pagina 1: 10.2.e, 10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002
pagina 2 en 3: 10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002
pagina 4 t/m 6: 10.2.e, 10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002
pagina 7 t/m 39: 10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002

Final Report S-2016-03411 AM

VALIDATION OF A GC/MS METHOD FOR THE IDENTIFICATION AND QUANTIFICATION OF NICOTINE RESIDUE IN THE TEST ITEM "VX1" AND ANALYSIS ON FIVE PRODUCTION BATCHES

Study program: S-2016-03411 AM

Contract n: QCH1071

Sponsor: [redacted]

Test facility: [redacted]

Test item: "VX1"

Study Director: [redacted] ..... Released on: Sept 24th, 2016

[redacted]

INDEX

[redacted]

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 [redacted] perform analyses in compliance with the principles of good laboratory practices [redacted]

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

[redacted]
Study Director
[redacted]

Sept 24th 2016
Date

[redacted]

QUALITY ASSURANCE STATEMENT

The study was assessed for compliance with the approved study program and the Standard Operating Procedures of [redacted]
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 [redacted] Management:

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

This report accurately reflects the raw data.

[redacted]

QA GLP
[redacted]

Sept 24th 2016
DATE

[redacted]

SUMMARY

The aim of the study was to set up and validate a GC/MS method for the identification and the quantification of the impurity (-)-Nicotine in the test item "VX1 validation" and then test this analyte in 5 different production batches of the test item "VX1".

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 µg/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 µg/ml (corresponding to 0.046-0.228 µg/ml of sample). The preparations were dissolved in 2-Propanol. The samples for accuracy were prepared at 0.021, 0.053 and 0.106 µg/ml of (-)-Nicotine reference standard having known purity, representing low, middle, and high controls, respectively. Mean percentage (%) recovery ± relative standard deviation % (RSD%) ranged from 94.78 ± 3.47, 96.25 ± 0.03, to 89.02 ± 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 "VX1".

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

INTRODUCTION

On behalf o[redacted]a study aimed to validate a GC-MS method for the identification and quantification of the impurity (-)-Nicotine in the test item "VX1 validation' was performed.
Subsequently, with this validated method we tested 5 different lots of the test item production "VX1".
The study was conducted at the [redacted]

EXPERIMENTATION	*START*	*END*	*RESEARCHER*
GC/MS method for the quantification of Nicotine and five batches analysis	September 22nd, 2016	September 24th, 2016	[redacted]

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.

[redacted]

FILING

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 [redacted] 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 [redacted]

TEST ITEM IDENTIFICATION

NAME:	VX1
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 and presents no risks to human or animal health or to environment.

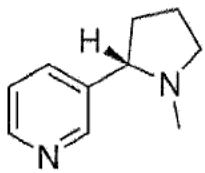
COMPOSITION DECLARED BY THE SPONSOR :

COMPONENTS	% (w/w)
extract of tobacco plants	N.A.
dibasic sodium phosphate dodecahydrate	3
monobasic potassium phosphate	0.08
sodium sulphite	0.2
PepMV VX1	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: 162 g mol^{-1}
FORMULA: $C_{10}H_{14}N_2$
STRUCTURE:



MAIN HAZARDS: danger
RISK CODES: H300, H310, H400, H410

[redacted]

ANALYZED SAMPLE FOR VALIDATION

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

Name	VX1 VALIDATION
Batch number	F
Manufacturing date	June 15th, 2016
Expiry date	December 15th, 2016
Receiving	EUITVI-82155
Date	Sept 02th, 2016
#ID	ACE-2016-00123817

ANALYZED SAMPLE FOR 5 BATCHES ANALYSIS

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

1.

Name	VX1
Batch number	A
Manufacturing date	June 15th, 2016
Expiry date	December 15th, 2016
Receiving	EUITVI-82155
Date	Sept 02th, 2016
#ID	ACE-2016-00134916

2.

Name	VX1
Batch number	B
Manufacturing date	June 15th, 2016
Expiry date	December 15th, 2016
Receiving	EUITVI-82155
Date	Sept 02th, 2016
#ID	ACE-2016-00134917

3.

Name	VX1
Batch number	C
Manufacturing date	June 15th, 2016
Expiry date	December 15th, 2016
Receiving	EUITVI-82155
Date	Sept 02th, 2016
#ID	ACE-2016-00134918

[redacted]

4.

