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Final Report S-2016-03209 AM

VALIDATION OF A GC/MS METHOD FOR THE IDENTIFICATION AND QUANTIFICATION OF NICOTINE RESIDUE IN THE TEST ITEM "VC1" AND **ANALYSIS ON FIVE PRODUCTION BATCHES**

Study program:	S-2016-03209 AM
Contract n:	M3O820160213-01
Sponsor:	
Test facility:	
Test item:	"VC1"
	· .
Study Director:	Released on: Sept 24 ¹⁴ ZolG

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COMPLIANCE WITH GOOD LABORATORY PRACTICE

I the undersigned declare that the studies described in this report have been conducted under my supervision and in compliance with the following standards of Good Laboratory Practice:

- OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring OECD principles of Good Laboratory Practice (as revised in 1997) - Environment Directorate -Organisation for Economic Co-Operation and Development, Paris 1998.
- Legislative decree n. 50 of March the 2nd, 2007. Enforcement of Community Directives 2004/9/CE e 2004/10/CE, concerning the inspection and verification of Good Laboratory Practice and the drawing of the legislative, regulatory and administrative dispositions relative to the application of Good Laboratory Practice rules, to the control of their application on the assays performed on the chemical substances (GU n.86 of April the 13th, 2007).
- United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Federal Register 22 December 1978, and subsequent amendments.
- Certification N. 038/2013 released by the Italian Ministry of Health on November 19th 2013 and Provisional Certificate released on November 20th 2015 authorizing perform analyses in compliance with the principles of good laporatory practices

There were no circumstances that may affected the quality or integrity of the study.

Study Director

Sept. 2414 2016



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QUALITY ASSURANCE STATEMENT

The study was assessed for compliance with the approved study program and the Standard Operating Procedures of Eurofins Biolab S.r.l.

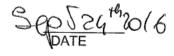
The study and/or the test facility were periodically inspected by the Quality Assurance unit according to the corresponding SOPs. These inspections and audit were carried out by the Quality Assurance unit, personnel independent of staff involved in the study.

The undersigned hereby certifies the dates on which the inspections have been carried out and reported to the Director of the Study and to

QAU INSPECTIONS		
PHASE	DATE	
Experimentation:		
-Audit process-based		
Validation of analytical methods (GC)	October 20 th - 23 th 2015	
Assay determination according to validated method (GC)	June, 07 th 2016	
-Audit study-based	//	
Documentation:		
- Study program	September, 24 th 2016	
- Raw data	September, 24 th 2016	
- Final report	September, 24 th 2016	

This report accurately reflects the raw data.







SUMMARY

The aim of the study was to validate a GC/MS method for the identification and the quantification of the impurity (-)-Nicotine in the test item "VC1 validation" and then test this analyte in 5 different production batches of the test item "VC1".

The GC/MS-SIM Mode method for the quantification of the impurity was validated according to SANCO3030/99 Rev.4 and the ECHA-14-G-10-EN and the following parameters were investigated:

- Specificity and Selectivity:
- Linearity;
- Accuracy;
- Precision and Repeatability;
- LOQ

The limit content for this impurity is provided as 0.1 mg/L (100 ppb), while the value expected from the preparation of the batches was 10-50 μ g/L (ppb).

For this reason, the study was set trying a range able to contain these values (40 ppb – 200 ppb).

The method for the (-)-Nicotine determination was implemented with an GC/MS - SCAN analysis in order to correctly identify the peak of analyte in the sample.

A sensitive and precise gas chromatography (GC-MS) technique was applied for determination of (-)-Nicotine. The calibration curve was found to be linear (r = 0.9953) over the concentration range of 0.023-0.114 μ g/ml (corresponding to 0.046-0.228 μ g/ml of sample). The preparations were dissolved in 2-Propanol. The samples for accuracy were prepared at 0.021, 0.053 and 0.106 μ g/ml of (-)-Nicotine reference standard having known purity, representing low, middle, and high controls, respectively. Mean percentage (%) recovery \pm relative standard deviation % (RSD%) ranged from 94.78 \pm 3.47, 96.25 \pm 0.03, to 89.02 \pm 0.24. Within-day precision and instrumental repeatability were also in acceptable range: 13.8% and 1.3% respectively.

The reported method for the estimation of (-)-Nicotine proved to be specific, linear, precise, repeatable and accurate.

With this validated method were then tested 5 different production batches of the test item "VC1".

"Results" section reports the values obtained in detailed tables.

INTRODUCTION	_		
On behalf of	a study aimed to val	lidate a GC-MS method	d for the identification
and quantification of the impurity (-)-Nico			
Subsequently, with this validated method		e test item production "	VC1" were tested.
The study was conducted at the Test Fa	acility	· ·	,
EXPERIMENTATION	START	END	RESEARCHER
GC/MS method for the quantification	September 20 th ,	September 23 rd ,	
of Nicotine and five batches analysis	2016	2016	

BIBLIOGRAPHY

- Guidance on the Biocidal Products Regulation: Volume I: Identity/physico-chemical properties/analytical methodology - Part A: Information Requirements - Reference: ECHA-14-G-10-EN - Publ. date: November 2014.
- SANCO/3030/99 rev. 4: Technical material and preparation: Guidance for generating and reporting methods of analysis in support of pre and post registration data requirements for Annex II (part A. section 4) and Annex III (part A. section 5) of Directive 91/414.





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The study program with possible amendments, raw data with possible deviations and a copy of the final report with possible revisions, will be stored in archives for a period of 10 years starting from the end of the study.

At the end of the study residual sample will be kept in the fridge until December 15th, 2016, the expiry date provided by the Sponsor.

The Sponsor, drawing up of a suitable contract, may request an extension of the conservation of all or part of the documents/products for a further period or their restitution.

PROCEDURES

All procedures used during this study are recorded in the

TEST ITEM IDENTIFICATION

NAME:

VC1

NATURE OF TEST SUBSTANCE:

Pesticide/Agrochemical

APPLICATION AREA:

Microbial pest control agent

STABILITY:

6 months

STORAGE:

Freezer (-18°C) and protected from light

SAMPLE DISPOSAL:

Not hazardous. The substances (plant extract) are non-toxic, non-radioactive, non-infectious presents no risks to human or animal health or to

environment.

COMPOSITION DECLARED BY THE SPONSOR:

COMPONENTS	% (w/w)
extract of tobacco plants	N.A.
dibasic sodium phosphate dodecahydrate	3
monobasic potassium phosphate	0.08
sodium sulphite	0.2
PepMV VC1	0.001-0.005

At pH 7.7 +/- 0.5

ANALYTE

NAME:

Nicotine

IUPAC NAME:

3-[(2S)-1-methylpyrrolidin-2-yl]pyridine

CAS:

54-11-5

MOLAR MASS: FORMULA:

162 g mol⁻¹

C₁₀H₁₄N₂

STRUCTURE:

MAIN HAZARDS:

danger

RISK CODES:

H300, H310, H400, H410



ANALYZED SAMPLE FOR VALIDATION

The sample, representative of the test item, is a frozen lightly green liquid contained in a 50 ml conical centrifuge plastic tube with a screw orange cup (4 tubes). The liquid consists of mild isolates of pepino mosaic virus (PepMV).

Name	VC-1 VALIDATION		
Batch number	F		
Manufacturing date	June 15 th , 2016		
Expiry date	December 15 th , 2016		
Receiving	EUITVI-82155		
Date	Sept 02 th , 2016		
#ID	ACE-2016-00123816		

ANALYZED SAMPLE FOR 5 BATCHES ANALYSIS

The samples, representative of the each production batch of test item, is a frozen lightly green liquid contained in a 15 ml conical centrifuge plastic tube with a screw blue cup (1 tube for each batch). The liquid consists of mild isolates of pepino mosaic virus (PepMV).

1.

VC-1		
A		
June 15 th , 2016		
December 15 th , 2016		
EUITVI-82155		
Sept 02 th , 2016		
ACE-2016-00123818		
	A June 15 th , 2016 December 15 th , 2016 EUITVI-82155 Sept 02 th , 2016	

2.

Name	VC-1		
Batch number	В		
Manufacturing date	June 15 th , 2016		
Expiry date	December 15 th , 2016		
Receiving	EUITVI-82155		
Date	Sept 02 th , 2016		
#ID	ACE-2016-00123819		

3.

Name	VC-1		
Batch number	С		
Manufacturing date	June 15 th , 2016		
Expiry date	December 15 th , 2016		
Receiving	EUITVI-82155		
Date	Sept 02 th , 2016		
#ID	ACE-2016-00134913		

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	•		

Name	VC-1		
Batch number	D		
Manufacturing date	June 15 th , 2016		
Expiry date	December 15 th , 2016		
Receiving	EUITVI-82155		
Date	Sept 02 th , 2016		
#ID	ACE-2016-00134914		

5.

