placed in the auto sampler of the spectrometer.

4.3.3 NMR acquisition parameters

A JEOL ECS-400 spectrometer operating at 9.4 T (400 MHz for proton) equipped with a direct type automatic tuning (Royal) probe was used for all data acquisition. For qualitative analyses, ¹H spectra were acquired for both solvent blank and the PAT sample using a pulse-acquire sequence with the parameters listed in Table 1.

Table 1 - Acquisition parameters for qualitative ¹H NMR.

| <i>Parameter</i> | <i>Value</i> |
|----------------------|--------------|
| Number of Transients | 32 |
| Receiver gain | 42 |
| Acquisition time (s) | 2.2 |
| Relaxation delay (s) | 5.0 |
| Pulse offset (ppm) | 5.0 |
| Spectral width (ppm) | 15.0 |
| Data points | 16384 |
| Temperature (K) | 298 |
| Spinning | Off |

¹³C-NMR experiments were conducted using an ordinary power gated sequence (pulse-acquire in ¹³C channel with proton decoupling both during acquisition and the relaxation delay) using the parameters listed in Table 2.

Table 2 - Acquisition parameters for ¹³C NMR.

| <i>Parameter</i> | <i>Value</i> |
|----------------------|--------------|
| Number of Transients | 3000 |
| Receiver gain | 60 |
| Acquisition time (s) | 1.04 |
| Relaxation delay (s) | 2.0 |
| Pulse offset (ppm) | 100 |
| Spectral width (ppm) | 250 |
| Data points | 32768 |
| Temperature (K) | 298 |
| Spinning | Off |

4.3.4 1D ¹H- and ¹³C-NMR spectra

The PAT material was slightly soluble in CDCl₃. It crystallised within the NMR tube over time at the concentrations used. It dissolved readily in D₂O. The initial ¹H NMR spectrum in D₂O was clean (Figure 2) however impurity/breakdown product began to be visible within 24 h (Figure 3). Similarly, over time breakdown products were observed after reanalysis of a solution of PAT in CD₃OD (Figure 4). Solutions of PAT in acetone-*d*₆ were stable and the ¹H NMR signals well resolved (Figure 5). A resonance from the OH proton at 6.34 ppm in acetone-*d*₆ is not observed in D₂O due to rapid exchange and only as an attenuated signal in CD₃OD. 1D ¹³C and Attached Proton Test (APT) spectra of PAT are shown in Figure 6 and 7 respectively.

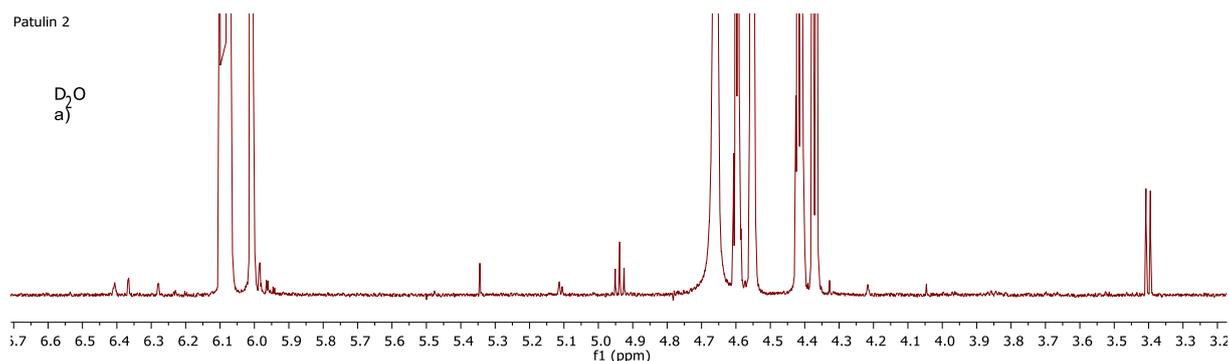


Figure 1 – ¹H NMR spectrum of the Patulin (OGO.180a):Initial spectrum in D₂O.

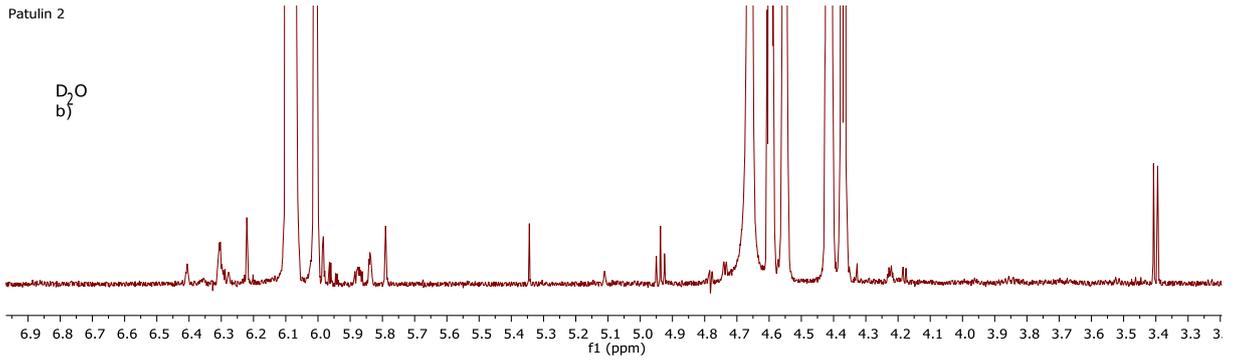


Figure 2 – ¹H NMR spectrum of Patulin (OGO.180a) after 24 h in D₂O.

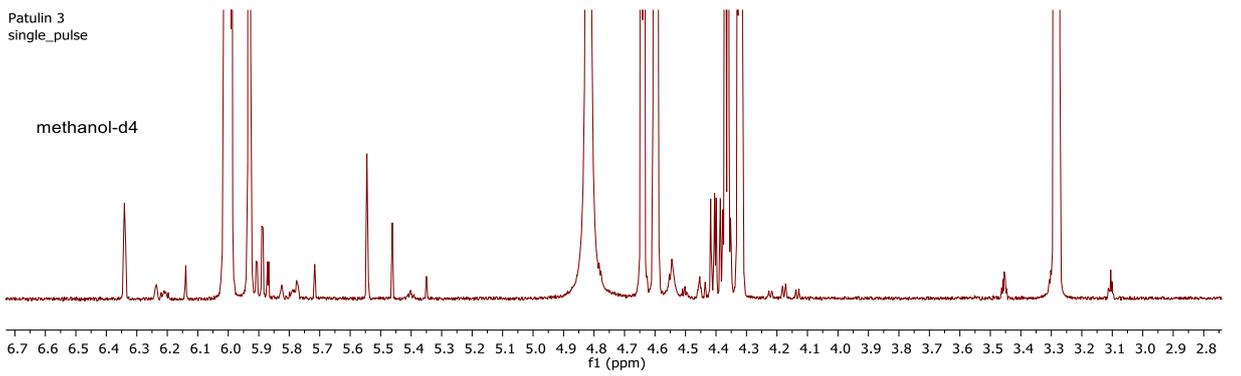


Figure 3 – ¹H NMR spectrum of Patulin after 24 h in CD₃OD.