Name	VX1
Batch number	D
Manufacturing date	June 15th, 2016
Expiry date	December 15th, 2016
Receiving	EUITVI-82155
Date	Sept 02th, 2016
#ID	ACE-2016-00134919

5.

Name	VX1
Batch number	E
Manufacturing date	June 15th, 2016
Expiry date	December 15th, 2016
Receiving	EUITVI-82155
Date	Sept 02th, 2016
#ID	ACE-2016-00134920

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	[redacted] Supelco)
Ref No	36733
USP Batch	SZBE205XV
Assay (% w/w)	99.1
Expire date	Sept. 02nd, 2019

[redacted]

Experimentation S-2016-03411 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 VX1 validation. The optimized method was 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 ($K_{b1} = 1\times10^{-6}$, $K_{b2} = 1\times10^{-11}$).

Nicotine is optically active, having two enantiomeric forms. The naturally occurring form of Nicotine is levorotatory with a specific rotation of $[\alpha]_D = -166.4°$ ((−)-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) 'VX1 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 µg/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.

[redacted]

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.
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.
LINEARITY	R	>0.99
	Confidence limits at 95% of intercept	contain the zero
ACCURACY	% Recovery	75-125% (% impurity 'nominal' < 0.1)
	Confidence range:	μ = Xaverage ± t ($s/n^{1/2}$) → contain the 100%
PRECISION	RSD% (*)	Horwitz : $RSD_R\% = 2^{[1 - 0,5 \log(C)]}$; *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.

(*) = 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

[redacted]

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: $\% RSD_R = 2^{(1-0.5\log C)} = 22.63$, *considering C equal to 0.00000010*

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

$\% RSD_r = \% RSD_R \times 0.67 = 15.2$

Precision: $RSD\% \leq RSD_r\%$

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.

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.

[redacted]

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

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 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 30% - 0.03 µg/ml of sample	30 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.

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).

[redacted]

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

CALCULATIONS

The content of analyte was calculated as follows:

$$C_{ST} = \frac{W_{ST} \times T_{ST}}{D_{ST} \times 100} \times 1000 \quad ;$$

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 µg/ml (respectively 0.04, 0.06, 0.08, 0.1 and 0.2 µg/ml of sample) :
$Y = a X + b$

$$\text{(-)-NICOTINE (µg/ml)} = [(Y - b)/a] \times D_C$$

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

$$\text{(-)-NICOTINE (\% µg/µg)} = \frac{[(Y - b)/a] \times D_C}{Wc} \times 100$$

Where:
D_C = 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.

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 injected with GC/MS technique - SCAN mode to confirm the analyte identity, while 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 50 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

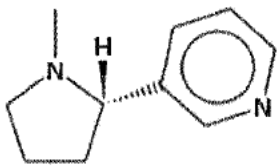
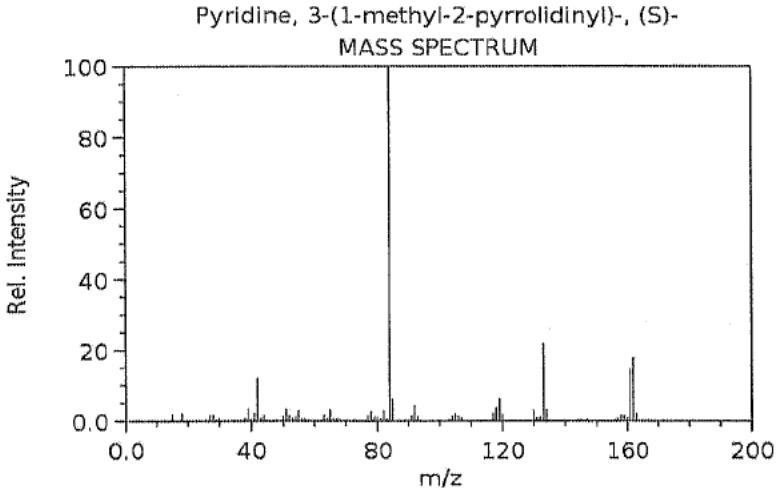
[redacted]

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.

	IDENTIFICATION	
	NAME / STRUCTURE	MASS SPECTRUM
1	Nicotine (cas 54-11-5) 	 NIST Chemistry WebBook (http://webbook.nist.gov/chemistry)

Group	Retention Time (min)	Selected Ions [a]			
		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.

[redacted]

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.

