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Bureau International des Poids et Mesures

Calibrant Assessment Guideline: Patulin (PAT)

BIPM CAG-04

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1. Scope

This document has been prepared to provide guidance on the preparation and value assignment of a calibration solution of **patulin (PAT)** in the mass fraction range of 10-100 mg/kg. The calibration solution is prepared by gravimetric dilution of a gravimetrically prepared stock solution having known PAT mass fraction and it is intended for use as a primary calibrator for PAT analysis.

The information summarized in the document was obtained as part of the BIPM Metrology for Safe Food and Feed Programme for capacity building and knowledge transfer on the production and characterization of reference materials for mycotoxin analysis.

2. Introduction

In collaboration with the National Institute of Metrology, China (NIM) and the National Metrology Institute of South Africa (NMISA), the BIPM initiated in 2016 a Capacity Building and Knowledge Transfer program for Metrology for Safe Food and Feed (MMCBKT) in Developing Economies (1). This project is designed to allow NMIs/DIs to work together to strengthen the worldwide mycotoxin metrology infrastructure; provide knowledge transfer to scientists developing capabilities in this area and to enable NMIs/DIs in developing regions to provide calibrants, matrix reference materials and proficiency test samples that support testing activities and laboratory services for mycotoxin analysis within their countries.

Calibration solutions prepared from well characterized, high purity compounds are the source of metrological traceability of most routine organic analysis results. The preparation and characterization of these solutions is therefore essential within the measurement infrastructure that supports the delivery of reliable results. It is particularly challenging in the case of the provision of standards to underpin mycotoxin testing in developing economies due to stringent export / import regulations, challenging logistics and high costs.

Patulin (PAT), a polyketide and unsaturated heterocyclic lactone, is a mycotoxin produced by a variety of moulds, particularly by fungi of the genus *Aspergillus* and *Penicillium*. Often found in rotting apples and apple products, PAT can also occur in various other moldy fruits, grains and other foods. Major human dietary sources of patulin are apples and apple juice made from affected fruit (2). The acute symptoms in animals include liver, spleen and kidney damage and toxicity to the immune system. For humans, nausea, gastrointestinal disturbances and vomiting have been reported. PAT is considered to be genotoxic however a carcinogenic potential has not been demonstrated yet (3). PAT is relatively stable when given a short-acting heat-treatment (pasteurization), especially in an acidic environment (4). The importance of monitoring PAT content in primary products and derived foodstuffs is reflected in the existence of regulations controlling the maximum limits for PAT in about 48 countries. A typical minimum residue level is 50 µg/kg in food and juices (5). Levels below this were established in the European Union with regard to solid apple products of 25 µg/kg and for in apple-based baby food/juices of 10 µg/kg (6, 7). The analytical difficulty and the importance

of controlling PAT in baby food and beverages support the need for 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.

The present guideline summarizes methods that can be used for the preparation and characterization of PAT calibration solutions. The method development and validation studies carried out within the BIPM MMCBKT program are the basis for the results and procedures described herein. The document is intended to be of use to other metrology institutes and reference measurement service providers needing to prepare and characterize their own PAT primary calibrator solution to underpin the metrological traceability of results. Stock and calibration solutions were prepared from a PAT source material. For the MMCBKT programme that material was value assigned in-house for purity. Methods for the characterization of PAT pure materials are described in a separate purity evaluation guideline (PEG) (8). The PAT solutions prepared gravimetrically from the MMCBKT source material were value assigned and dispensed into glass ampoules and flame sealed. A range of analytical methods were developed to quantify the mass fractions of PAT and related structure impurities in solution in order to evaluate the homogeneity and stability of the materials, as well as to verify the gravimetrically assigned PAT solution mass fraction value.

3. Properties of PAT solutions

3.1 Hazards identification

The substance poses high potential risks for human health if handled inappropriately. It is acutely toxic by inhalation, in contact with skin and if swallowed (H300, H315). Recommendations for handling high-purity solid samples of PAT are given in reference (9).

DISCLAIMER: The safety recommendations given in this section are based on literature reported best practice and are not verified by the BIPM.

3.1.1 **Protective measures**

Wear respiratory protection. Avoid dust formation. Avoid breathing of vapours, mist or gas. Wear protective gloves, goggles and clothing. Take special care to avoid skin exposure if handling solutions and work in adequately ventilated areas. Wash hands thoroughly after handling.

3.1.2 **Emergency procedures**

General advice: Immediately call a POISON CENTER or doctor/physician. Show this safety data sheet to the doctor in attendance. Move out of dangerous area.
If inhaled: Move person 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 min 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.

3.1.3 **Spillage / Projections**

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.

3.2 Physical and chemical properties

Common Name:	**Patulin (PAT)**
Synonyms:	4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one or other trivial names: clavacin, clavatin, clairformin, expansin, gigantin, mycoin C_3, leucopenin, penicidin and penantin
CAS Registry Number:	149-29-1
Molecular Formula:	$C_7H_6O_4$
Molar Mass:	154.12 g/mol
Monoisotopic mass:	154.02660867 g/mol
Melting point:	111.0 °C (9)
Appearance:	White crystalline powder
Solubility:	Soluble in water. Soluble in organic solvents, acetonitrile, ethanol, diethyl ether, acetone, benzene, and ethyl or amyl acetate etc. (9)
pKOW	−0.26 ± 0.09 at pH of 7.3 (10)**Error! Bookmark not defined.**
UV maximum:	277 nm in ethanol (9)

3.3 Structure

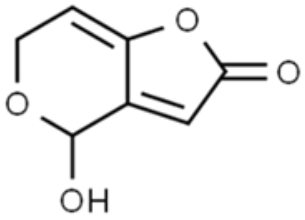

Figure 1. Chemical structure of patulin (PAT). $C_7H_6O_4$. Mw = 154.12 g/mol.

4. Methods for the characterization of PAT solutions

This section of the guideline describes the methods developed during the BIPM MMCBKT program for the characterization of PAT stock and calibration solutions prepared from the source PAT material. The methods are the basis for the stability and homogeneity studies and for the analytical confirmation of the PAT mass fraction value assigned gravimetrically.