Name	VC-1
Batch number	E
Manufacturing date	June 15 th , 2016
Expiry date	December 15 th , 2016
Receiving	EUITVI-82155
Date	Sept 02 th , 2016
#ID	ACE-2016-00134915

The test item and the information concerning its was provided by the Sponsor. All data related to the test item are under the responsibility of the Sponsor and have not been verified by the Test Facility.

REFERENCE STANDARD

The (-)-Nicotine reference standard consists of a liquid contained into an amber bottle.

Name	(-)-Nicotine Pestanal (See Annex#1)	
Ref. Article	Sigma-Aldrich (Supelco)	
Ref No	36733	
USP Batch	SZBE205XV	
Assay (% w/w)	99.1	
Expire date	Sept. 02 nd , 2019	



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Experimentation S-2016-03209 AM: GC/MS method for the quantification of Nicotine and five batches analysis

Before starting with the validation activity a set up method, in a not-GLP session, was performed in order to find a suitable method for the quantification of the impurity (-)-Nicotine in the test item VC-1 The optimized method was subsequently validated according to SANCO3030/99 Rev.4 guidelines and to the Guidance on the Biocidal Products Regulation ECHA-14-G-10-EN.

INFORMATION

Nicotine is a hygroscopic, oily liquid that is readily soluble in alcohol, ether or light petroleum. It is miscible with water in its base form between 60 °C and 210 °C. As a nitrogenous base, Nicotine forms salts with acids that are usually solid and water-soluble.

Nicotine is readily volatile (vapor pressure 5.5 Pa at 25°C) and dibasic ($K_{b1} = 1 \times 10^{-6}$, $K_{b2} = 1 \times 10^{-11}$).

Nicotine is optically active, having two enantiomeric forms. The naturally occurring form of Nicotine is levorotatory with a specific rotation of $[\alpha]_D = -166.4^{\circ}$ ((-)-Nicotine). The dextrorotatory form, (+)-Nicotine is physiologically less active than (-)-Nicotine. (-)-Nicotine is more toxic than (+)-Nicotine. The salts of (+)-Nicotine are usually dextrorotatory. The hydrochloride and sulphate salts become optically inactive if heated in a closed vessel above 180 °C.

On exposure to ultraviolet light or various oxidizing agents, Nicotine is converted to Nicotine oxide, Nicotinic acid (vitamin B3), and Methylamine.

EXPERIMENTAL PROCEDURE - VALIDATION

TEST METHOD

Gas chromatography with mass detector (GC/MS)

Parameters under investigation

Specificity/Selectivity

As part of the validation of the method, it was necessary to confirm the identity of the compound and provide that there were no relevant interferences, as required by SANCO/3030/99 rev. 4 guidelines.

For this reason, initially the solvent, the reference standard and the test sample were injected with GC/MS – SCAN Mode.

Specificity represent the capability of the method to estimate unequivocally the analyte in presence of the other components of the final product. In order to demonstrate the specificity of the method, the following solutions were separately injected into the chromatographic system:

- 1) Blank (Solvent)
- 2) (-)-Nicotine reference standard
- 3) 'VC1 validation' test sample

The specificity of the method was confirmed during the method validation.

A GC/MS-SCAN Mode confirmatory technique was used to demonstrate the method selectivity.

Linearity

Linearity refers to the ability of a detection system to produce an acceptable correlation between the instrumental response and the concentration of the analyte in the sample. The linearity of the method was assessed on the standard solutions at 40% (LOQ), 60%, 80%, 100% and 200% respectability of the limit concentration, equal to 100 μ g/L. The concentrations of analyte was plotted against area. The linear regression coefficient, the slope and the intercept of the line fitting the data were calculated, along with the confidence interval at 95% for the intercept.

Accuracy

Accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted as a conventional true value and the value found with the method applied.

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Two reconstituted samples (two for each level) were prepared at the concentrations corresponding to 40% (LOQ), 100% and 200% of theoretical value.

Recovery was calculated for each level and it was also determined the confidence interval of the global recovery. The accuracy was reported as mean recovery ± relative standard deviation.

Precision

Precision of an analytical procedure refers to the closeness of agreement between mutually independent test results obtained with the same method on identical test item in the same laboratory by the same operator using the same equipment, within short intervals of time. Precision was obtained performing the assay determination of 6 samples and is expressed as RSD% of the test results.

Repeatability

Repeatability expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Repeatability was obtained injecting a sample 6 times and is expressed as RSD% of the test results.

LOQ

The limit of quantitation (LOQ) is set at least 10 times above the blank value (expressed as Signal to Noise S/N), thus presenting a greater probability that a value at the LOQ is "real" and not just a random fluctuation of the blank reading. The LOQ is defined as the concentration at which all acceptance criteria indicated in table 1 of this study are met. The LOQ is the lowest validated level.

Acceptability criteria

The acceptability criteria for all the above-described parameters, according to SANCO/3030/99 rev. 4 and ECHA-14-G-10-EN guidelines, are summarized in the following table:

PARAMETERS	Measurement Unit	Acceptability criterion
SPECIFICITY	Chromatograms verification	No peak of blank or interferes with that of each analyte. Any interference < 3 % can be neglected.
	R	>0.99
LINEARITY	Confidence limits at 95% of intercept	contain the zero
ACCURACY	% Recovery	75-125% (% impurity 'nominal' < 0.1)
ACCONACT	Confidence range:	μ = Xaverage ± t (s/n ^½) \rightarrow contain the 100%
_		Horwitz: $RSD_R\% = 2^{[1 - 0.5 \log(C)]}$;
PRECISION	RSD% (*)	C is the analyte concentration as a fraction. In this case C = 0.00000010 for Nicotine (100 ppb).
		RSD% ≤ Horwitz x 0.67 = 15.2
LOQ	% of analyte	The peak of Nicotine in the lowest solution will be a S/N ≥ 10. This concentration falls in the linearity and accuracy range.





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(*) = The acceptability of the value of RSD%, resulting from the precision, is based upon the Horwitz equation, an exponential correlation between the relative standard deviation (RSD $_{R}$) and the concentration (C) of the analyte expressed as fraction, regardless of the analyte nature, of the matrix and of the method of measurement employed:

Horwitz equation: % RSD_R = $2^{(1-0.5logC)}$ = 22.63, considering C equal to 0.00000010

The modify Horwitz values, shown below, is used as reference in accordance with the indications of the SANCO guideline.

 $% RSD_r = % RSD_R x 0.67 = 15.2$

Precision; RSD% ≤ RSD_r%

Analytical sequence

The analytical sequences for the quantification of the active ingredient in the precision and accuracy tests were characterized by:

- injections of two standard solutions (STD1 and STD2) containing analyte at 100% in order to evaluate the suitability of reference standard solutions to be used for the quantification;
- 6 injections of one standard solution (STD1) in order to evaluate the repeatability of the chromatographic system;
- injections of one standard solution containing analyte at 100% at the end of the analytical sequence (STD1check) in order to evaluate the suitability of complete analytical sequence;
- injections of six different sample solutions (S1, S2...S6) in order to evaluate the precision of the method.
- 6 injections of one sample solution (S1) in order to evaluate the repeatability of the method in presence of the matrix.
- injections of two reconstituted sample solution (REC SAMPLE1 and REC SAMPLE2) for each accuracy level (50%, 100% and 150%) in order to evaluate the suitability of recovery values.

Analytical acceptability criteria

The analytical acceptability criteria, according to instrumental characteristic, are summarized in the following table:

Parameters	Measurement Unit	Acceptability criteria
System suitability of standard solutions	% agreement between response factors (Fr) of standard solutions	% agreement STD1 vs STD2 = [Ass (Fr _{STD1} - Fr _{STD2}) / average (Fr _{STD1} and Fr _{STD2})x100] ≤ 5% % agreement STD1 vs STD1check = [Ass (Fr _{STD1} - Fr _{STD1check}) / average (Fr _{STD1} and Fr _{STD1check})x100] ≤ 5%
System suitability of enriched solutions	% Agreement between Recovery % values	%agreement REC SAMPLE 1 vs REC SAMPLE 2 = [Ass(%recovery recs1 – %recovery recs2) / average (%recovery recs1 and %recovery recs2)x100] ≤ 5%

The analytical sessions were considered acceptable because the criteria of the system suitability test, reported in the table above, proved to be satisfied.

Fauinment

- Gas chromatograph HP 7820 A split/splitless injector, provided with MSD 5977E detector
- Capillary column Restek, Rtx®-5 Amine (30 m, 0.25 mm ID, 1.0 µm film thickness) cat. 12353 Serial 993086
- Standard laboratory equipment

The GC instrument used is qualified every year according internal procedure.