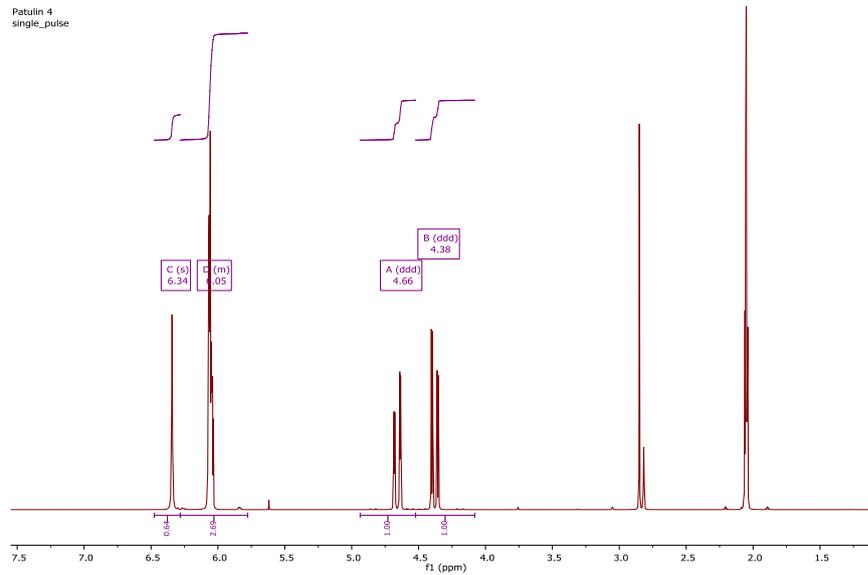


Figure 4 – ¹H NMR spectrum of Patulin (OGO.180a) in acetone-*d*₆.
Peaks below 3 ppm are due to residual water and non-deuterated solvent

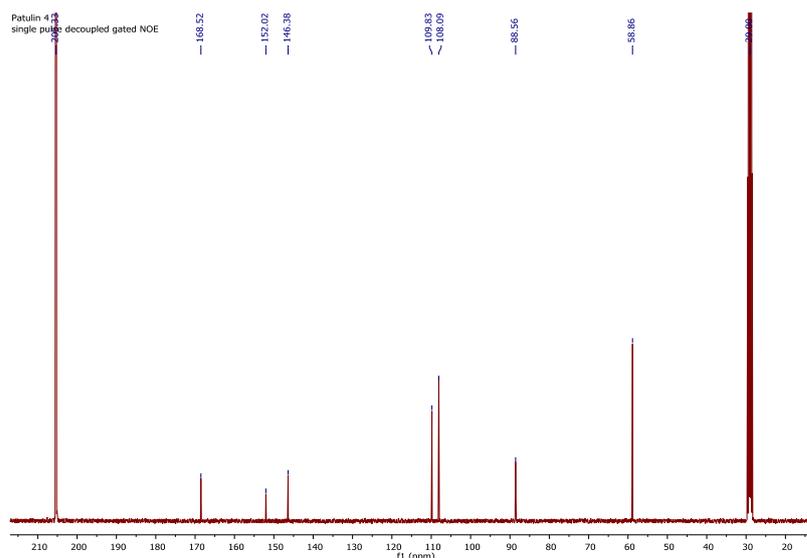


Figure 6 – ^{13}C NMR spectrum of Patulin (OGO.180a) in acetone- d_6 .

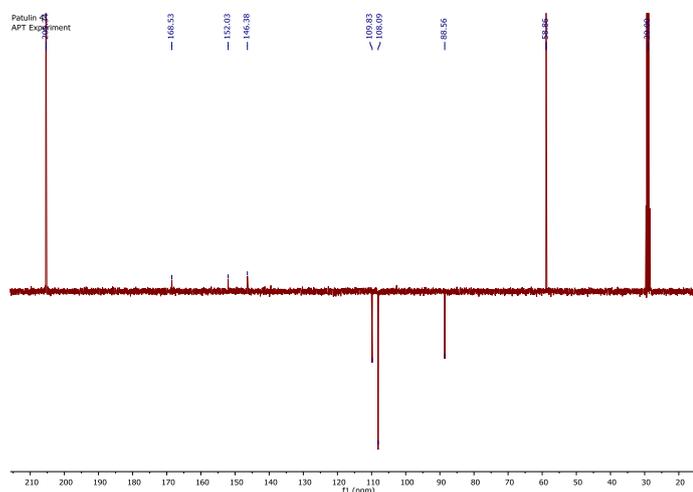


Figure 5 – ^{13}C APT spectrum of Patulin. Up = CH_2/C_q ; Down = CH/CH_3 . Solvent: Acetone- d_6 .

4.3.5 2D NMR spectra

To confirm the identification and stereochemical assignment of the structure, a series of two-dimensional (2D) NMR experiments were undertaken. These included homonuclear (^1H - ^1H) correlated spectroscopy (COSY) and heteronuclear single-quantum correlation (^{13}C - ^1H) (HSQC). The 2D-spectra obtained for the PAT material OGO.180 are reproduced in Annex 7.1.

The peak assignments based on the combined data obtained at the BIPM are compiled in Table 3. They are self-consistent, agree with literature assignments and establish that the NMR properties of the primary component in OGO.180 are fully consistent with the structure of PAT.

Table 3 – ¹H and ¹³C NMR assignments for PAT

| Position | ¹ H-NMR (ppm, integral) | ¹³ C-NMR (ppm, APT) | COSY (ppm ¹ H J-coupled) | HSQC (¹ H- ¹³ C direct) |
|-------------|---------------------------------------|-----------------------------------|--|---|
| 2 | | 168.5, Cq | | |
| 3 | 6.04, 1H | 109.8, CH | - | 109.8 ppm |
| 3a | - | 152.0, Cq | - | - |
| 4 | 6.03, 1H | 88.5, CH | 6.34 | 88.5 ppm |
| 6 | 4.38/4.66, 2H | 58.9, CH ₂ | 6.01 | 58.9 ppm |
| 7 | 6.01, 1H | 108.1, CH | 4.38, 4.66 | 108.1 ppm |
| 7a | | 146.4, Cq | - | - |
| 4-OH | 6.34 | | 6.03 | - |

4.3.6 UV-VIS spectrum

A reported¹³ UV-VIS spectrum of PAT in methanol is reproduced in Figure 8.