DISCLAIMER: Commercial instruments, software and materials are identified in this document in order to 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.

4.1 PAT and related structure impurities analysis by LC-DAD

A method based on liquid chromatography inline coupled to diode array detection (DAD) was developed for the quantification of patulin in the source material (BIPM ref. OGO.180a). Potentially occurring related structure impurities ascladiol (ASC) and deoxypatulinic acid (DPA) have only been screened by inline connected tandem mass spectrometry (MS/MS) for verification as they have not been found to be present by qNMR. Details on the method development and validation are described in the purity evaluation guideline (8). A commercial standard was purchased from A Chemtek Inc., First Standard for the main compound patulin (PAT) (Figure 1). The purity of PAT was assessed by qNMR while for the rest of impurities it was taken from information in the supplier's certificates. PAT (BIPM ref. OGO.180a) obtained from A Chemtek Inc., First Standard were used to prepare solutions that served as the basis for the LC method development. The method was validated in-house for the performance characteristics of linearity, precision and limits of detection and quantification.

4.1.1 **Materials**

- Acetonitrile. HPLC gradient grade (HiPerSolv Chromanorm VWR);
- Ultrapure water (Milli-Q);
- PAT stock (BIPM ref. OGP.035) and calibration (BIPM ref. OGP.036) solutions;
- PAT standard (First Standard via NIM China).

4.1.2 **Sample preparation**

Ampoules of the stock or calibration solution were vortexed before opening and 0.5 mL aliquots of solution were transferred to glass injection vials and placed in the autosampler at 4 °C for immediate analysis.

4.1.3 **Instrumentation**

Liquid chromatography system Agilent 1100 HPLC equipped with a diode array detector (DAD) and coupled to a Sciex 4000 Qtrap mass spectrometry detector.

4.1.4 **Liquid chromatography parameters**

Column:	Shiseido Capcell PAK C18 MG S-5 (250 x 4.6 mm, 5 µm)	
Column temperature:	30 °C	
Detector	DAD 276 nm and ref. 400 nm (MS/MS for verification)	
Mobile phase:	A) Acetonitrile/water (5/95, v/v) with 0.1 % formic acid B) Acetonitrile with 0.1 % formic acid	
Operation mode:	Gradient (inclusive cleaning gradient)	
Solvent gradient	Time (min)	Mobile phase B
	0	0 %
	20	0 %
	22	95 %
	24	95 %
	26	0 %
	40	0 %
Flow rate:	0.8 mL/min	
Injection volume:	5 µL	
Duration:	40 min	

4.1.5 **DAD detection 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.

4.1.6 **MS/MS detection parameters**

For verification purposes, the 4000 QTRAP was operated in positive electrospray ionization (ESI) mode. The capillary voltage was set at 4500 V and the source temperature at 550 °C. Nitrogen was used as the ion source gas, curtain gas and collision gas. The Gas 1 and Gas 2 of the ion source were set at 55 psi and 60 psi, respectively. The curtain gas (CUR) was set at 40 psi. The Collision Gas (CAD) was set at "Mid". The declustering potential (DP), being the accelerating current from atmospheric pressure into high vacuum, 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 1 lists the optimized transitions and conditions for multiple reaction monitoring (MRM) detection and quantification of PAT and potential structurally related impurities as ascladiol (ASC) and deoxypatulinic acid (DPA).

Table 1. Transition ions and MS/MS parameters for the detection of PAT and potential selected impurities in MRM mode. Transitions marked with an asterisk were used for quantification purposes.

	Q1 (*m/z*)	Q3 (*m/z*)	Time (ms)	CE (V)
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

4.1.7 **Data analysis**

Data was evaluated using Analyst 1.6.3 software (SCIEX). Peak integration was verified manually for all samples and standards. Peak areas were extracted for quantification and uncertainty evaluation.

5. Characterization summary of the PAT stock solution

5.1 Preparation and value assignment

The PAT stock solution (OGP.035) was prepared gravimetrically by dissolving about 200 mg of PAT powder material (OGO.180a) in 1 L of acetonitrile with 0.1 % (v/v) formic acid. Mettler Toledo balances MX5 and XP10002S were used for the mass determination of OGO.180a and the final solution, respectively. Table 2 summarizes the preparation of the stock solution and the mass fraction assignment, calculated according to Equation 1. The purity of OGO.180a was determined in-house by quantitative NMR corrected for related structure impurities, as described in a separate Purity Evaluation Guideline (8).

Table 2. Experimental data corresponding to the preparation of the PAT stock solution and the calculated mass fraction.

***PAT* stock solution preparation**			
	Weighed mass (m)	**Buoyancy (*b*)**	**m x b**
PAT powder (mg)	204.967	1.000635	205.097
Stock solution (g)	780.310	1.001386	781.392
Purity ± U (mg/g)	995.1 +3.9/-4.2		
Mass fraction (µg/g)	**261.19**		

$$w_{stock} = \frac{m_p \cdot b_p \cdot w_p}{m_{sol} \cdot b_{sol}} \qquad \textit{Eq. 1}$$

Where:
m_p: observed mass of PAT powder
b_p: buoyancy correction of powder weighing
w_p: mass fraction of PAT powder
m_{sol}: observed mass of stock solution
b_{sol}: buoyancy correction of solution weighing

The uncertainties from input quantities in Equation 1 were combined (Eq. 2) and the final uncertainty was calculated (Table 3). A minor uncertainty component, u(V), was included to account for the potential solvent loss due to evaporation during sample preparation and weighing. The buoyancy mass correction and its uncertainty were calculated as described by Reichmuth *et al*. (11).

$$u(w_{stock}) = w_{stock} \cdot \sqrt{\left[\frac{u(m_p)}{m_p}\right]^2 + \left[\frac{u(b_p)}{b_p}\right]^2 + \left[\frac{u(w_p)}{w_p}\right]^2 + \left[\frac{u(m_{sol})}{m_{sol}}\right]^2 + \left[\frac{u(b_{sol})}{b_{sol}}\right]^2 + \left[\frac{u(V)}{V}\right]^2} \quad Eq.2$$

Table 3. Individual uncertainty components contributing to the final combined uncertainty of the PAT stock solution mass fraction.