Figure 1: Blank mass chromatogram

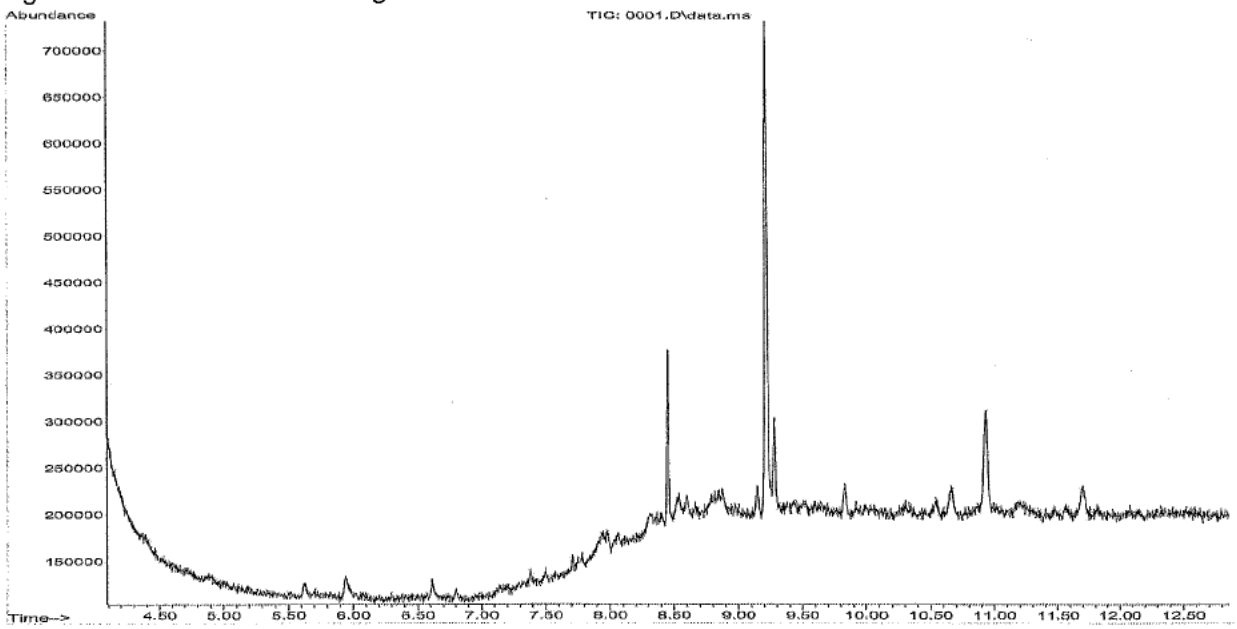