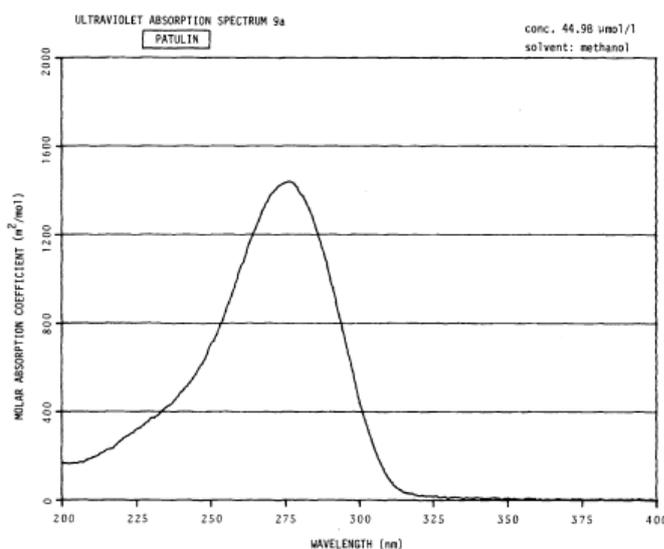


Fig. 8: UV-VIS spectrum of Patulin in methanol (46 µmol/L)

4.3.7 Residual solvent content by NMR

The ¹H NMR spectrum of the BIPM patulin material was examined for signals due to residual solvent.¹⁶ Signals due to the presence of residual solvent (dichloromethane and ethanol) were observed in the material.

4.3.8 Mass spectrometry

Reference MS data for PAT are available by searching under the entry for “patulin” from open access databases including the [European Mass Bank](#),¹⁷ the [MassBank of North America](#)¹⁸ and [PubChem](#).¹⁵ From studies undertaken at the BIPM, the MS parameters of PAT and a number of related structure materials in a positive electrospray ionization mode were optimized by direct infusion of single analyte standard solutions.

Using our spectrometer, optimized intensity was obtained at an ionspray voltage of 4500 V, source temperature of 550 °C with nitrogen as the ion source gas, curtain gas and collision gas. The optimal pressures for Gas 1 and Gas 2 of the ion source were 55 psi and 60 psi. For curtain gas (CUR) the pressure was set at 40 psi. For the collision gas (CAD) the “Mid” setting was used. The declustering potential (DP) was set at -60 V. The entrance potential (EP) was set at -8.5 V. The collision cell exit potential (CXP) was set at -16 V. Table 4 summarizes the optimized transitions and conditions for multiple reaction monitoring (MRM) detection and quantification of PAT and its potential structurally related impurities ascladiol (ASC) and deoxyapatulinic acid (DPA) under these conditions.

Table 4 PAT and Related Structure MRM parameters

| Component | Q1 Mass (Da) | Q3 Mass (Da) | Time (msec) | CE (volt) |
|-----------|--------------|--------------|-------------|-----------|
| PAT | 153.0 | 109.0 | 50 | -17 |
| ASC | 155.0 | 125.0 | 50 | -20 |
| DPA | 155.0 | 111.0 | 50 | -15 |
| | 155.0 | 63.0 | 50 | -30 |

5. Purity of Patulin

5.1 Introduction

The general approach developed during the BIPM MMCBKT programme for the purity assignment of mycotoxins was applied to the PAT source material. A quantitative NMR (qNMR) measurement^{19,20} provided a value for the mass fraction in the material of PAT and related impurities containing a signal at chemical shift of the quantification signal – in this case at δ 6.0 ppm. This value was corrected if necessary for contributions due to PAT-related impurity content, which were quantified separately by LC. The presence of related structure impurities were investigated using an LC-DAD method and the assignments were checked using LC-MS/MS based on the ionization parameters described in section 4.3.8. This approach has the advantage of requiring a significantly smaller amount of the toxic source material than would be required for a conventional mass balance purity assignment. In the particular case of the material investigated at BIPM only trace levels of one inherent impurity was identified by LC-MS/MS.

The identity of the primary component in the material was established as PAT using the combination of 1D- and 2D-NMR techniques described in Section 4.3.1 – 4.3.5 above.²¹ A check for residual solvent impurity content in the material obtained by NMR also indicated that there were detectable amounts of ethanol and dichloromethane present. This identification was verified with the UV-Vis spectrophotometric and mass spectrometric properties (Section 4.3.8) of the material, which were consistent with reported values.

The initial assignment of the PAT mass fraction content of OGO.180 by qNMR is described in Section 5.2. The application of methods for the detection and quantification of any PAT-related impurity content of the material by LC-UV(DAD) and LC-MS/MS are described in Section 5.3. In the case of the material investigated, no significant level of impurity of this type was found and so no correction was required and the “raw” qNMR value gave the final assignment of the “true” PAT

content of the material.

Supporting analyses undertaken to detect other impurity classes are summarized in Section 5.4 and 5.5. The final purity assignment of the BIPM PAT material is summarized in Section 5.6.

DISCLAIMER: Commercial NMR and LC instruments, software, materials and reagents are identified in this document in order to fully describe some procedures. This does not imply a recommendation or endorsement by the BIPM nor does it imply that any of the instruments, equipment and materials identified are necessarily the best available for the purpose.

5.2 qNMR

5.2.1 Materials

Chemicals

- Patulin (PAT); BIPM Reference OGO. 180a
- Supplier: First Standard, Product No. 1ST7213, Lot ALT601343
- Dimethylterephthalate (DMTP); BIPM Reference OGE.022b was used as the qNMR internal standard²². The mass fraction content of DMTP in the material was assigned at the BIPM by qNMR measurements using CRMs as internal standard as 999.3 ± 0.8 mg/g ($k = 2$).

NMR Solvents:

- Acetone-*d*₆; BIPM Reference OGS.029

Deuterated acetone was purchased from a commercial supplier and was used without further treatment. NMR tubes were HG-Type: high-grade class, 8 inch, 5 mm diameter rated for use with 600 MHz spectrometers fitted with PE caps.

5.2.2 qNMR Sample preparation

Gravimetric operations were performed using a Mettler Toledo XP2U ultramicrobalance. Prior to all weighing operations the repeatability of the balance was assessed for suitability to the preparation of qNMR samples by repeat mass determinations of an empty weighing boat. The general recommendations for qNMR sample preparation by Yamazaki *et al*²³ were followed.