Unc. source	$\frac{u(m_p)}{m_p}$	$\frac{u(b_p)}{b_p}$	$\frac{u(w_p)}{w_p}$	$\frac{u(m_{sol})}{m_{sol}}$	$\frac{u(b_{sol})}{b_{sol}}$	$\frac{u(V)}{V}$	u_{rel} (%)	$u(w_{stock})$ µg/g	$U(w_{stock})$ µg/g (*k*=2)
Value (%)	0.0023	0.0016	0.26	0.0028	0.0012	0.0050	0.26	0.68	1.36

The 1 L flask containing the stock solution was agitated thoroughly and about 125 mL were used to prepare the calibration solution (section 6). The rest of the stock solution was stored at -20 °C until ampouling, which took place within 24 h of the preparation. The ampouling process was similar to that of the calibration solution and it is described in detail in section 6.1.

5.2 Stability study

The present section provides a summary of the stock solution isochronous stability study results. A detailed description of the study design and evaluation is given for the characterization of the calibration solution (section 6.2). The main component PAT was measured by LC-DAD.

For the main component PAT, no calibration was performed so peak areas (LC-DAD) were directly normalized to the PAT peak area (276 nm) in the reference samples, respectively. Data were evaluated as a function of the storage time at each of the studied temperatures.

Preliminary studies demonstrated that PAT is not stable in solution in acetonitrile unless stabilized with a weak organic acid (0.1 % formic acid has been used). These findings are supported by literature (4). The acidified stock solution was found to be stable over the entire study period of 8 weeks at storage temperatures of 4 °C, 22 °C and 40 °C .

It was concluded that the material was suitably stable for short-term transport provided it was not exposed to light and to temperatures significantly in excess of 40 °C for more than two to four weeks. To minimize the potential for changes in the material composition, long-term storage is recommended at -20 °C in the dark.

Stability studies were undertaken of the PAT content of the material. Isochronous

stability studies confirmed that the material was stable for the purpose of the comparison provided it is stored and handled as recommended. Standard uncertainty contributions due to stability (u_{lts}) of 1.02 % for PAT by LC-DAD (276 nm) have been used for establishing the uncertainty budget of the assigned value of the main component to cover the period from characterization until comparison performed. The uncertainty contribution of 1.02 % is a very conservative estimate based on storage of 20 weeks at 4 °C and in the dark.

5.3 Homogeneity study and combined uncertainty

The homogeneity study for the *PAT* stock solution is analogous to that of the calibration solution, which is reviewed in detail in section 6.3. The present discussion is therefore limited to a summary of the results. *PAT* in the ten selected homogeneity samples was measured by LC-DAD (276 nm detection wavelength).

Homogeneity evaluation was performed by single factor ANOVA, allowing for the separation of the variation associated with the method (s_{wb}) from the actual variation between ampoules (s_{bb}), which is an estimate of the uncertainty associated to batch inhomogeneity. This maximum relative standard uncertainty contribution due to inhomogeneity was 0.13 % for PAT (Table 4).

Table 4: Homogeneity results of the PAT stock solution OGP.035 using the LC-DAD method.

	PAT (276 nm)
N	30
s_{wb} (%)	0.35
s_{bb} (%)	0.13
u^*_{bb} (%)	0.11
$\mathbf{u_{bb}}$ (%)/$\mathbf{s_{bb}}$ (%)[(1)]	**0.13**
F	1.39
F_{crit}	2.39

[(1)] Higher value (u^*_{bb} or s_{bb}) was taken as uncertainty estimate for potential inhomogeneity

The homogeneity (section 5.3, u_{bb}) and stability (section 5.2, u_{lts}) uncertainty contributions for the main component *PAT* obtained by LC-DAD were combined with the uncertainty from the gravimetric value assignment – see $u(w_{stock})$ in section 5.1 – to produce a final estimate of the mass fraction uncertainty of the batch (Table 5).

Table 5. Combination of the uncertainty from the gravimetric value assignment and the uncertainty from between-ampoule homogeneity and stability to estimate the final uncertainty of the PAT mass fraction in the batch of the stock solution OGP.035.

$u(w_{stock})_{rel}$ (%)	u_{bb} (%)	u_{lts} (%)	$u(comb)_{rel}$ (%)	w_{stock} µg/g	U(comb) µg/g (*k = 2*)
0.26	0.13	1.02	1.06	261.2	5.6

6. Preparation and characterization of the PAT calibration solution

6.1 Preparation and ampouling

The *PAT* calibration solution (BIPM reference: OGP.036) was prepared by gravimetric dilution of 125 mL of the stock solution with acidified acetonitrile (0.1 % FA) to a final volume of 1 L. The solution was stored at 4 °C until ampouling, which took place within 24 h of the preparation. A 500 mL bottle and 1-10 mL bottle-top dispenser (Dispensette, Brand GmbH) were rinsed twice with the calibration solution and a stainless-steel flat tip syringe needle was fitted at the outlet of the dispenser to ensure that all solution is discharged at the bottom of the ampoule.

10 mL glass ampoules were selected for a filling volume of 4 mL to ensure that sufficient head space remains above the liquid and therefore minimize the risk of accidental ignition of the solvent during the sealing process. An Ampoulmatic (Bioscience Inc) system connected to propane and oxygen cylinders was used to ampoule the batch. The flow of both gases was adjusted to produce a bright blue flame at the neck of the ampoules.

The ampoules were filled with 4 mL of OGP.036, one at a time, to minimize the impact of evaporation of acetonitrile. A refrigerant (Jelt Refroidisseur 5320) was sprayed onto the lower portion of the ampoule before being placed in the ampouling carousel to further reduce the ignition risk. After flame sealing, ampoules were allowed to cool down at room temperature in an upright position and have been labelled according to the order of filling.