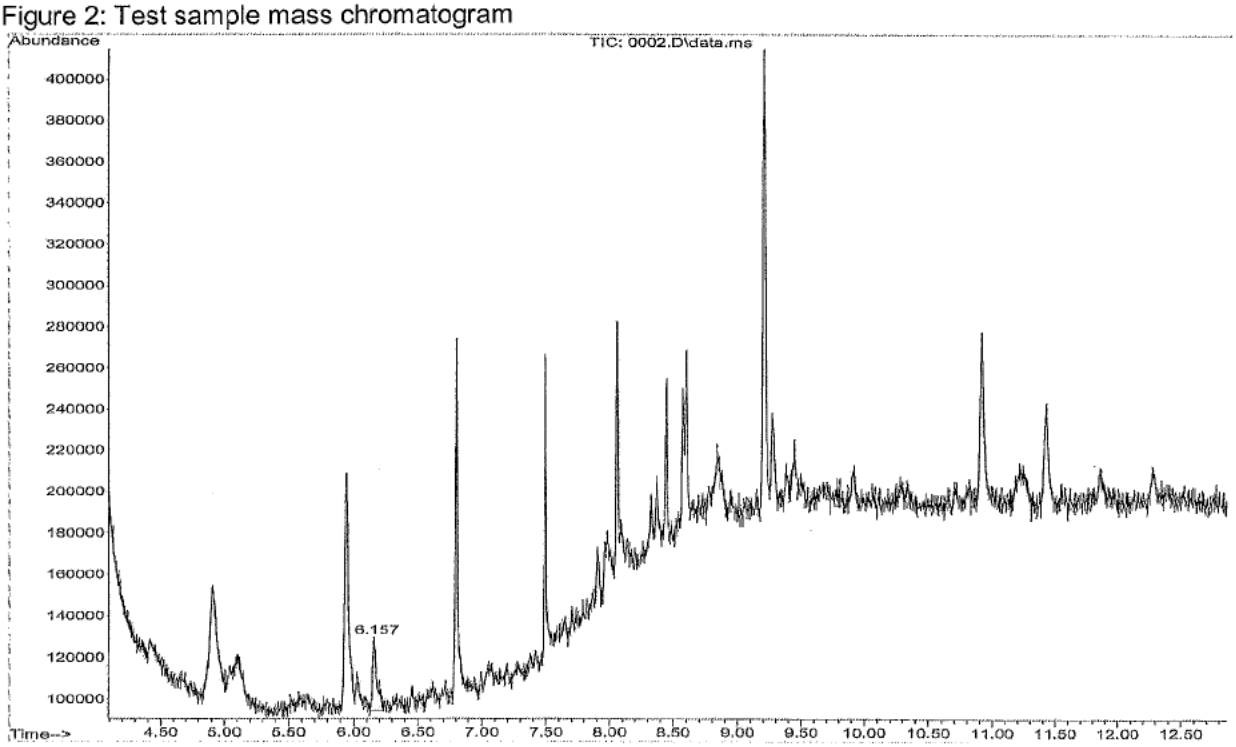
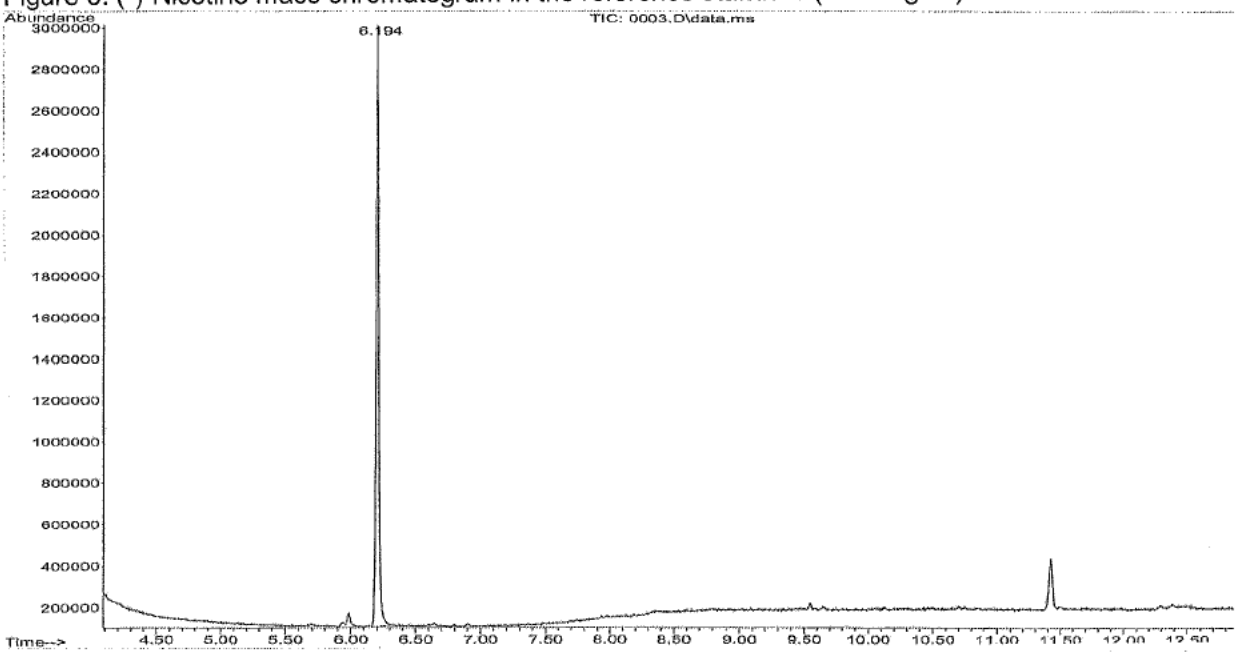