Reagent

2-propanol - IPA (Sigma-Aldrich, code 190764, lot n. BCBR0008V)

All standard and reagents are of high purity analytical grade. The validity of each product was checked before starting the analyses.

Working solutions preparation

Specificity

<u>Blank</u>

2-Propanol.

(-)-Nicotine reference standard - mother solution (conc. ~ 2 mg/ml)

About 100 mg of (-)-Nicotine reference standard were quantitatively weighed into a 50 ml volumetric flask and brought to volume with 2-Propanol (Solution A).

(-)-Nicotine reference standard - intermediate solution (conc. ~ 0.01 mg/ml)

100 µl of (-)-Nicotine reference standard mother solution were quantitatively transferred into a 20 ml volumetric flask and brought to volume with 2-Propanol (Solution B).

(-)-Nicotine reference standard - LOQ solution (conc. ~ 0.02 µg/ml)

40 μl of (-)-Nicotine reference standard (Solution B) were quantitatively transferred into a 20 ml volumetric flask and brought to volume with 2-Propanol.

Test sample

About 1.0 ml of test sample was quantitatively weighed into a 2.0 ml volumetric flask and brought to volume with 2-Propanol.

Linearity

(-)-Nicotine reference standard - mother solution (conc. ~ 2 mg/ml)

About 100 mg of (-)-Nicotine reference standard were quantitatively weighed into a 50 ml volumetric flask and brought to volume with 2-Propanol (Solution A).

(-)-Nicotine reference standard - intermediate solution (conc. ~ 0.01 mg/ml)

100 µl of (-)-Nicotine reference standard mother solution were quantitatively transferred into a 20 ml volumetric flask and brought to volume with 2-Propanol (Solution B).

Calibration:

Level 40% - 0.04 µg/ml of sample	40 µl of Nicotine mother solution B	to 20 ml with 2-Propanol
Level 60% - 0.06 µg/ml of sample	60 µl of Nicotine mother solution B	to 20 ml with 2-Propanol
Level 80% - 0.08 µg/ml of sample	80 μl of Nicotine mother solution B	to 20 ml with 2-Propanol
Level 100% - 0.1 µg/ml of sample	100 µl of Nicotine mother solution B	to 20 ml with 2-Propanol
Level 200% - 0.2 µg/ml of sample	200 µl of Nicotine mother solution B	to 20 ml with 2-Propanol

Precision/Repeatability

Sample preparation (six preparations)

About 1.0 ml of test sample was quantitatively weighed into a 2.0 ml volumetric flask and brought to volume with 2-Propanol.

The sample must be thawed slowly in refrigerator (5 ± 3 °C) and prepared just before the injections.





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Precision was obtained performing the assay determination of these 6 samples and is expressed as RSD% of the test results.

Repeatability was obtained injecting the first sample 6 times and is expressed as RSD% of the test results.

Accuracy

(-)-Nicotine reference standard - mother solution (conc. ~ 2 mg/ml)

About 100 mg of (-)-Nicotine reference standard were quantitatively weighed into a 50 ml volumetric flask and brought to volume with 2-Propanol (Solution A).

(-)-Nicotine reference standard - intermediate solution (conc. ~ 0.01 mg/ml)

100 µl of (-)-Nicotine reference standard mother solution were quantitatively transferred into a 20 ml volumetric flask and brought to volume with 2-Propanol (Solution B).

Calibration:

STD - Level 1 (40%)	40 μl of Nicotine mother solution B	to 20 ml with 2-Propanol
STD - Level 2 (60%)	60 μl of Nicotine mother solution B	to 20 ml with 2-Propanol
STD - Level 3 (80%)	80 μl of Nicotine mother solution B	to 20 ml with 2-Propanol
STD - Level 4 (100%)	100 µl of Nicotine mother solution B	to 20 ml with 2-Propanol
STD - Level 5 (200%)	200 µl of Nicotine mother solution B	to 20 ml with 2-Propanol

Enriched sample 40% (two preparations)

1.0 ml of test sample was quantitatively weighed into a 2.0 ml volumetric flask. 1.0 ml of Reference standard - Level 3 was quantitatively transferred and the solution was brought to volume with 2-Propanol.

Enriched sample 100% (two preparations)

1.0 ml of test sample was quantitatively weighed into a 2.0 ml volumetric flask. 1.0 ml of Reference standard - Level 5 was quantitatively transferred and the solution was brought to volume with 2-Propanol.

Enriched sample 200% (two preparations)

- 1.0 ml of test sample was quantitatively weighed into a 2.0 ml volumetric flask. 20 µl of Reference standard
- Solution B were quantitatively transferred and the solution was brought to volume with 2-Propanol.

LOQ

The LOQ is the analyte concentration with S/N ratio of at least 10 and was verified on the working standard solutions at 0.02 µg/ml.

To conduct excellently this delicate parameter, in addition to verify that this level was in the linearity range and met the accuracy in presence of matrix, we were carried out three other preparations starting from different mothers to measure the reproducibility of its response.

(-)-Nicotine reference standard - mother solution (conc. ~ 2 mg/ml) - Three preparations

About 100 mg of (-)-Nicotine reference standard were quantitatively weighed into a 50 ml volumetric flask and brought to volume with 2-Propanol (Solution A).

(-)-Nicotine reference standard - intermediate solution (conc. ~ 0.01 mg/ml) - Three preparations

100 µl of (-)-Nicotine reference standard mother solution were quantitatively transferred into a 20 ml volumetric flask and brought to volume with 2-Propanol (Solution B).





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(-)-Nicotine reference standard - LOQ solution (conc. $\sim 0.02~\mu g/ml$) - Three preparations 40 μl of (-)-Nicotine reference standard - solution B were quantitatively transferred into a 20 ml volumetric flask and brought to volume with 2-Propanol.

Five batches analysis

As required by Guidance on Regulation (EU) No 528/2012 guidelines, it was necessary to determine the analytical profile of five representative production batches of test item.

For this reason, five production batches were analysed, confirming the repeatability of the production process.

Sample preparation

About 1.0 ml of test sample was quantitatively weighed into a 2.0 ml volumetric flask and brought to volume with 2-Propanol.

The sample must be thawed slowly in refrigerator (5 ± 3 °C) and prepared just before the injections.

GC-MS METHOD - SIM MODE

According to good mass spectrometric analysis, a minimum of 3 ions (ideally with an m/z ratio of >100) must be used for identification/quantification.

Instrumentation	GC-MS
Column	Rtx®-5 Amine, 30m x 0.25mm x 1.0 μm
Detector (Aux 2)	MSD, 280°C
Source	230°C
Quadrupole	150°C
MS mode	SIM (84 m/z, 161 m/z, 162 m/z)
Threshold	100
Flow	Helium, 1.1 ml/min
GC oven program	100°C for 0 min, rate 20°C/min to 200°C for 0 min, rate 35°C/min to 300°C for 5 min.
Run Time	12.86 min
Injector temperature	290°C
Injection volume	1 μl - Split 1:2
Solvent Delay	4.00 min
RETENTION TIME	(-)-Nicotine ~ 6.2 min



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CALCULATIONS

The content of analyte was calculated as follows:

$$C_{ST} = \frac{W_{ST} \times T_{ST}}{D_{ST} \times 1000}$$
;

Where:

C_{ST} = standard concentration (mg/ml)

W_{ST} = standard weight (g)

D_{ST} = dilution of analyte in working standard solution (ml)

 T_{ST} = standard assay (% w/w)

A calibration line was obtained after injections of diluted reference standard solutions at the concentrations of 0.02, 0.03, 0.04, 0.05 and 0.1 μ g/ml (respectively 0.04, 0.06, 0.08, 0.1 and 0.2 μ g/ml of sample) : Y = a X + b

(-)-NICOTINE (
$$\mu$$
g/ml) = [(Y - b)/a] x D_C

Where:

Y = A_{ST} = Area of the analyte in working standard solution

 $X = C_{ST} = Conc.$ (µg/ml) of the analyte in working standard solution

A_{ST} = analyte area in the working standard solution (pA*s)

a = slope

b = intercept

(-)-NICOTINE (%
$$\mu g/\mu g$$
) = $U(Y - b)/aJ \times D_C$
 W_C

Where:

 $D_C = \text{sample dilution (ml)}$

W_C = sample weight (μg)

The quantifications are made on the 84 m/z fragment, being the most abundant. The other two fragmentation ions are measured as verification of the abundances.





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EXPERIMENTAL PROCEDURE - GC/MS CONFIRMATORY TECHNIQUE

TEST METHOD - GC/MS

Gas chromatography with mass detector (GC/MS)

Equipment

- Gas chromatograph HP 7820 A split/splitless injector, provided with MSD 5977E detector
- Capillary column Restek, Rtx®-5 Amine (30 m, 0.25 mm ID, 1.0 μm film thickness) cat. 12353 Serial 993086
- Standard laboratory equipment

The GC instrument used is qualified every year according internal procedure.