To test for possible leaks, ampoules were placed into a vacuum drying oven (Haraeus) in an upright position and vacuum (approximately 50 mbar) was applied for at least 4 hours. The ampoules then remained in the sealed oven overnight, after which they were visually inspected for changes in the solution levels. Inadequately sealed ampoules were noted and discarded while the rest of the batch was stored at -20 °C.

6.2 Stability study

6.2.1 **Study design**

Stability studies consider the impact of temperature and time to simulate potential transport conditions and/or storage conditions. Any significant influence of light, UV-radiation, moisture, etc. is excluded provided that the storage facilities and transport/packaging conditions are appropriate (12).

The stability study of OGP.036 followed an isochronous design (13) with a reference temperature of -20 °C and study temperatures of 4 °C, 22 °C and 40 °C and storage in the dark. Selected sample units were transferred from study temperatures to the reference temperature every two weeks until the end of the eight-week study.

The sample units were selected using a random stratified sampling scheme from each of the quartiles of the approximately 200-unit batch. The study was composed of three units (one unit as reserve) at the reference temperature and twelve units (four units as reserve) at each of the study temperatures, requiring 39 samples in total (Table 6).

Table 6: Temperatures, time points and sample units selected for the stability study of OGP.036 (reserve units in brackets).

Temperature	Time (weeks)	Units
-20 °C (reference temperature)	n.a.	017,129,(076)
4 °C dark	2	050,113,(069)
	4	025,176,(120)
	6	014,159,(102)
	8	028,165,(073)
22 °C dark	2	006,193,(106)
	4	009,152,(082)
	6	020,184,(146)
	8	038,187,(115)
40 °C dark	2	037,161,(117)
	4	018,191,(083)
	6	044,181,(112)
	8	012,140,(066)

6.2.2 **Stability study measurements**

Two samples of each time point and temperature conditions were measured under repeatability conditions (same day and run) in a randomised manner using the LC-DAD(-MS/MS) for *PAT*. Ampoules were vortexed before opening and two aliquots were transferred into separate injection vials to have duplicate measurements of each sample (4 measurements for each condition) by LC-DAD(-MS/MS). Representative XIC and DAD chromatograms of OGP.036 samples are shown in Figure 2.

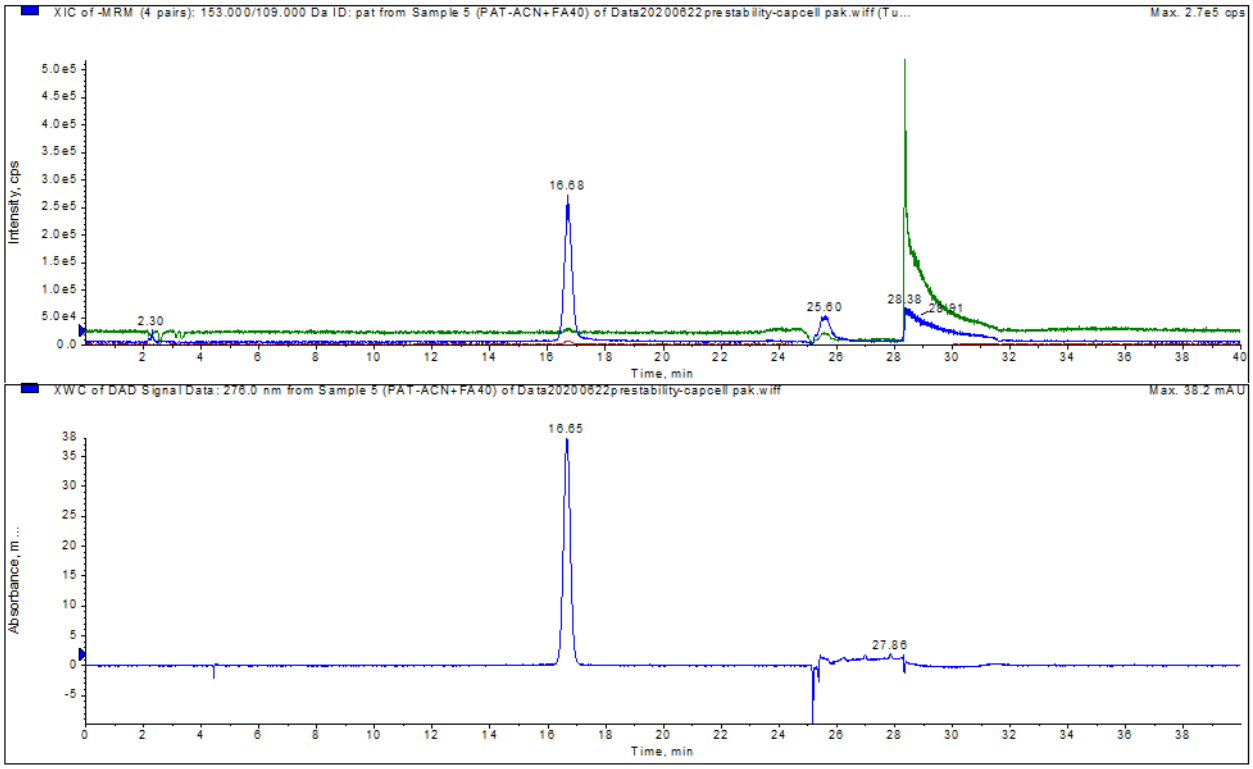

Figure 2. LC-MS/MS extracted ion chromatogram (top) and LC-DAD chromatogram at 276 nm (bottom) of a representative sample of OGP.036.

6.2.3 **Stability data evaluation**

Calculated mass fraction values of *PAT* by LC-DAD at 276 nm were normalized to the respective average values of the reference samples (stored at -20°C) to render results comparable. As a first evaluation step, normalized data were plotted according to the injection sequence to discard any potential analytical drift. The slope of the fitted regression line for *PAT* was not significant (t-test) at the 95 % confidence level (Figure 3).