Figure 2: Test sample mass chromatogram



[redacted]

Figure 3: (-)-Nicotine mass chromatogram in the reference standard (0.01 mg/ml)

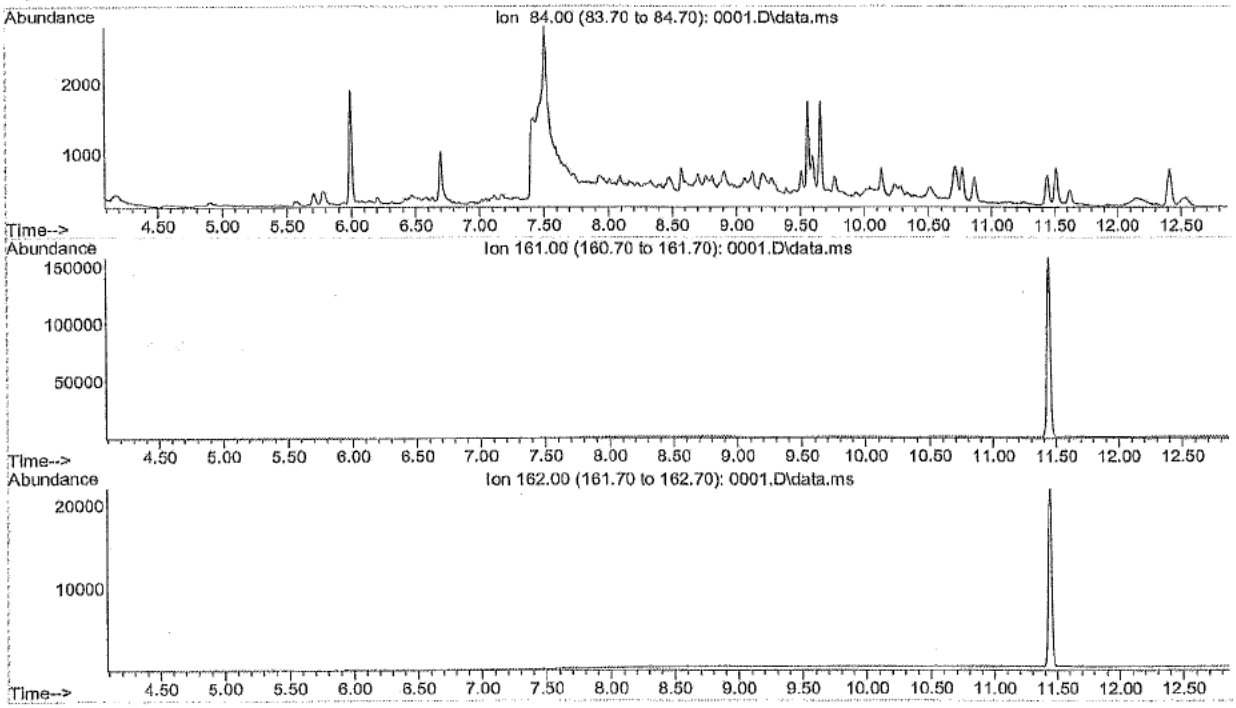


[redacted]

Specificity

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

Figure 1: Blank chromatogram (Solvent)



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. ≈ 6.2 min).