In the initial study, using deuterated acetone as solvent, three separate samples were prepared for analysis. The individual sample sizes were (5-9) mg for the PAT material and (2-4) mg for the qNMR internal standard used (DMTP). Each sample was separately weighed into an aluminium weighing boat and in order to avoid contact of the solvent with the metal boat the contents of both were transferred into a common glass vial and each emptied boat was reweighed. The amount of PAT and DMTP transferred into the common vial was determined by difference and this value was used for qNMR calculations. 1 mL of deuterated solvent was added to the vial and the sample solution was mixed in a vortex shaker and checked visually for completeness of dissolution. Approximately 800 μ L of this solution was transferred into an NMR tube (HG-Type: high-grade class, 8 inch, 5 mm o.d., with PE cap) using a glass pasteur pipette.

5.2.3 Choice of solvent and quantification signals

When the ^1H qNMR spectrum was examined, eight impurity signals were detected. The choice for the patulin quantification peak was either the overlapping multiplet corresponding to the three methine hydrogens (Figure 1, H-3, H-4 and H-7) or the H-6 methylene resonating as two separate multiplets corresponding to one hydrogen each at 4.63 and 4.35 ppm. The H-6 methylene signals were selected as being less subject to potential interference. An impurity peak at 4.42 ppm is found in the region that is included in the quantitative calculation. However, the integral of this impurity was less than 0.08 % of the integral value for the main component. qNMR purity assignments were made against the aromatic hydrogen signal at 8.1 ppm from DMTP as shown in Figure 9.

DMTP was used as the internal standard since its singlet resonance from the four equivalent aromatic protons at chemical shift 8.1 ppm occurs in a clean region in the PAT spectrum. The 90° pulse calibration was established at $5.83 \mu\text{s}$ and the longest measured T_1 time constant for the quantified peaks was 5.9 seconds for the DMTP aromatic peak. A relaxation delay of 89 seconds between pulses, corresponding to fifteen times the longest T_1 , was applied for quantification studies. The excitation pulse offset was set at 6.28 ppm and a signal window of 400 ppm was used. The experiments were undertaken using ^{13}C -gated decoupling-on mode. Use of ^{13}C -decoupling simplifies each resonance by removing contributions from ^{13}C satellite peaks, thereby making it easier to establish a baseline for integration.

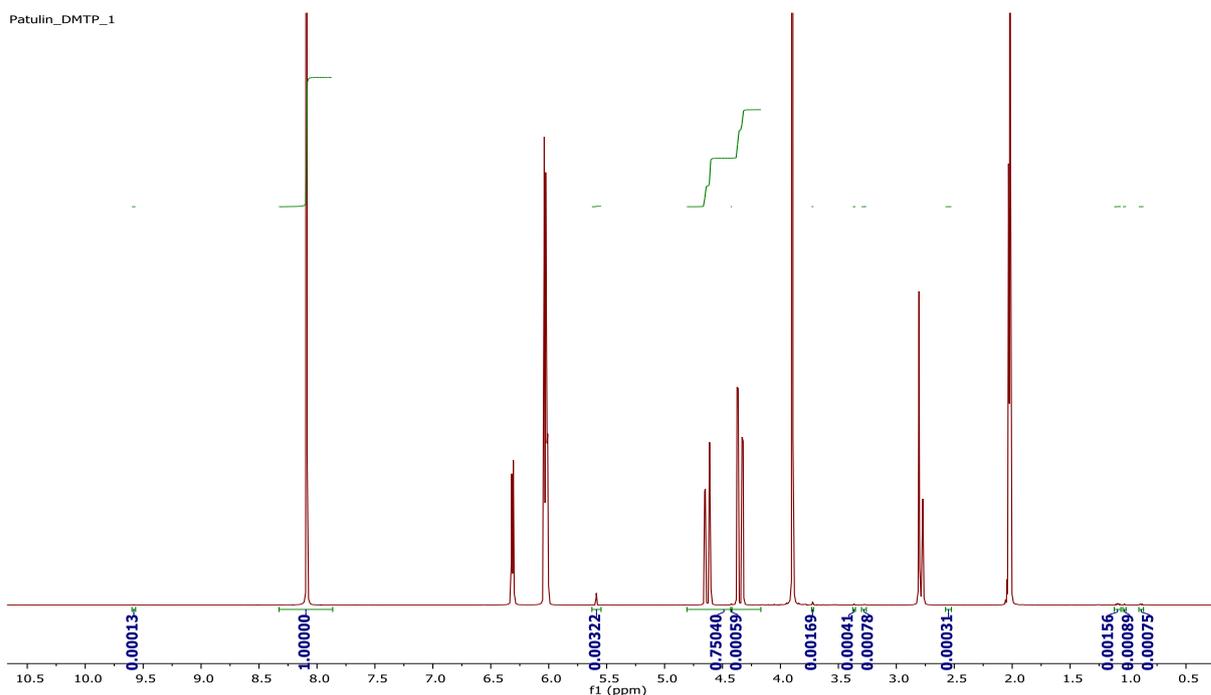


Figure 9 – qNMR spectrum of OGO.180a and DMTP with integrated peaks indicated. Singlets at $\delta = 8.1$ and 3.8 ppm from DMTP. Signals < 3 ppm from non-deuterated solvent and water

5.2.4 NMR acquisition parameters

A JEOL ECS-400 spectrometer operating at 9.4 T (400 MHz for proton) equipped with a direct type automatic tuning (Royal) probe operating Delta software was used for all NMR data acquisition.

The general recommendations for optimizing spectrometer performance, determining the relevant NMR experiment parameters and undertaking a qNMR experiment as described in the BIPM Internal Standard Reference Data report for the use of DMTP for qNMR measurements²² were followed, with the exception that for this assignment the acquisition was carried out with ¹³C-decoupling activated to eliminate satellite peaks and simplify the integration process. The final qNMR acquisition parameters used for PAT are summarized in Table 5.

| <i>Parameter</i> | <i>Value</i> |
|----------------------------------|--------------|
| PAT Sample size (mg) | 5 – 9 |
| DMTP Sample size (mg) | 2 – 4 |
| Number of Transients | 64 |
| Receiver gain | 38 |
| Acquisition time (s) | 4 |
| Relaxation delay (s) | 89 |
| Pulse offset (ppm) | 6.3 |
| Spectral width (ppm) | 400 |
| Data points | 799565 |
| Temperature (K) | 298 |
| ¹³C-Decoupling | On |
| Spinning | Off |
| Integral ratio (PAT:DMTP) | 0.25 – 0.48 |

Table 5 - Acquisition parameters for qNMR of PAT.

The integration range used start and end points fifty Hertz beyond the limit of each signal.

5.2.5 Value assignment and measurement uncertainty

Results from four samples analyzed in triplicate were obtained. A sample of the acetone-*d*₆ solvent was also analyzed under the same conditions. In the “blank” spectra signals were observed due to impurities in the solvent. One has a chemical shift that overlaps with the integration region of the aromatic signal of DMTP, and another with one region used for PAT. The raw integration values for the DMTP and PAT signals were corrected to take into account these contributions.