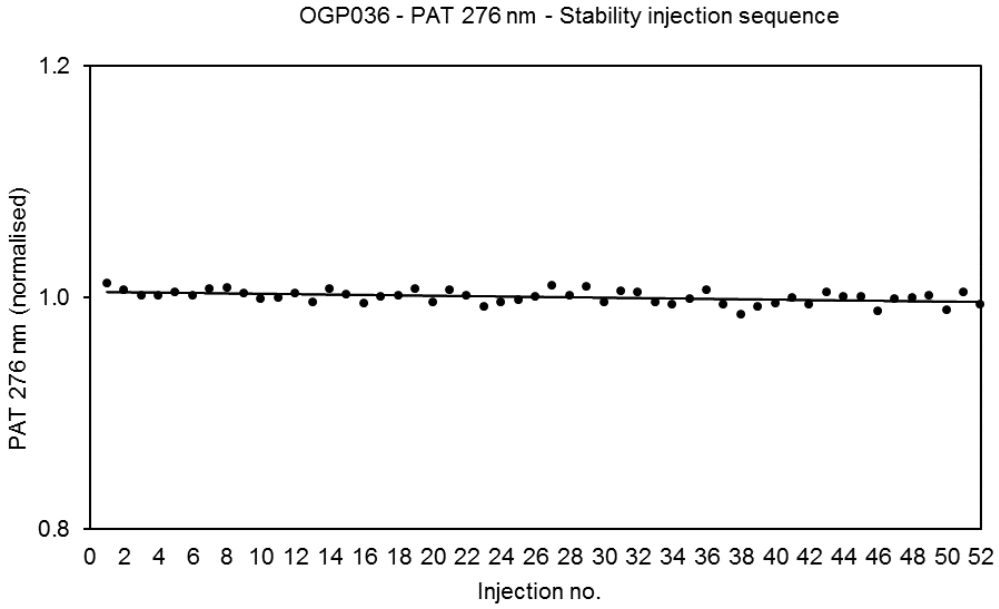

Figure 3. OGP.036 stability data analysis to identify potential trends in the analytical sequence. Data correspond to normalised mass fractions of the main compound PAT determined by LC-DAD.

For each temperature, regression lines of the normalised values versus storage time were calculated. The fitted regression model was tested for overall significance (loss/increase due to storage) using an F-test (95% confidence level). The LC-DAD(-MS/MS) stability results of the main component at each of the studied temperatures are shown in Figure 4.

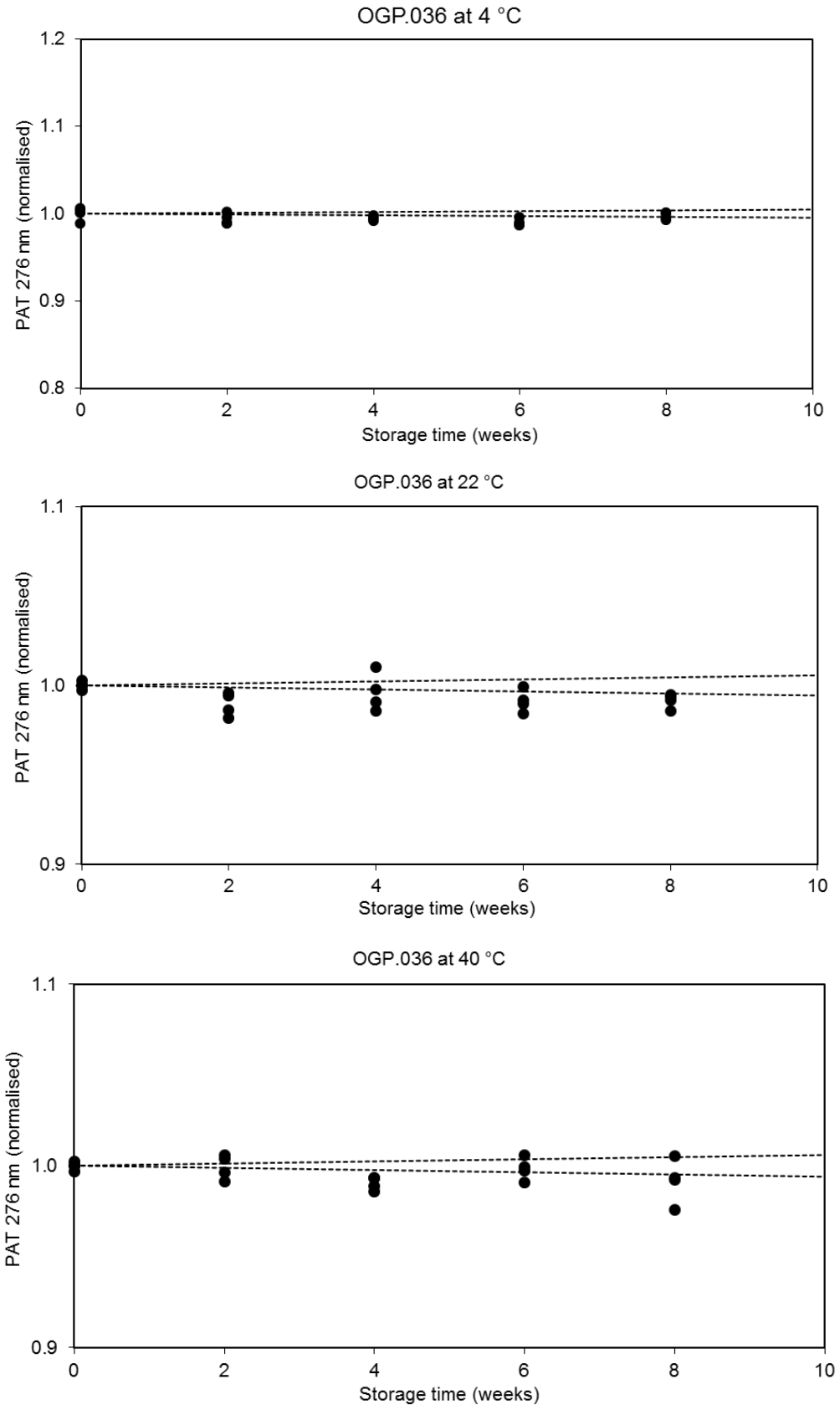

Fig 4. Stability results of OGP.036 for the main compound PAT at the three studied temperatures (top 4 °C, middle 22 °C and bottom 40 °C). Data correspond to normalised peak areas of PAT measured by LC-DAD.