Analysis

The method was implemented with an analysis GC/MS in order to correctly identify the peaks of the analyte in the sample and in the raw material.

For this reason the standard solution (Solution B) and the test sample preparations were initially injected with GC/MS technique - SCAN mode, then the analysis was carried out and validate in SIM mode (after selection of the characteristic fragments).

Working solutions preparation

Blank

2-Propanol.

(-)-Nicotine reference standard - mother solution (conc. ~ 2 mg/ml)

About 100 mg of (-)-Nicotine reference standard were quantitatively weighed into a 50 ml volumetric flask and brought to volume with 2-Propanol (Solution A).

(-)-Nicotine reference standard - intermediate solution (conc. ~ 0.01 mg/ml)

100 µl of (-)-Nicotine reference standard mother solution were quantitatively transferred into a 20 ml volumetric flask and brought to volume with 2-Propanol (Solution B).

Test sample

About 1.0 ml of test sample was quantitatively weighed into a 2.0 ml volumetric flask and brought to volume with 2-Propanol.

These solutions were injected using the following GC/MS method.

Instrumentation	GC-MS
Column	Rtx®-5 Amine, 30m x 0.25mm x 1.0 μm
Detector (Aux 2)	MSD, 280°C
Source	230°C
Quadrupole	150°C
MS mode	SCAN (30-200 amu)
Threshold	100
Flow	Helium, 1.1 ml/min
GC oven program	100°C for 0 min, rate 20°C/min to 200°C for 0 min, rate 35°C/min to 300°C for 5 min.
Injector temperature	290°C





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Injection volume	1 µl - Split 1:2
Solvent delay	4.00 min

The following parameters were evaluated:

1) characteristic MS ions: characteristic MS ions were extrapolated by mass spectra.

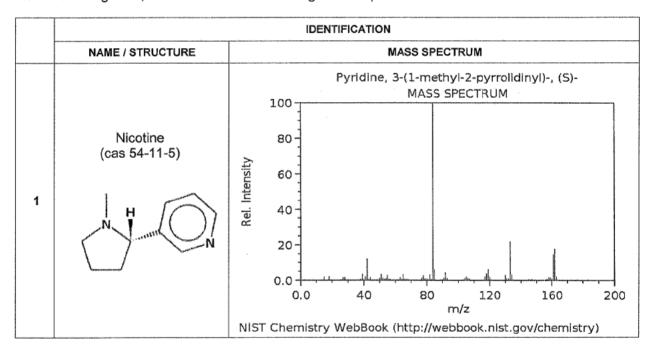
2) identification: identity of each peak were identified by mass spectra analyses and interpretation of the MS spectra performed by using the NIST/EPA/NIH (version-11.0).

3) Probability %: the correspondence between the mass spectra obtained by analysis and those contained in the NIST/EPA/NIH Library version-11.0 is expressed through the parameter "probability %." This value indicates how the unknown substance is correctly identified from the reference library. Values greater than 90% indicate a good correlation, while values below 50% indicate that there is a substantial difference between the compound analysed and the reference library. Differences of ± 5% in the values of probability are not considered significant.

According to the conditions previously described, the peaks belonging to (-)-Nicotine was clearly visible. The recognition by the GC/MS library has allowed to characterize this principal peak present in the solutions. This peak were under mentioned. In the range time (4.0 min) the solvent (2-propanol) fell and to avoid mass source damage the solvent delay was set.

For this reason, in this zone, other possible compounds were not recognized.

In the following table, identifications of the investigated compound is summarized.



	Retention Time	Selected lons ^a			
Group	(min)	1	2	3	4
Nicotine	1.7	84 (100)	133 (22) ^b	161 (16)	162 (M) ^c (18)

^a Dwell times should be adjusted to produce a cycle time of about 4 scans/sec

^b Percent relative abundance with respect to the ion of highest abundance.

^c M is the Molecular Ion.



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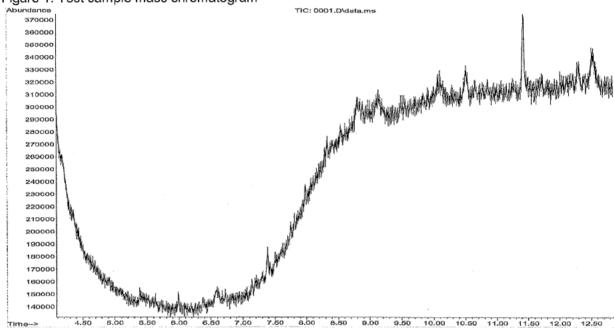
RESULTS

VALIDATION METHOD PARAMETERS

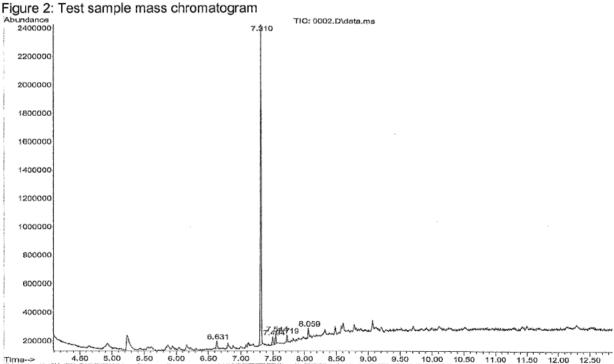
Selectivity

The method was implemented with an analysis GC/MS in order to correctly identify the peak of the analyte (-)-Nicotine in the reference standard and in the sample. The GC/MS-SCAN Mode has proved itself as confirmatory technique.

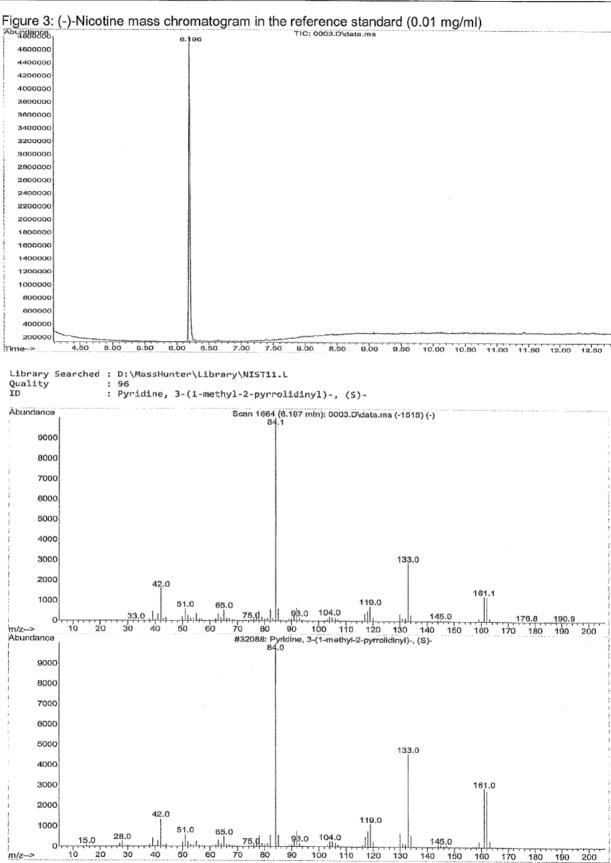












1	
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Specificity

5.00

Time-->

5.50

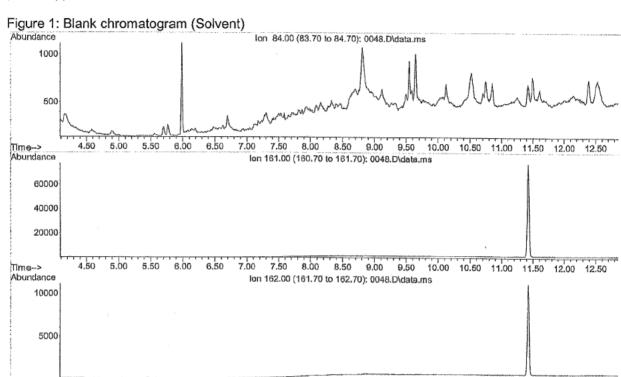
6.00

6.50

7.00

7.50

The method proved to be specific; in fact it has been verified that the test solution do not interfere with the peak of (-)-Nicotine.



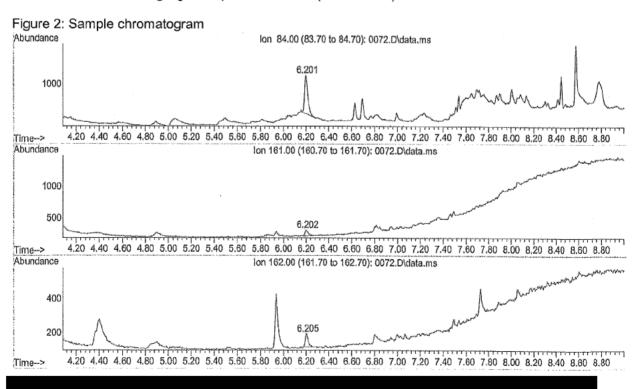
Since mass chromatogram was normalized on the major peak, a narrower time range (4.0 - 9.0 min) was selected to better highlight the peak of interest (rt. $\approx 6.2 \text{ min}$).