An example of the measurement uncertainty budget for one assignment of PAT content is given in Table 6. The integral ratio used is the mean of the twelve values. The contributions to the overall standard uncertainty of the assignment are also listed. The standard deviation of the mean of the integral ratio (Integral A/Integral S), with the ratios for independent samples normalized to take into account their different precise compositions, was assigned the standard uncertainty of the precision of the integration ratio.

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| Uncertainty source | Value | Uncertainty Evaluation Type | Standard Uncertainty | Sensitivity coefficient | Relative Uncertainty |
|------------------------------------|---------------|-----------------------------|----------------------|-------------------------|----------------------|
| I_A | 1 | - | - | - | - |
| I_S | 1 | - | - | - | - |
| Integral A/Integral S | 1.3260 | A | 0.00031 | 0.751073225 | 2.51E-04 |
| Analyte signal ^1H Nuclei | 1.9996 | B | 0.0003 | -0.498059859 | 1.49E-04 |
| IS signal ^1H Nuclei | 3.9992 | B | 0.0003 | 0.249037166 | 7.47E-05 |
| Analyte Molar Mass (g/mol) | 154.129 | B | 0.006 | 0.006461604 | 3.75E-05 |
| IS Molar Mass (g/mol) | 194.186 | B | 0.0085 | -0.005128683 | 4.35E-05 |
| Analyte Sample Mass (mg) | 4.3889 | B | 0.00124 | -0.226918536 | 2.82E-04 |
| IS Sample Mass (mg) | 2.0780 | B | 0.00124 | 0.479268765 | 5.95E-04 |
| IS Purity (g/g) | 0.9993 | B | 0.0004 | 0.996618126 | 3.99E-04 |
| Assigned value (g/g) | 0.9959 | | 0.00081 | | 8.15E-04 |
| | | | | Combined Uncertainty | 0.000812 |
| | | | | ν_{eff} | 439 |
| | | | | k | 2 |
| | | | | Expanded Uncertainty | 0.00162 |

| | | | |
|------------------------------|--------|---|--------|
| Analyte mass fraction (g/g): | 0.9959 | ± | 0.0016 |
| Analyte purity (% mass): | 99.59 | ± | 0.2 |

Table 6: Example qNMR uncertainty budget for PAT

The relative contributions of each component to the combined standard measurement uncertainty are presented in Figure 10:

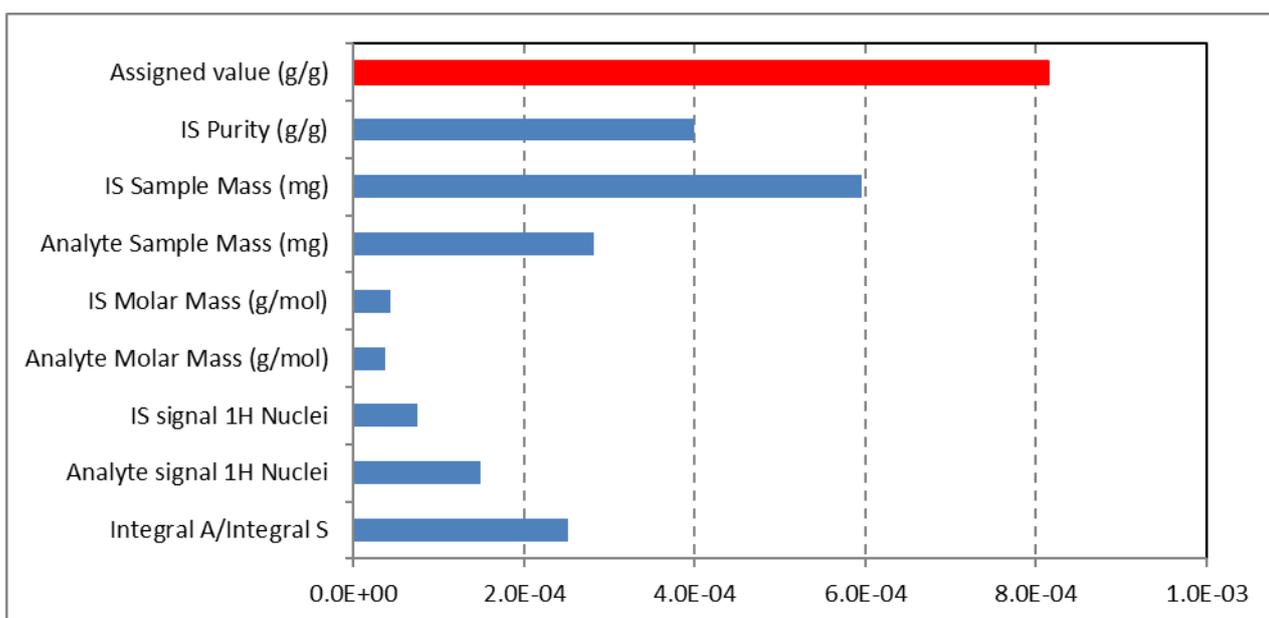


Figure 10 – Relative uncertainty contributions to the uncertainty budget for PAT purity

Note that at the mass sizes used for the sample preparation, the contributions to the uncertainty of the purity assignment from gravimetric operations, and also from the purity of the internal standard are greater in magnitude than that from the precision of the integral ratio determination.

The estimate for PAT content of the material from this experiment was 996 ± 2 mg/g.

A second estimate was obtained subsequently by another operator using a separately prepared sample. The estimate was 994 ± 3 mg/g after correction for contributions due to the solvent impurity peaks.²⁴

For the overall PAT content a consensus value was assigned as the weighted mean of the two independent assignments. This gave the estimate of the PAT content in OGO.180 as 995.1 ± 4.0 mg/g.

5.3 LC-DAD and LC-MS/MS

5.3.1 Apparatus

The liquid chromatography (LC) system used consisted of an Agilent 1100 series micro vacuum degasser, binary pump, thermostatted standard autosampler, thermostatted column compartment and diode array detector (DAD). An Applied Biosystems 4000 Qtrap hybrid tandem mass spectrometer (MS/MS) was coupled to the LC system employing a Sciex TurbolonSpray (TIS) source and a Valco 10-position valve. A direct flow injection was used for optimization studies.

5.3.2 Materials

- Patulin (PAT). BIPM Reference OGO.180
- Ascladiol (ASC)
- Deoxyapatulinic acid (DPA)
- all purchased from First Standard China via NIM China

Pure water was obtained from a MilliQ RiOs gradient ultrapure device.