The acidic solution of *PAT* (OGP.036) was stable for 8 weeks at all storage temperatures of 4 °C, 22 °C and 40 °C, as evidenced by the absence of a significant trend (F-test, 95 % confidence level). It should be noted that the beginning formation of a related structure impurity has been observed by LC-MS/MS at the storage temperature of 40 °C only. However, the peak that started to form after 2 weeks at 40 °C at a retention time of 8.2 min was too small to allow identification or quantification.

Overall, the LC-DAD measurements results of the OGP.036 stability samples indicated that shipment conditions should not exceed 40 °C for about two weeks. Long-term storage is recommended at -20 °C as a precautionary measure.

6.2.4 **Stability uncertainty contribution**

The uncertainty contribution of stability over the comparison period was estimated from the isochronous stability studies (13). The influence of the measurement variance of the estimate of the degradation rate is calculated by use of the standard deviation of the slope of the regression line (14). Divergent straight uncertainty lines (dotted lines in Figure 4) are calculated by use of the standard deviation as conservative estimate of the degradation rate. Twenty weeks was chosen as time frame for the comparison period and u_{lts} of 0.94 % is directly obtained from the divergent straight uncertainty lines at the time point twenty months storage time for PAT at 4 °C (Figure 4, middle). However, long-term storage is recommended at -20 °C as a precautionary measure.

6.3 Homogeneity study

6.3.1 **Study design**

Homogeneity between ampoules was evaluated to ensure that the assigned value of the calibration solution was valid for all units of the material, within the stated uncertainty. It was therefore necessary to determine this between-unit variation and incorporate it in a combined uncertainty estimate (12).

Ten ampoules were selected from the OGP.036 batch following a randomly stratified sampling scheme. They were measured under repeatability conditions using an LC-DAD method for the main compound *PAT*.

6.3.2 **Homogeneity study measurements**

The selected ampoules were allowed to equilibrate at room temperature and were vortexed before opening. They were analysed in a random order to ensure that any trends in the ampouling process could be distinguished from potential trends in the analytical sequence. Three aliquots per ampoule were transferred into glass injection vials for LC-DAD analysis.

6.3.3 **Homogeneity data evaluation**

Peak area values were normalized with respect to the average result for each of the studied compounds. Linear regression functions were calculated for the normalized values arranged in ampouling and analysis order. The slopes of the lines were tested for significance at approximately 95 % confidence level to discard the presence of trends. Figure 5 shows the LC-DAD measurements for main compound PAT at 276 nm displayed according to the order of analysis and of ampouling. No significant trends were found in the analytical sequences.

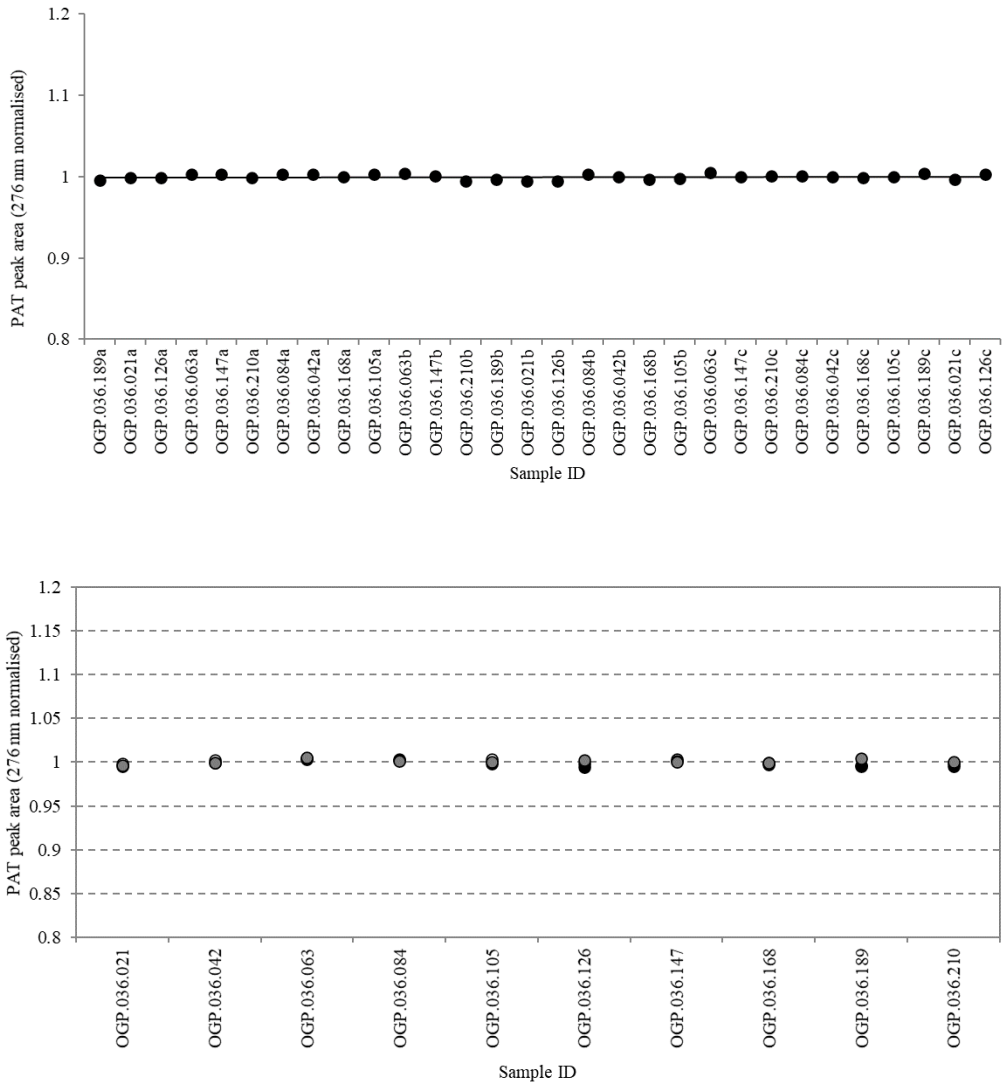

Figure 5. Homogeneity results of OGP.036 as determined by LC-DAD at 276 nm plotted according to the analysis (top) and ampouling (bottom) order.