8.00

8.50

9.00

9,50 10.00 10.50 11.00 11.50 12.00 12.50



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Figure 3: (-)-Nicotine chromatogram (Reference standard 0.02 µg/ml - LOQ level)

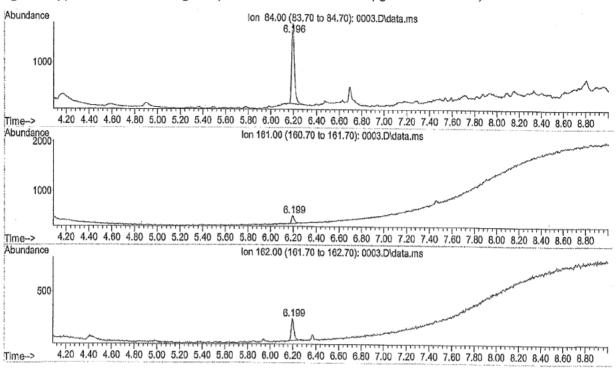
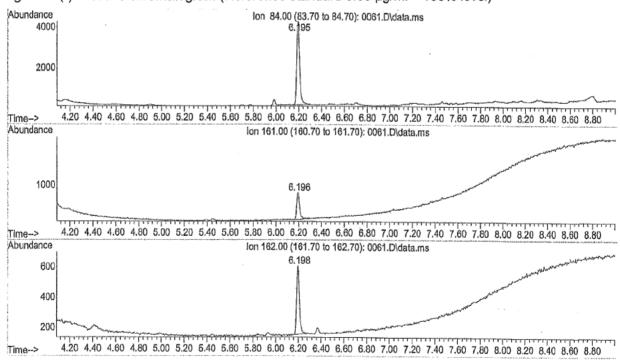
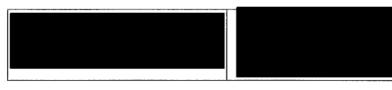


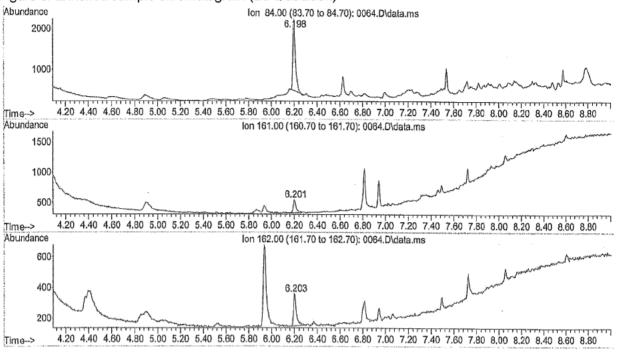
Figure 4: (-)-Nicotine chromatogram (Reference standard 0.05 μg/ml – 100% level)





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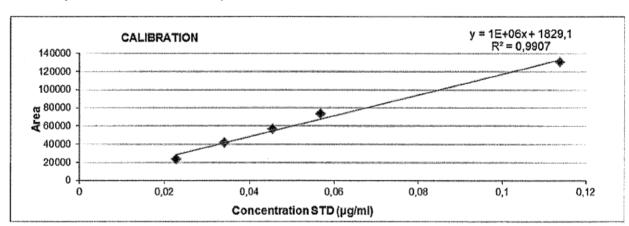




Linearity

Method Linearity was tested on the (-)-Nicotine reference standard on 5 different concentration levels from LOQ (40%) to 200% of the theoretical amount of analyte in the sample.

All acceptance criteria (R > 0.99 and/or the confidence interval at 95% for the intercept contains zero) were satisfied (see Annex#2: excel sheet).



(-)-Nicotine Linearity	
R 0.9953	
Slope	1154294.9906
Intercept	1829.0928

Confide	nce interval at 95% for	[-0.0112;0.0112]
	the intercept	



<u>Accuracy</u>

Method accuracy was tested on spiked samples prepared at three concentration levels for analyte (40% (LOQ), 100% and 200% of the theoretical amount of analyte in the sample). Two fortified samples (two for each level) were prepared (see Annex#4: excel sheet).

		(-)-Nicotine	e	
	Recovery %	Average Recovery %	95% confidence interval lower limit	95% confidence interval upper limit
Reconstituted sample at 40%	94.78			•
Reconstituted sample at 100%	96.25	93.35	89.60	97.10
Reconstituted sample at 200%	89.02			

The measured values were compared with the 'expected' value of 100% using the Student's t-test and the choice of null hypothesis was appropriate to the data set. For the t-tests the following Equation A was used:

Equation A:

$$t_{cal} = \frac{\left| \overline{x} - \mu \right|}{s / \sqrt{n}}$$

where

x = mean of test results of a sample

 μ = "true" or reference value

s = standard deviation of test results

n = number of test results of the sample.

To compare the mean of a data set with a reference value normally the "two-sided t-table of critical values" is used ($t_{cal} \le t_{tab}$). The applicable number of degrees of freedom here is:

$$df = n-1$$

The value for t calculated with Equation A did not exceed the critical value in the table, therefore the data were taken to belong to the same population: there is no difference and the "null hypothesis" is accepted (with the applicable probability of 95%).

The acceptance criterion (Recovery $_{active\ ingredient}$ = 95%-105%, % and/or the confidence interval at 95% for the recovery contains 100%) proved to be satisfied.

Precision

Method precision was proved preparing 6 different samples in the same analytic session. The RSD% of the percentage assay (% w/w) was calculated for the analyte on the test sample. Results are reported below even if they are an estimate, being the found values below the LOQ (see Annex#3: excel sheet). The experimental RSD% respects the acceptance criteria.

Analyte	Assay (% w/w)	Acceptance criteria	RSD%
(-)-Nicotine content	0.0000024	RSD% _{NICOTINE} ≤ 15.2% ^(*)	13.9

^{(*) =} The Horwitz equation is an exponential correlation between the relative standard deviation (RSD_R) and the concentration (C) of the analyte expressed as fraction, regardless of the analyte nature, of the matrix and of the method of measurement employed:



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Repeatability was obtained injecting a sample 6 times and is expressed as RSD% of the test results. Results are reported below even if they are an estimate, being the found values below the LOQ. The method proved to be repeatable (see Annex#3: excel sheet).

Analyte in the preparation S1	Assay (% w/w)	RSD%
(-)-Nicotine content	0.0000011	1.3

As you can see the sample undergoes degradation over time. Repeatability is fact was proven by injecting the first vial of precision, put on the GC sampler.

LOQ

The LOQ is defined as the concentration at which all acceptance criteria indicated in table "Acceptability criteria" (page 11 of this report) of this study are met. The LOQ is the analyte concentration at which the S/N ratio is at least 10 and corresponds to the lowest validated level

The method proved that the concentration corresponding to 0.02 µg/ml (or 0.04 µg/ml on the sample) had a signal to noise ≈ 35 and fall in the linearity and accuracy range (see Annex#6: excel sheets).

The measured precision is within the recommended values, given by Horwitz modification values.

It is then possible to derive the reproducibility standard deviation, σ_R , from the approximate form of the Horwitz equation. $\sigma_R = 0.02^* \, \text{C}^{0.8495}$ which as you can see directly puts in relation σ_R with the analyte concentration.

Next, multiplying σ_R for the coverage factor, you get the expanded uncertainty.

Before using the reproducibility standard deviation for the calculation of the expanded uncertainty, it is necessary to verify that its close repeatability standard deviation (Sr) is compatible with σ_R obtained from the Horwitz equation.

It have to check the condition $1/2 \sigma_R \le Sr \le 2/3 \sigma_R$.

You can have a better repeatability standard deviation, occurring $1/2 \sigma_R > Sr$.

In our method there is the condition in which our values are below the lower limit ($\sigma R \times 0.5$).