5.3.3 HPLC method

A method based on liquid chromatography coupled to diode array detection (DAD) was developed for the quantification of PAT in the material (OGO.180a). Potential related structure impurities ascladiol (ASC) and deoxyapatulinic acid (DPA), whose identity was confirmed by tandem mass spectrometry (MS/MS), were also used in method development although they were not subsequently found to be present in the material. There was also no evidence in the NMR studies of the significant presence of either of these compounds. The purity of PAT was assessed by qNMR while for the other materials it was taken from information in the supplier's certificates. The method was validated in-house for the parameters of linearity, precision and limits of detection and quantification. Chromatographic separation was performed at 25 °C using a Kinetex EVO C18 100Å, (250 x 4.6 mm, 2.6 µm) column. The chromatographic conditions used for the separation of the compounds were:

| | |
|----------------------|--|
| Column: | Shiseido Capcell PAK C18 MG S-5 (250 x 4.6 mm, 5 µm) |
| Column temperature: | 30 °C |
| Detector 1: | DAD 276, 267 and 254 nm, ref. wavelength 400 nm. |
| Detector 2: | MS/MS (see above) |
| Mobile phase: | A) acetonitrile/H ₂ O Milli Q (5/95) with 0.1 % formic acid B) acetonitrile with 0.1 % formic acid |
| Operation mode: | Gradient |
| Solvent gradient: | |
| Flow rate: | 0.8 mL/min |
| Injection volume: | 5 µL |

5.3.5 (DAD) parameters

The absorption wavelength used for the detection of the main component PAT was 276 nm (step and slit widths 2 nm and 4 mm, respectively). The wavelengths of 267 nm and 254 nm were recorded for verification.

5.3.6 MS/MS parameters

The optimized MRM detection parameters are described in section 4.3.8

5.3.7 LC-DAD-MS/MS results – OGO.180

Using the HPLC-DAD-MS/MS analysis described in Section 5.3.3 above with UV detection at 276 nm and MS/MS detection using EMS-IDA-EPI ionization, analysis of a solution of the OGO.180 material identified no significant related structure impurities present in the PAT material.

To avoid contamination of the sensitive LC-MS/MS instrument by the high content of PAT component, after passing through the DAD detector the mobile phase was diverted to waste during elution of the major component to allow for improved detection and analysis of any potential impurities. Figures 11 and 12 show chromatograms by UV (276 nm) and MS/MS detection.

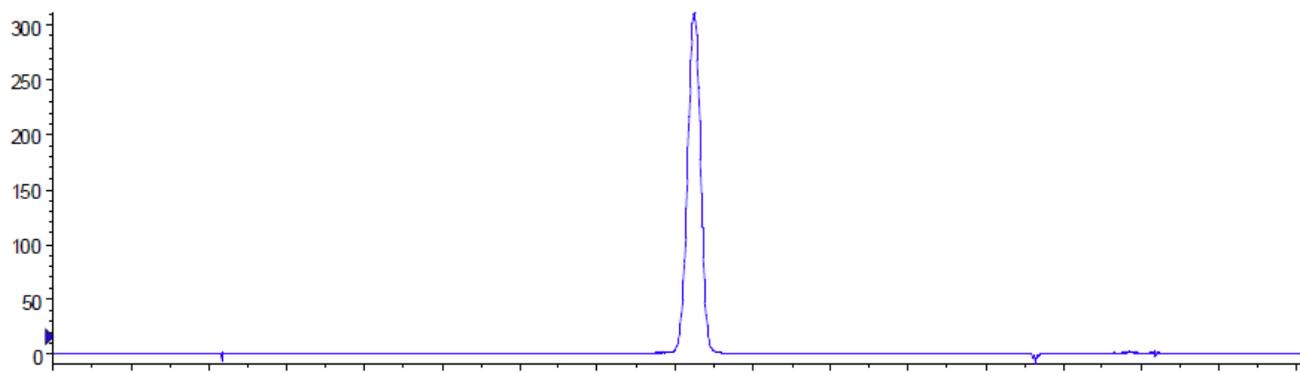


Figure 11 Chromatogram with UV detection at 276 nm of PAT stock solution

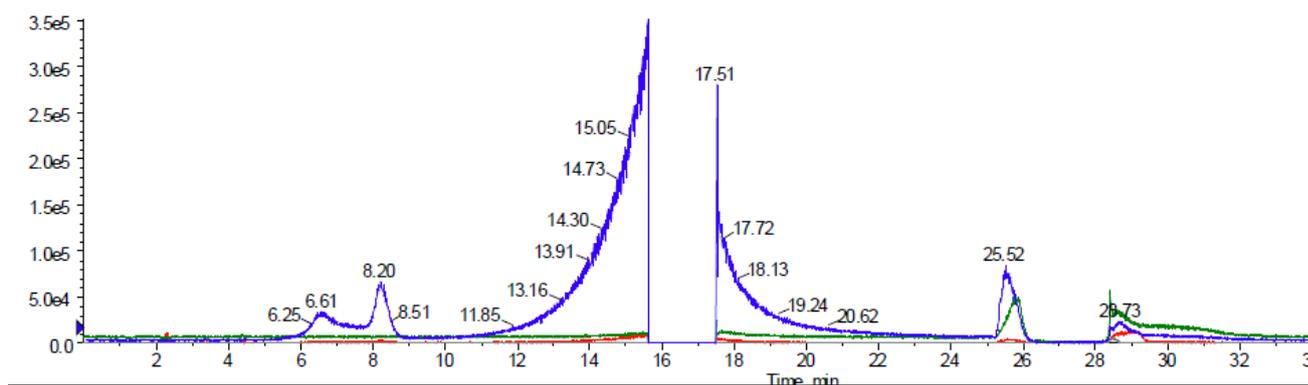


Figure 12 TIC by MS/MS detection of PAT solution in LC-EMS-IDA-EPI mode. PAT peak diverted to waste.