Quantification of between-unit heterogeneity was undertaken by analysis of variance (ANOVA), which allows for the separation of the variation between ampoules (s_{bb}) from that associated with the method repeatability (s_{wb}). These variances are calculated as follows (14):

$$S_{bb}^2 = \frac{MS_{btw} - MS_{with}}{n} \qquad \textit{Eq. 3}$$

$$S_{wb}^2 = MS_{with} \qquad \textit{Eq. 4}$$

where MS_{btw} and MS_{with} are the mean sums of squares between- and within-units obtained by the ANOVA evaluation and n is the number of replicates per ampoule (n = 3).

The standard deviation between the sample units is used as the estimator for the between-units variability. The measurement variation sets a lower limit to this estimator reflected in MS_{btw} being smaller than MS_{with}. This does not imply that the material is perfectly homogeneous, but only shows that the study set-up was not adequate to detect evidence of heterogeneity. In this case, the maximum heterogeneity that could be hidden by the intrinsic variability of the method, u^*_{bb}, is calculated according to the equation below (14):

$$u_{bb}{}^* = \sqrt{\frac{MS_w}{n}} \cdot \sqrt[4]{\frac{2}{p(n-1)}} \qquad \textit{Eq. 5}$$

where p is the number of measured ampoules (p=10) and n is the number of measurement replicates per ampoule (n = 3).

The final uncertainty from homogeneity (u_{bb}) is estimated as s_{bb} or u^*_{bb}, depending on which of these is larger. This uncertainty is presented in Table 7 for every measured compound using the LC-DAD method. The F-test at the approximately 95 % confidence level did not detect significant differences between ampoules for any of the studied compounds. Therefore, the *PAT calibration solution* (OGP.036) can be regarded as homogeneous. The homogeneity uncertainty contribution (u_{bb}) of 0.17 % of the main compound *PAT* was considered to establish the overall uncertainty for the *PAT calibration solution* (section 6.4)

Table 7: Homogeneity uncertainty results of OGP.036 from data generated by LC-DAD (276 nm) for main compound PAT.

	PAT (276 nm)
N	30
s_{wb} (%)	0.26
s_{bb} (%)	0.17
u^*_{bb} (%)	0.08
$\mathbf{u_{bb}}$ (%) or $\mathbf{s_{bb}}$ (%)[(1)]	**0.17**
F	2.35
F_{crit}	2.39

[(1)] Higher value (u^*_{bb} or s_{bb}) was taken as uncertainty estimate for potential inhomogeneity

6.4 Mass fraction value assignment and uncertainty

The preparation of the calibration solution and the mass fraction assignment, w_{cal}, are shown in Table 8. Mettler Toledo balances AX504 and $XP_1$0002S were used for mass determinations.

Table 8. Experimental data corresponding to the preparation of the PAT calibration solution and the calculated mass fraction.

PAT calibration solution preparation			
	Weighed mass (m)	**Buoyancy (*b*)**	**m x b**
PAT stock sol. (mg)	94.63	1.001386	94.761
Calibration sol. (g)	779.22	1.001386	780.230
***w*(stock) ± u (µg/g)**	261.2 ± 0.68		
$\boldsymbol{w_{cal}}$ **(µg/g)**	**31.72**		

The PAT mass fraction of OGP.036, calculated according to Equation 6, was 31.72 µg/g. The associated uncertainty was calculated by considering the input quantities and related uncertainties represented in the Ishikawa diagram of Figure 6.

$$w_{cal} = \frac{m_{stock} \cdot b_{stock} \cdot w_{stock}}{m_{sol} \cdot b_{sol}} \qquad \textit{Eq. 6}$$

Where:

m_{stock}: weighed mass of PAT stock solution

b_{stock}: buoyancy correction of stock solution weighing

w_{stock}: PAT mass fraction of the stock solution

m_{sol}: weighed mass of calibration solution OGP.036

b_{sol}: buoyancy correction of calibration solution weighing

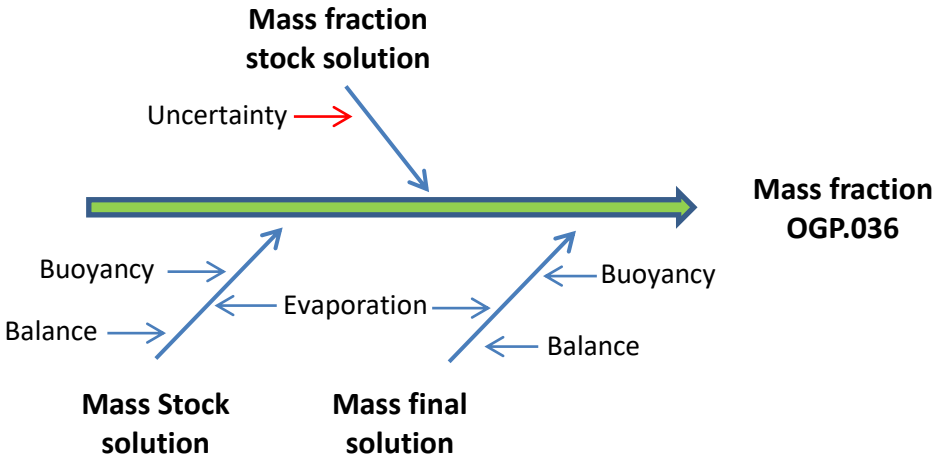

Figure 6. Ishikawa diagram indicating the input quantities contributing to the final uncertainty of the PAT mass fraction of the calibration solution OGP.036.