% w/w	(-)-Nicotine
Xmedium	0.0000024
$Ue = K * \sigma R$	Non applicable

However, since our analyte content is affected by errors, in excess or defect, we prefer to give an estimate interval that expresses this error in the equation below (obtained from 6 preparations - see Annex#5: excel sheet):

$X = Xmedium \pm t*Sr/RADQ(n)$

% w/w	(-)-Nicotine
Xmedium	0.0000024
± t*Sr/RADQ(n)	± 0.000004



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DETERMINATION OF (-)-NICOTINE CONTENT IN FIVE BATCHES OF THE TEST ITEM "VC1"

		(-)-Nicotine (*)
ACE-2016-00123818	А	0,021 µg/ml
ACE-2016-00123819	В	0,017 µg/ml
ACE-2016-00134913	С	0,017 µg/ml
ACE-2016-00134914	D	0,014 µg/ml
ACE-2016-00134915	E	0,020 µg/ml
	Average (µg/ml)	0,018
**************************************	SD (µg/ml)	0,003
	RSD%	14,4

^{(*) (-)-}Nicotine LOQ = $0.04 \mu g/ml = 0,000004 \%$ w/w, according to Validated method S-2016-03209 AM See Annex#4 for individual data and calculations.

DEVIATION

No deviation has been recorded from study program.

CONCLUSIONS

The method described in this study proved to be specific, linear, precise and repeatable and was successfully validated.

The (-)-Nicotine content in five production batches of test item was < LOQ validated value (0.04 µg/ml).

ANNEXES

ANNEX	TITLE
N.1	NICOTINE - REFERENCE STANDARD COA
N.2	LINEARITY - EXCEL SHEET
N.3	PRECISION-REPEATABILITY EXCEL SHEET
N.4	ACCURACY AND 5 BATCHES ANALYSIS — EXCEL SHEET
N.5	STATISTIC - EXCEL SHEET
N.6	LOQ - EXCEL SHEET





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ANNEX#1: NICOTINE - REFERENCE STANDARD CoA

CERTIFICATE OF ANALYSIS

Sigma-Aldrich Laborchemikalien GmbH D-30918 Seelze

Telefon: +49 5137 8238-150

Seelze, 17.09.2014/541285/14/18728

Order-No.: Customer-No.:

Order-Code:

Quantity:

Production Date: 02.Sep.2014 Expiry Date: 02.Sep.2019

Article/Product: 36733 Batch: SZBE205XV

(-)-Nicotine PESTANAL®

Reference Material (RM)

1. General Information

Formula: C10H14N2 Molar mass: 162.23 g/Mole

CAS-No.: [54-11-5] Recomm. storage temp.: roomtemp.
Usage : Insecticide

The estimated uncertainty of a single measurement of the assay can be expected to be $0.5\,\%$ relative (confidence level = 95%, n= 6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR) Assay (GC) Refractive index (n 20/D)

Refractive index (n 20/D) Date of Analysis complying 99.1 area % 1.5278 17.Sep.2014

3. Advice and Remarks

- The expiry date is based on the current knowledge and holds only for proper storage conditions in the originally closed flasks/ packages.
- Whenever the container is opened for removal of aliquout portions of the substance, the person handling the substance must assure, that the integrity of the substance is maintained and proper records of all its handlings are kept. Special care has to be taken to avoid any contamination or adultoration of the substance.
- We herewith confirm that the delivery is effected according to the technical delivery conditions agreed.
- Particular properties of the products or the suitability for a particular area of application are not assured.
- . We guarantee a proper quality within our General Conditions of Sales.

Sigma-Aldrich Laborchemikalien GmbH Quality Management SA-LC

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GLC-Method

Analytical Department

Article Article-No

: (-)-Nicotine : 36733 : SZBE205XV Batch

: SZBE205XV : SP-1701, 30m, 0,32mm i.D., 1.0µm Film : 280°C : 280°C - FID : 150°C to 250°C (10°C/min) hold 20min : 1:100 : 1ml He/min Column

Inj.-Temp. Det.-Temp. Oven-Temp.

Split Flow Inj.v. Evaluation $0,2\mu l$ uncorrected Operator : Schulz

CHROMATOGRAMM pA 350 300 250 200 150 100 10.005 19,486 50 9.192

Area Percent Report

#	Meas. Re	Height	Area	Area %
1	L 8.60	8886.7	30157.3	99.16
2	8.96	1.0	5.2	0.02
3	9.19	7.1	27.1	0.09
4	9.92	3.1	8.2	0.03
5	10.00	35.6	99.0	0.33
6	10.25	1.1	5.0	0.02
7	7 10.61	2.5	13.7	0.04
	3 13.31	5.0	19.2	0.06
9	16.56	11,1	61.8	0.20
1.0	19.49	2.3	16.1	0.05

2/3 OSA 20.99.16

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ndd 0 ψ Pulse Sequence: PROTON (s2pul) Solvent: cdcll Data collected on: Sep 15 2014 16 repetitions OBSERVE H1, 399.8818869 MHz DATA PROCESSING FidFile: 14_18728_PROTON_01 Seelze-NMR-vnmrs400 Archive directory: /home/vnmr1/vnmrsys/data Temp. 26.0 C / 299.1 K Sample #2, Operator: vnmrl Relax, delay 5.000 sec Fulse 45.0 degrees Acq, time 2.281 sec Width 7183.9 Hz Total time I min 56 sec Sample directory: 14_18728_20140915 14_18728 Data Collected on: FT Size 32768 19

#36733 Ch.:SZBE205XV

(-)-Nicotin PESTANAL Sample Name:

3/3 Od co. 99.16

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ANNEX#2: LINEARITY - EXCEL SHEET

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STD	Nicotine	0,1147	(%)	(ml) 50,0	(mg/ml) 2,2734	(m)) 0,1	(ml) 20,0	(mg/ml) 0,0114
STANDARD JOB SOLUTIONS:	1.							
STD Level	STD Volume taken	Dilution (ml)	STD Concentration (µg/ml)	(Rt= 6.20 min, m/z84)	STD Area (average)	RF ([STD]/Area)	RF (average)	Assesment % (between injections)
40% (LOQ)	40,0	20,0	0,023	23485	23359	9,68E-07 9,79E-07	9,73E-07	1,08
%09	60,0	20,0	0,034	41539	41093	8,21E-07 8,39E-07	8,30E-07	2,17
80%	80,0	20,0	0,045	55810 56316	56063	8,15E-07 8,07E-07	8,11E-07	06'0
100%	100,0	20,0	0,067	71429	73007	7,96E-07 7,62E-07	7,79E-07	4,32
200%	200,0	20,0	0,114	129271	130519	8,79E-07	8,71E-07	1,91
					A A PLACE THE STREET IN LAST THE THE STREET PROPERTY OF THE STREET OF TH		8,53E-07 8.8	Average RSD%
	CALIBRATION		$y = 1E+06x+1829,1$ $R^2 = 0,9907$	29,1			and the state of t	
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0,114	130519,0	0,111	98,1	(Brl)		\		AND A STATE OF THE PROPERTY OF
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SSreg	0,01	0,000047	SSread		•		The same state of the same sta	
		3,182	t 95% (n-2 degrees of freedom)					
	Confidence limit b (95%)	it b (95%)		0	0,02 0,04	90'0	0,1	
	-	0.544.0	_					

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ANNEX#3: PRECISION-REPEATABILITY - EXCEL SHEET

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		21-	Sep-16	THE RESIDENCE OF THE PROPERTY	THE RESERVE THE PROPERTY OF TH		742-742-1711-1712-1713-1713-1713-1713-1713-171	
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370.1	0.1	20.0	0,011		The second secon			
STD 2	0,1	20,0	0,011					
STANDARD JOB SOLUTIONS:								
C. C.	Sampling	Dilution	STD Concentration	STD Area	STD Area	RF	뀸	Assesment %
SIDLEVE	(rd)	(mi)	(ju/Brl)	(Rt= 6.20 min, m/z 84)	(average)	([STD]/Area)	(average)	(between injections)
Level 40%	40.0	20.0	0.023	23485	23358,5	9,68E-07	9,73E-07	1,08
				23232		9,735-07		
Level 60%	0.09	20,0	0,034	41539	41092,5	8,21E-07	8,30E-07	2,17
Level 80%	80.0	20,0	0,045	55810	56063,0	8,15E-07	8,11E-07	06'0
				25,420		0,0/E-0/		
Level 100%	100,0	20,0	0,057	74585	73007.0	7,62E-07	7,79E-07	4,32
Level 200%	200,0	20,0	0,114	129271	130519,0	8,79E-07 8,63E-07	8,63E-07	1,93
							8,51E-07	Average
Slope	1.					***	8,8	RSD%
Intercept						and the same of th		
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PRECISION				OIN	Nicotine Estimation Value	ne en		
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1	1,0163	15376	7	0,023	0,0000023		The second section is a made of a set of the second declaration in the second by the state of the second section is	
8	1,0129	13909	7	0,021	0,000021	-		
6	1,0157	18621	2.0	0,029	0,0000029	LOQ (0.04 uq/ml)		
च्ये :	1,0169	16325	·	0,025	0,0000025)	The state of the s	
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STD 1 check	100.0	20.0	0.057	73272	7,76E-07	7.76E-07	0.19	0.31
				13131	/,//E-0/			