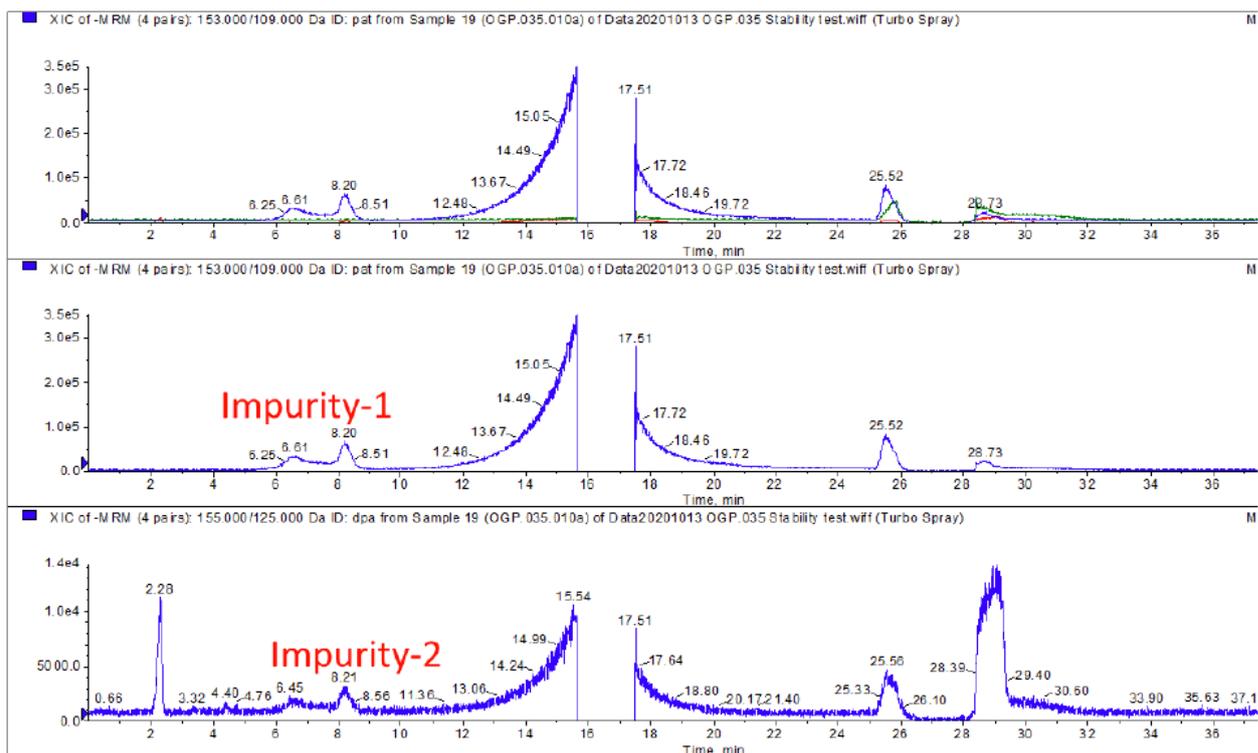


Figure 13 Comparison of impurities in OGO.180 solution by LC-MS/MS (injection volume 5 μ L)

Impurity 1 was detected for the mass transition 153/109, a mass transition also found in PAT. Impurity 2 was detected at the mass transition 155/125, a mass transition reported for ASC. Impurity 1 was inherent in the material. Impurity 2 was only produced after storage at 40 °C. Both were present at negligible levels and were not quantified.

5.4 Water

Water content measurements by coulometric Karl Fischer titration were carried out on the OGO.180a PAT material. The challenges with handling PAT in solid form due to its toxicity and fears of contaminating equipment meant that use of a heated oven to release any water from the material was precluded. A protocol was implemented to avoid as much as possible exposure to the material in powder form.

Individual samples were weighed into a tared GC vial and the vial was sealed as soon as the gravimetric measurement was completed. Solvent (anhydrous acetonitrile, purity > 99.9%) was added via syringe into the sealed vial immediately prior to the measurement and the vial was weighed. After dissolution of the PAT solid the bulk of the resulting solution was withdrawn by syringe and injected directly into the coulometric titration cell containing the KFT reagent. By measuring the mass changes of the vial and the syringe before and after transfer of the PAT solution it was possible to calculate the mass introduced into the titration cell.

A series of “blank” measurements were obtained by injection of solvent only to establish the background level of water introduced in the injection process. A reference material having a nominal water content of $103 \pm 3 \mu\text{g/g}$ water in hexane was used to validate the sensitivity and accuracy of the instrument to quantify low levels of water.

The injection of five separate samples of PAT in solution in acetonitrile, each containing ca 1 mg of solid in ca 200 mg of solvent, gave results that were not statistically different from the results obtained after injection of blank solvent. On the basis of these results it was determined that the material did not contain a quantifiable level (< 0.1 mg/g) of water.

5.5 Residual solvent

After analysis of minor signals detected in the ¹H qNMR experiment, the presence of dichloromethane and ethanol as residual solvents were present in the sample. The approximate concentrations of dichloromethane and ethanol were found to be 2 mg/g and 2.8 mg/g respectively.

5.6 Purity assignment

The value for PAT in the material determined as described in 5.2.5 is 995.1 ± 3.9/4.2 mg/g.

The only significant impurity identified in the OGO.180 material was residual organic solvent. The amounts of dichloromethane and ethanol present assigned by qNMR are consistent with the assigned content of the primary component, patulin. No significant amount of related structure impurity or water was quantified.

| Secondary component | Mass fraction (mg/g) | Measurement Method | Verification Method |
|------------------------|----------------------|--------------------|---------------------|
| PAT related impurities | - | LC-UV | LC-MS/MS |
| VOCs | 4.8 ± 0.22 | qNMR | |
| Water | - | KFT | TGA |