The standard uncertainties of the input quantities of Figure 6 were combined (Eq. 7) to produce the uncertainty of the calibration solution mass fraction, $u(w_{cal})$ (Table 9). The uncertainty of the stock solution does not comprise homogeneity and stability contributions because the aliquot of the stock solution was taken directly and without delay from the 1 L bottle to produce the calibration solution. The evaporation uncertainty, u(V), accounts for potential solvent losses during the weighing of the stock solution and of the final solution. The buoyancy mass correction and its uncertainty were calculated as described by Reichmuth *et al*. (11).

$$u(w_{cal}) = w_{cal} \cdot \sqrt{\left[\frac{u(m_{stock})}{m_{stock}}\right]^2 + \left[\frac{u(b_{stock})}{b_{stock}}\right]^2 + \left[\frac{u(w_{stock})}{w_{stock}}\right]^2 + \left[\frac{u(m_{sol})}{m_{sol}}\right]^2 + \left[\frac{u(b_{sol})}{b_{sol}}\right]^2 + 2 \cdot \left[\frac{u(V)}{V}\right]^2} \qquad \textit{Eq. 7}$$

Table 9. Individual uncertainty components contributing to the final combined uncertainty of OGP.036 mass fraction.

Unc. source	$\frac{u(m_{stock})}{m_{stock}}$	$\frac{u(b_{stock})}{b_{stock}}$	$\frac{u(w_{stock})}{w_{stock}}$	$\frac{u(m_{sol})}{m_{sol}}$	$\frac{u(b_{sol})}{b_{sol}}$	$\frac{u(V)}{V}$	u_{rel} (%)	$u(w_{cal})$ µg/g	$U(w_{cal})$ µg/g (k = 2)
Value (%)	0.019	0.0012	0.260	0.0028	0.0012	0.005	0.261	0.08	**0.16**

The homogeneity (Table 7, section 6.3, u_{bb}) and stability (section 6.2, u_{lts}) uncertainty contributions of 0.17 % and 0.94 %, respectively for the main component *PAT* obtained by LC-DAD were combined with the uncertainty $u(w_{cal})$ corresponding to the gravimetric value assignment – to produce a final estimate of the mass fraction uncertainty of the calibration solution batch (Table 10).

Table 10. Combination of the uncertainty from the gravimetric value assignment, the uncertainty from between-ampoule homogeneity and the stability uncertainty to estimate the final uncertainty of the PAT mass fraction in the batch of the calibration solution OGP.036.

$u(w_{cal})_{rel}$ (%)	u_{bb} (%)	u_{lts} (%)	$u(comb)_{rel}$ (%)	w_{cal} µg/g	$U(comb)$ µg/g (k = 2)
0.261	0.17	0.94	0.99	31.72	0.62

The PAT mass fraction value and associated expanded uncertainty (k = *2*) of the calibration solution batch OGP.036 was 31.72 ± 0.62 µg/g.

6.5 Mass fraction value verification by analytical methods

The *PAT* mass fraction value assigned gravimetrically to the calibration solution OGP.036 was verified by an independent analytical method to gain additional confidence in the certified value. The LC-DAD method described in section 4 was used for this purpose. Ideally, a different PAT calibrant of certified purity should be used for calibration so that results are completely independent. In the absence of such calibrant, a partially independent calibration solution was prepared from the same original source material (OGO.180a).

Figure 7 shows the mass fraction value verification of an ampoule of OGP.036 material. The value assigned gravimetrically (section 6.4) was compared to the analytical values obtained using the LC-DAD method. The agreement between the pairs of methods values is conveniently assessed using the degrees of equivalence (DoE):

$$DoE = w(cal)_{meth} - w(cal)_{grav} \qquad Eq.\ 8$$

where $w(cal)_{meth}$ and $w(cal)_{grav}$ are the mass fractions calculated using the analytical and the gravimetric methods, respectively.

The standard uncertainties of the gravimetric (including the homogeneity and stability components) and analytical values add in quadrature to yield the combined uncertainty of the DoE value. The expanded uncertainty bar (k = 2) crossing zero indicate the agreement of the analytical measurements (LC-DAD) with the gravimetrically assigned value, taking into account the uncertainty associated at an approximately 95 % confidence level.

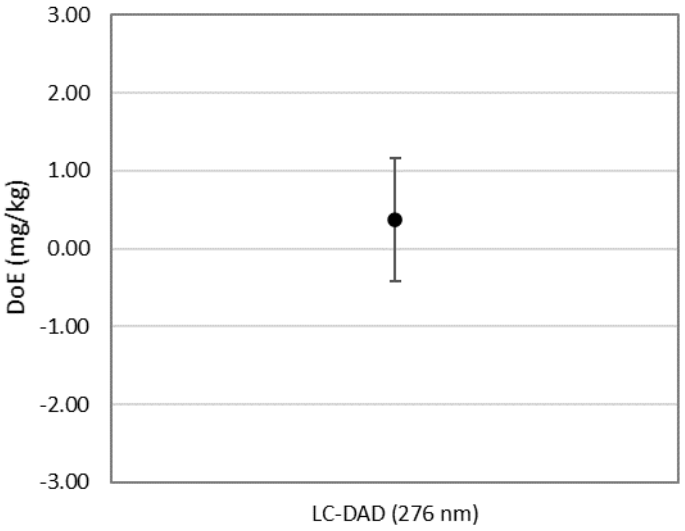

Figure 7. Degrees of equivalence (DoE) plot between the gravimetrically assigned value of OGP.036 and the analytical values obtained by LC-DAD. The error bar represents the expanded uncertainty of 0.79 mg/kg (k = 2) of the DoE value of 0.37 mg/kg.

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