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			THE RESERVE THE RESERVE THE PROPERTY OF THE PR		AAA James Aaa			1
REPEATABILITY			· reter	Nice	Nicotine Estimation Value	er		
Sample preparations	Weight	Area	Sample Diluition Volume	Nicotine	Nicotine			
	(a)	(Rt=6.20 min, m/z 84)	(ml)	conc. (µg/ml)	%		THE PROPERTY OF THE PROPERTY O	
1		8568		0,012	0,00000115		I SELECTE AND PRODUCTION AND AND ADDRESS OF AN ADDRESS IN cast to the other whomever many as an assessment or a	
2		8552		0,012	0,00000115		THE PROPERTY OF THE PROPERTY O	
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4	200	8683	2,0	0,012	0,00000117	\ LOG (0,04 ug/m)	N IN PRINCEN OF THE SPORTSCHARMING, BARRIES, BARRIES, IN LA AAALAN,	
c)		8527		0,012	0,00000114		NATIONAL STATEMENT OF THE PROPERTY OF THE PROP	THE RESERVE THE PROPERTY OF TH
ဖ		8510		0,012	0,00000114		THE REPORT OF THE PROPERTY OF	
Madded William of Madded and Associated Asso		8541		0,012	0,0000011	Average		
		1,1		1,3	1,3	RSD%	THE PROPERTY OF THE PROPERTY O	
STANDARD SOLUTION 2				Committee of the Spiritual Control of the Advisor of the Control o				
	Sampling	Volumetric flask	Conc.	Area	ı	ü	Assessment in	Accessment
Standard Solution	(h)	(m)	(mg/mf)	(Rt= 6.20 min, m/z 84)	(Conc/Area)	(Average)	(%)	STD1 - STD2
STD 3	0,000	0.00	0.053	69578	7,66E-07	7 0.1		
2	0,001	Λ'ΛΖ	CCO,C	69297	7 AGE 07	/,6/E-U/	0,40	1,52

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ANNEX#4: ACCURACY AND 5 BATCHES ANALYSIS - EXCEL SHEET

Provincial Control C	S-2016-03209AM		8-2	016-03209AM	And the second s							
	SAMETER TO VALIDATE		4	COURACY				ar maara			:	:
Control Cont	DUCT			VC-1			, i					
Weight District Weight District Di	NDARD MOTHER SOLUTIONS											
Column C	Reference	Weigh	Assay	Dilution	Conc. 1							
Sampling Sampling Distance Cont. Con	Nicotine	0,1066	1,00	50,	2,113				THE RESERVE THE VALUE OF THE SECOND S			
Simplified Sim		201.00	999	8	2,239							
District	Reference	Sampling	Dilution	Conc. 2	The state of the s	n contraction of		793111111111111111111111111111111111111				
Column C	STD 1	0,1	20,0	0,011								:
Column C	STD2	0,1	20,0	0,011	And the state of t				THE THE RESIDENCE OF THE PARTY	The state of the s		
Sampling Dialoca Sign December Sign De	DARD JOB SOLUTIONS:	The state of the s	3-10-10-10-10-10-10-10-10-10-10-10-10-10-	A STATE OF THE STA	S to Manufacture a contraction of the contraction o				F 87-1 1 111 111 111 111 111 111 111 111 11			
100 100	STD Level	Sampling (u)	Dilution (m)	STD Concentration (µg/m)	STD Area (Rt= 6.20 min, m/z 84)	STD Area	RETURN THE	(Average)	Assesment %			
1,000 20	Level 40%	40,0	20,0	0,021	20726	20993,0	1,02E-06	1,01E-06	2,64			:
Part	Level 60%	0,09	20.0	0,032	37512	37611,0	8,45E-07	8,43E-07	0,63			
100,0 200 200 0.0039 0.0130 0.0120	Level 80%	80,0	20,0	0.042	51816	52510,5	8,16E-07	8,05E-07	2,65			
2000 200 200 200 130046 110054 110	Level 100%	100,0	20,0	0.053	67100	67612,5	7,87E-07	7,81E-07	1,52			414-41-41-41-41
Silope 11300003,8079 Bildere Silope Si	Level 200%	200,0	20,0	0,108	120546	119413,5	8,765-07	8,93E-07	1,88			
Intercept 1906,05825 Intercept Interce	edols								Average RSD%			
Fig. 0,5877 Conc.	Intercept Correlation (R)		The state of the s	The second secon				-		: :	:	
Second Sample Volumetic flack Conc. Area Fr. Fr. Asso sens crit. Measure Mea	, E		To provide the second s									
Sampling Volumetric flask Cone. Alea Cone. Alea Cone. Alea Cone. C	To all the second secon											
100 (iii) (iiii) (iii) (iii) (iii) (iii) (iii) (iii) (iii)	Standard Solution	Sampling	Volumetric flask	Cone	Area	ů.	ů	Association in				
100,0 20,0 0.0659 0.06	Semidard Solution	(h)	(ml)	(ing/m)	(Rt= 6.20 min, m/z 84)	(Conc/Area)	(Average)	(%)				
Ports Rectric Area Correct Area Analyte Assay Recovery/Level Assassment % one, (parm) Recovery/Level Resonery/Level Assassment % one, (parm) Recovery/Level Resonery/Level Assassment % one, (parm) Recovery/Level Resonery/Level Resonery/Level Resonery/Level Resoner (parm) Resonery/Level Reso	STD 1 check	100,0	20,0	0.053	68685	8,04E-07 7,69E-07	7,87E-07	4,49	69'0			
Pack+1	4.5							And the second s	A STATE OF THE PARTY OF THE PAR			
tione Weight Theoretical conc. Sample Dilutition Volume Area Correct Area Analyte Assay Recovery Recovery Recovery (%) (average) (certain file) (RP-6.20 mt, mz. 8-1) (average) (certain file) (RP-6.20 mt, mz. 8-1) (average) (certain file) (certai	to subtract (Area)	8541	The state of the s									
Weight Theoretical conc. Sample Dituition Volume Acea Correct Acea Analyte Assay Recovery	HED SOLUTION	7 1 d.							The part will be served revenued at the table to take an in-			
Control	Sample preparations	Weight	Theoretical conc.	Sample Diluition Volume	Area	Correct Area	Analyte Assay	Ш	Recovery/Level			
1,0109 0,021 2,000 1,0109 0,000 2,		(8)	(m/brt)	(ml)	(Rt= 6.20 min, miz 84)	(Rt- 6.20 min, m/z 84)		(%)	(average)	nee whed)		
1,009 0,053 0,054 0,057 0,05	100-1	1,0109	0,021		28090	19549	11	93,14	94,78	3.47		
1,0103	100%-1	1,0099	0,053	c	73604	65063	1	96.23				
1,1050 0,105 1,1050 0,105 1,1050 0,105 0,054 88,12 89,02 0,24 1,1050 1,1050 1,1050 0,054 88,91 89,12 89,02 0,24 1,1050	100%-2	1,0103	0,053	2,7	73624	65083	ΙI	96,26	96,25	50,0	ALIBERTAL SALLES AND ALL SALLES AND ALL SALLES AND ALL SALLS AND ALL SAL	
NAL RECOVERY Confidence interval: t\(\alpha\) \(\text{sq.}\) \(\text	200%-2	1,0112	0,106		113705	105164		89,12	89,02	0,24		
AACCEPTABILITY CRITERIA PADO(n) μ (+) μ (+) μ (+) ACCEPTABILITY CRITERIA 83.55 μ = Xaverage ±t (s/n/s) 2.571 2.4S 89.60 97.10 SYSTEM SUITABILITY CRITERIA 3.58 3.58 Agreement % of STD+STD Agreement % of STD+STD Agreement % of STD+STD1check 5.83 LTcals for bias Xaverage - μ (sd²* h) ≤ tab Agreement % of STD+STD1check 6.65 8.76 -0.76 VAILD Agreement % of STD+STD1check	-			man a company of the first of t								
89,35	GLOBAL REC			Confidence Interval:	t _{0.025} (n=6-1;95%)	RADQ(n)	C) rl	(+) n		AO	CEPTABILITY CRITE	RIA
3,83 toalc = paverage - pl safth toalc ≤ tab Agreement % of STD1-STD2 Agreement % of STD1-STD2 Agreement % of STD1-STD2 Agreement % of STD1-STD2 to STD1-STD2 Agreement % of STD1-STD2 to STD1-STD2 to STD1-STD2 to STD1-STD2 to STD2	% w/w average Standard deviation	38,35	THE RESERVE THE PARTY OF THE PA	μ=Xaverage ±t (s/n½)	2,571	2,45	89,60	97,10		SYSTEM SUITA	BILITY (s)	1 1
1 Catic = Vaverage - µi/(sdr*n) ≤ ttab -6,65 8.76 - 0,76 VALID Agreement % between recovery level	global RSD%	3,83	The second section in the second section is the second sec	t-Tests for bias:		al-mos	olega	Help < Hely		Agreement % of	STO1-STO2	VALID
	A Paris		AND THE PERSON NAMED IN COLUMN 2 IN COLUMN	t calc = [Xaverage - µl/(sd⁴√n) ≤ ttab	L	8,76	-0.76	VALID		Agreement % be	stween recovery level	VALID