Table 7 Estimates for Impurity content in BIPM source material OGO.180

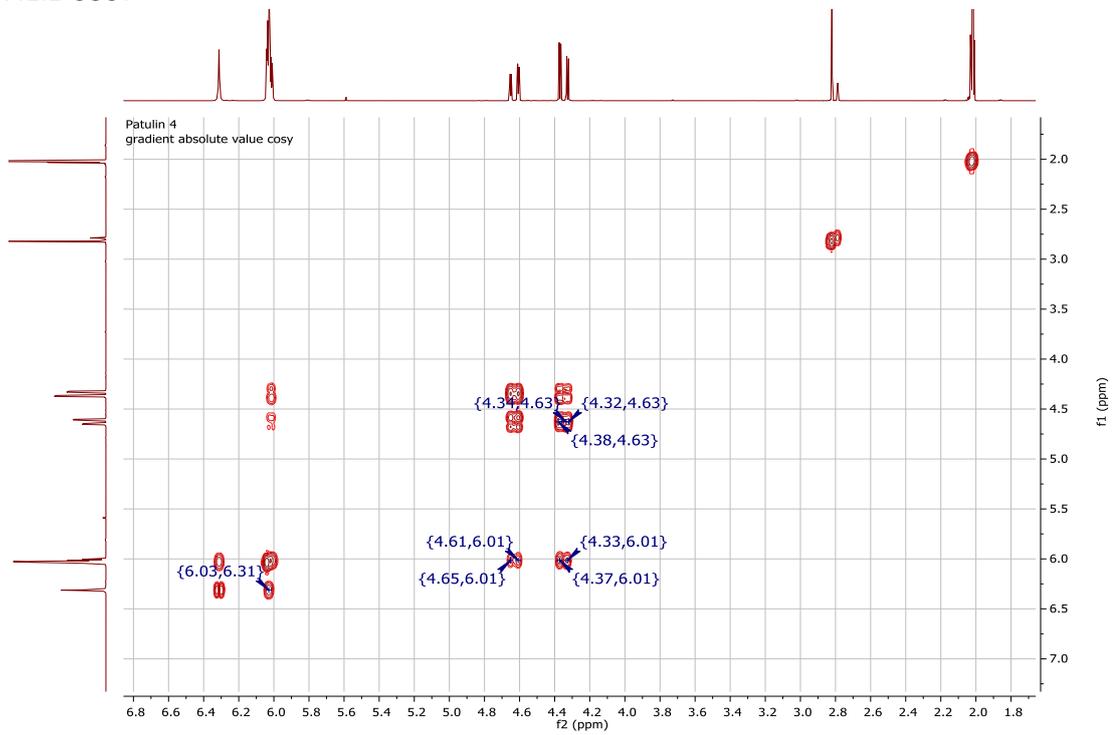
6. Acknowledgements

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7. Annexes

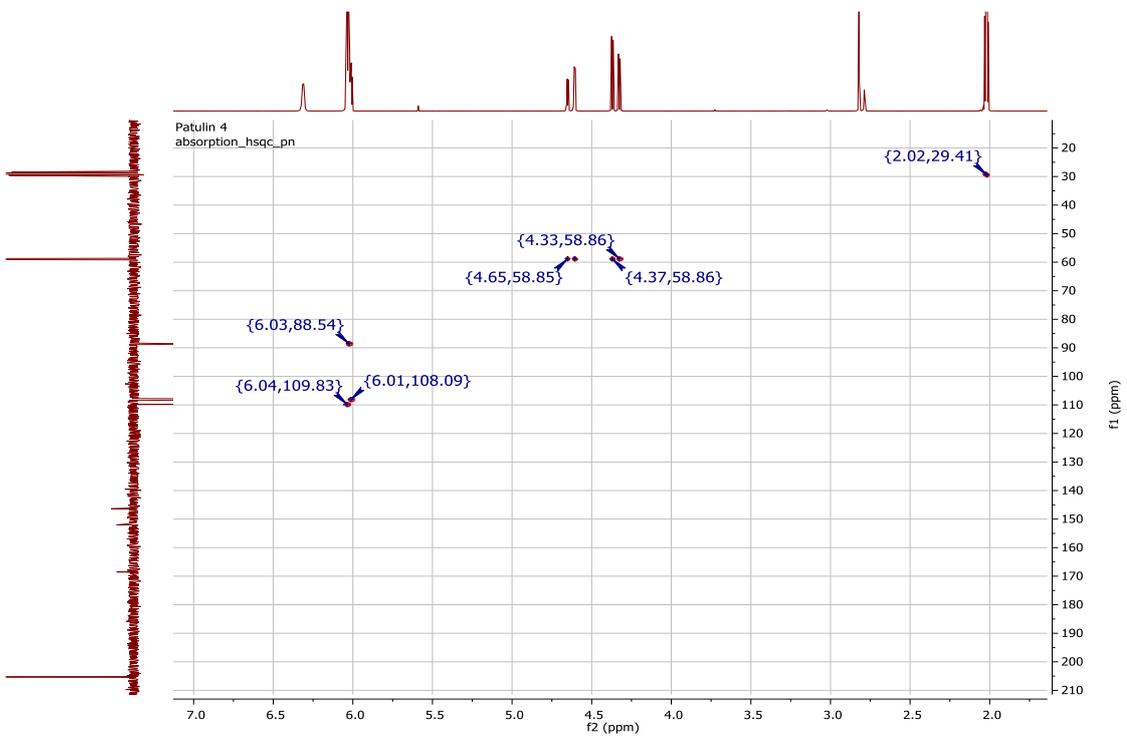
7.1 2D-NMR spectra of PAT

7.1.1 COSY



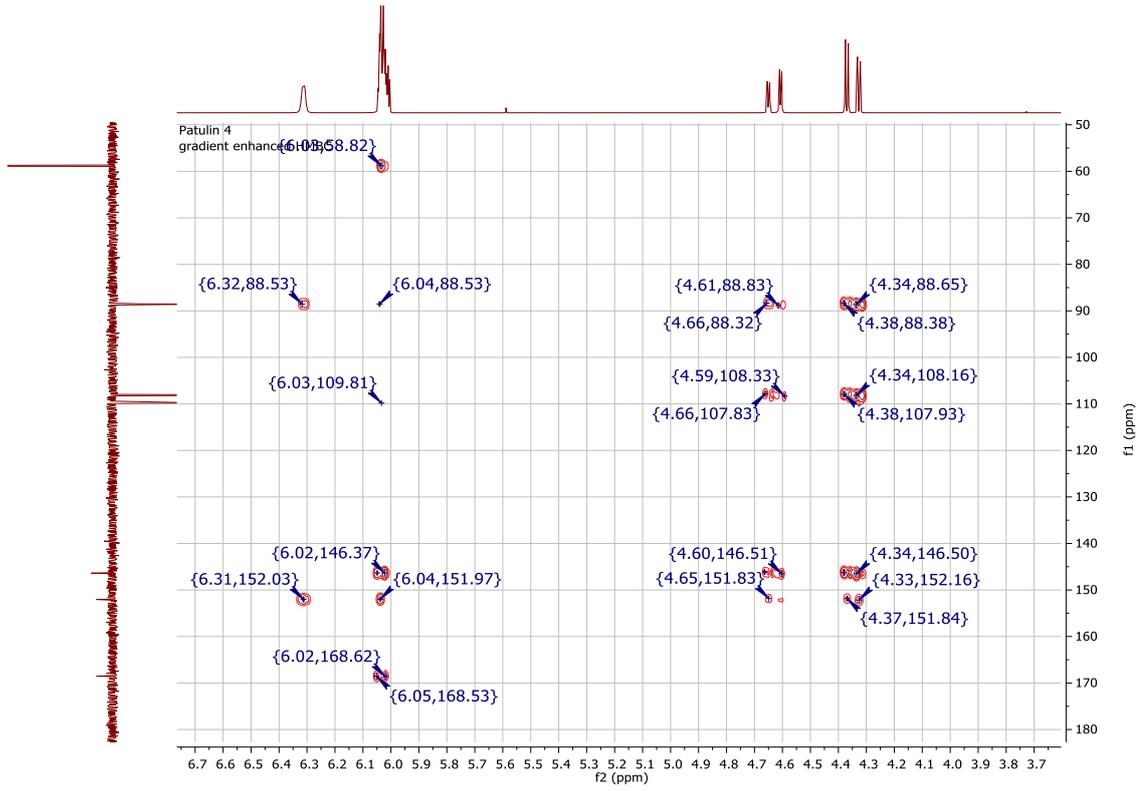
COSY spectrum of PAT

7.1.2 HSQC



HSQC spectrum of PAT

7.1.3 HMBC



HMBC spectrum of PAT

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