4M 35 116								The second secon				1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	1991 7 7 7 111 1 1 1 1 1 1 1 1 1 1 1 1 1		C. S. and Mildered Mr. All for community or
S-2016-03209 AM English 33 of 35 Sept 24 th , 2016						THE RESERVE AND ADDRESS OF THE PERSON OF THE					a hidrary and hidrary and an area and an area and a second	t inj Assessment	L		34.5
a que de la constante de la co		Street Street,	-	Market Ma	CHARLES AND STREET, ST	Child his make a second on	Andrew Andrews			-		Assessment -inj	8		0,48
Report No.: Version: Page: Print date:	Analyte Assay	conc. (ua/ml)			V LOQ (0,04	(IIII/Bn		Average	RSD%	To the second se	A CONTRACTOR OF THE PERSON OF	ī	(Average)	Time to	/0-mon*/
	Analyte Assay	8	0,0000021	0,0000017	0,0000017	0,0000014	0,0000019	0,0000018	14,2	THE COLUMN STREET, ST. LEWIS CO.,	The state of the s	ů.	(Conc/Area)	7,53E-07	7 575.07
	Analyte Assay	conc. (ug/ml)	0,021	0,017	0,017	0,014	0,020	0,018	14,4	The first state of the first sta		Area	(Rt= 6.20 min, m/z 84)	76338	7506/
	Sample Diluition Volume	(m)			2,0					III III III III III III III III III II		Conc.	(mg/m)	0000	regio
	Area	(Rt= 6.20 min, m/z 84)	13913	11584	11784	10146	13089	**************************************				Volumetric flask	(m)	000	20,02
	Weight	(6)	1,0140	1,0120	1,0115	1,0082	1,0113				V Pr Pr. V VIV. VI M Miles and Miles and Miles	Sampling	(h)	100.0	none.
FIVE BATCHES ANALYSIS	Sample preparations	Sample preparations	VC-1 lot A	VC-1 lot B	VC-1 lot C	VC-1 lot D	VC-1 lot E	and the same of the same same same same as a second of the same same same same same same same sam			STANDARD SOLUTION 2	Standard Solution		STES	1



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ANNEX#5: STATISTIC - EXCEL SHEET

STUDY NUMBER		S-2016-03209 AM			MR DR All all did in make him a bount in mount measurement and on any stray of the stray property.	A PART OF THE PART	of the same of the
DATE		21 September 2016				Statement and section of the section	
PARAMETER TO VALIDATE		HORWITZ					The second secon
PRODUCT		VC1				Carlo de la companya del companya de la companya de la companya del companya de la companya de l	- Commercial Commercia
Sample ID	Unit	Set data		anagija.			
ACE-2016-00123816	w/w %	0,0000023		ngenous.	t student (two sided)	d):	
	Company of the control of the contro	0,0000021		torrup	n-1	%56	%66
		0,0000029			2	4,303	9,925
		0,0000025		404131	3	3,182	5,841
		0,0000028	- THE THE REPORTED BATTERS IN THE CASE AND ADDRESS OF THE WINDS AND ADDRESS OF THE PARTY OF THE		4	2,776	4,604
		0,0000021		A11A4280	5	2,571	4,032
				a > 1 9 4 5	9	2,447	3,707
				-4 MAC	7	2,365	3,499
				56701800 56787980	σ	2,306	3,355
				000 bits	6	2,262	3,250
				ecetop.	10	2,228	3,169
				Marian	11	2,201	3,106
THE PROPERTY OF THE PROPERTY O	number of tests	9		60x50.00 (40x00.0x	A lot some problems		
t value (95%, n-1)	w.enipan		2.571	700000			No. one of the case
Media		Xmedium	0,0000024	// // // // // // // // // // // // //	Acceptability of the value of RSD%:	e value of RSE	3%:
Standard deviation (Sr)	ANY VERSION AND ADDRESS OF THE PROPERTY OF THE PROPERTY OF THE ADDRESS OF THE ADDRESS OF THE PROPERTY OF THE P	√[∑(Xi-Xmedium)²/(n-1)]	3,38E-07	% w/w	RSD% ≤ %RSDr	VALID	THE PERSON NAMED IN COLUMN TWO IS NOT THE PERSON NAMED IN COLUMN TO THE PERSON NAMED IN COLUMN T
Variance (Sr²)		[[\(\(\times\)\)]	1,14E-13	% w/w		Livide	PROPERTY AND ADDRESS OF THE PROPERTY OF THE PR
Limit of repeatability r		√2 * Sr * t _(0,95, n-1)	0,0000012	w/w %		of the latest	
RSD%		Sr/media*100	13,87		Significance repeatability 1/2 oR	tability 1/2 oR	! ≤ Sr ≤ 2/3 σR:
	A MARKAT SPENIES SERVICE SERVI				1/2 σR ≤	S	
Definition of C (mass/mass)	And the state of t		0,00001	w/w %	0,00000	0,000	0,00000
Horwitz equation (%RSD _R):	A PROPERTY OF THE PROPERTY OF	%RSD _R = 2 (1-0.5*logC)	22,63	0			
standard deviation according to Horwitz (S _R):	Horwitz (S _R):	S _R =0.02* C ^{0.8495}	0,00000		Expanded uncertainty:	inty:	
σ_{R} (%):		σ _R (%) = S _R * 100	0,00000	%	= 3	K*oR≡	Not applicable
Horwitz corrected (%RSDr):		%RSDr = %RSD _R *0.67	15,16				
			And the second s			Marini prison	A SAL A AMERICAN
	RESULTS.	$X = X$ medium $\pm U_E$	± UE	0,0000024			
		$X = X medium \pm t*Sr/RADQ(n)$	'n/RADQ(n)	0,0000024	± 0,0000004		

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-					

ANNEX#6: LOQ - EXCEL SHEET	

DATE PARAMETER TO VALIDATE PRODUCT					~			
PRODUCT		22-5	22-Sep-16	5				
PRODUCT		Ĭ	LOQ			AND AND ADDRESS OF STREET STRE	S. Albanda Madelliki albanda ana anakada manana anakada	The second second with the second consistence of the second secon
		>	VC-1			And the second s		BITTER AND ARTHUR THE THEORY OF A SET AND A SET AND ASSESSMENT OF A SERVICE OF A SE
PROPERTY OF THE REAL PROPERTY OF THE PROPERTY								
STANDARD MOTHER SOLUTIONS:								
Reference	Weigh	Assay	Dilution	Conc. 1				The second section is not being the second second second section of the second
	(6)	(%)	(FLL)	(mg/ml)		THE RESERVE THE PERSON NAMED IN COLUMN 2 I		- Separate and the services of the separate of
Nicotine	0,1049	99,1	50	2,079			The same of the sa	
Nicotine	0,1069	99,1	50	2,119		The second control of		
Nicotine	0,1075	99,1	20	2,131				The state of the s
	2.5864			- contra				
Reference	Sampling	Dilution	Conc. 2					THE RESIDENCE OF THE PROPERTY
	(ml)	(ml)	(mg/ml)			THE RESERVED TO SECURITION OF		A SECTION OF THE PARTY AND THE
STD 1	0,1	20,0	0,010	The second secon				PATRICIA DE LA CALIFORNIA DE LA CALIFORN
STD 2	1,0	20,0	0,011				ACTION AND ACTION AND INSTITUTION OF THE ACTION AND ACTION AND ACTION AND ACTION AND ACTION A	
STD 3	0,1	20,0	0,011					
STANDARD JOB SOLUTIONS:								
STD Level	Sampling	Dilution	STD Concentration	STD Area	STD Area	R	RF	Assesment %
	(H)	(m)	(ju/grl)	(Rt= 6.20 min, m/z 84)	(average)	([STD]/Area)	(average)	(betw een injections)
40% (LOQ) n.1	40,0	20,0	0,021	21281 21547	21414,0	9,77E-07 9,65E-07	9,71E-07	1,24
40% (LOQ) n.2	40,0	20,0	0,021	21950 21392	21671,0	9,65E-07 9,90E-07	9,78E-07	2,57
40% (LOQ) n.3	40,0	20,0	0,021	21754 21741	21747,5	9,79E-07 9,80E-07	9,80E-07	90'0
The principle of the state of t	The second section and the second	NA 1930 A ATT - A T- A T- A T- A T- A T- A T-		On the state of th		20	Average	
7 TO 10 TO 1			The state of the s			1,0	RSD%	