Figure 2: Sample chromatogram

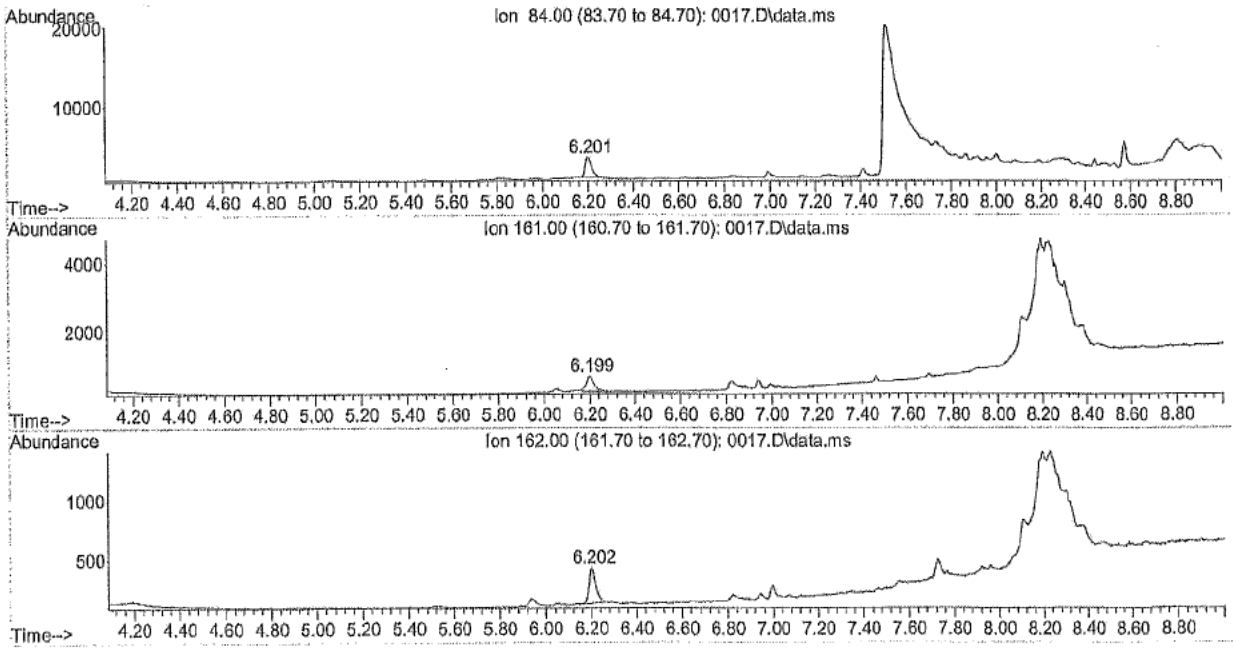


Figure 3: Nicotine chromatogram (Reference standard 0.02 µg/ml – LOQ level)

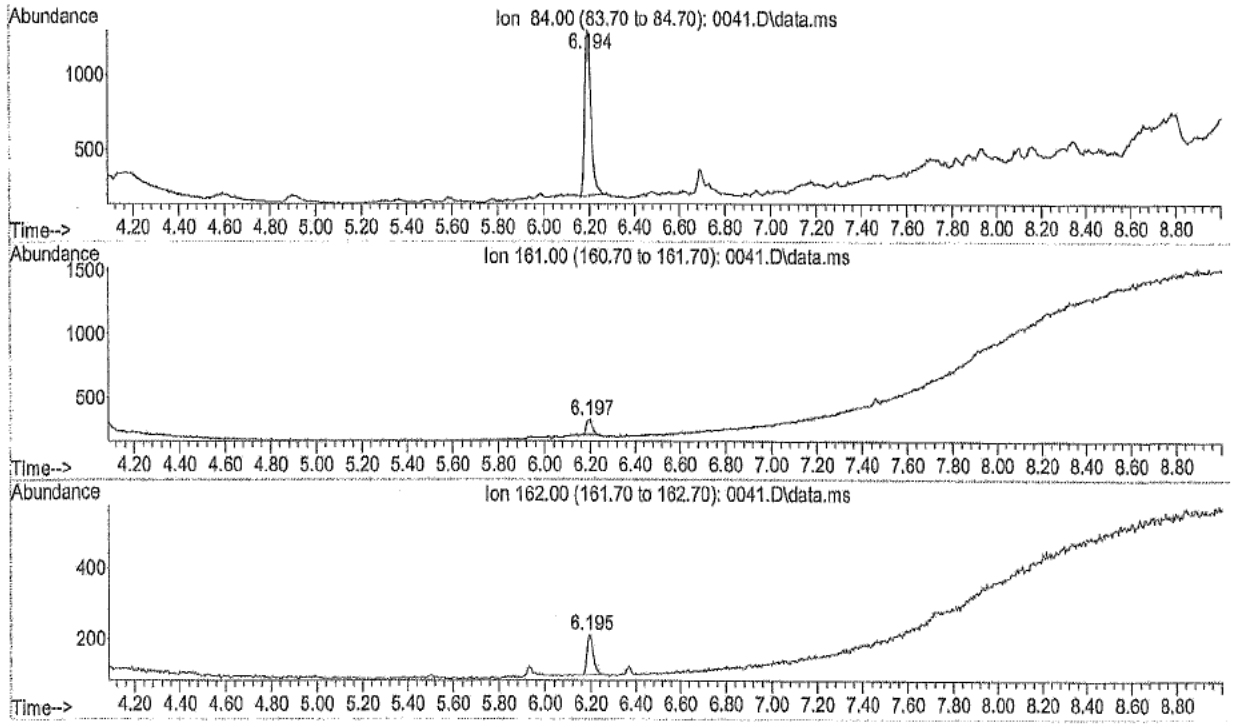


Figure 4: Nicotine chromatogram (Reference standard 0.05 µg/ml – 100% level)

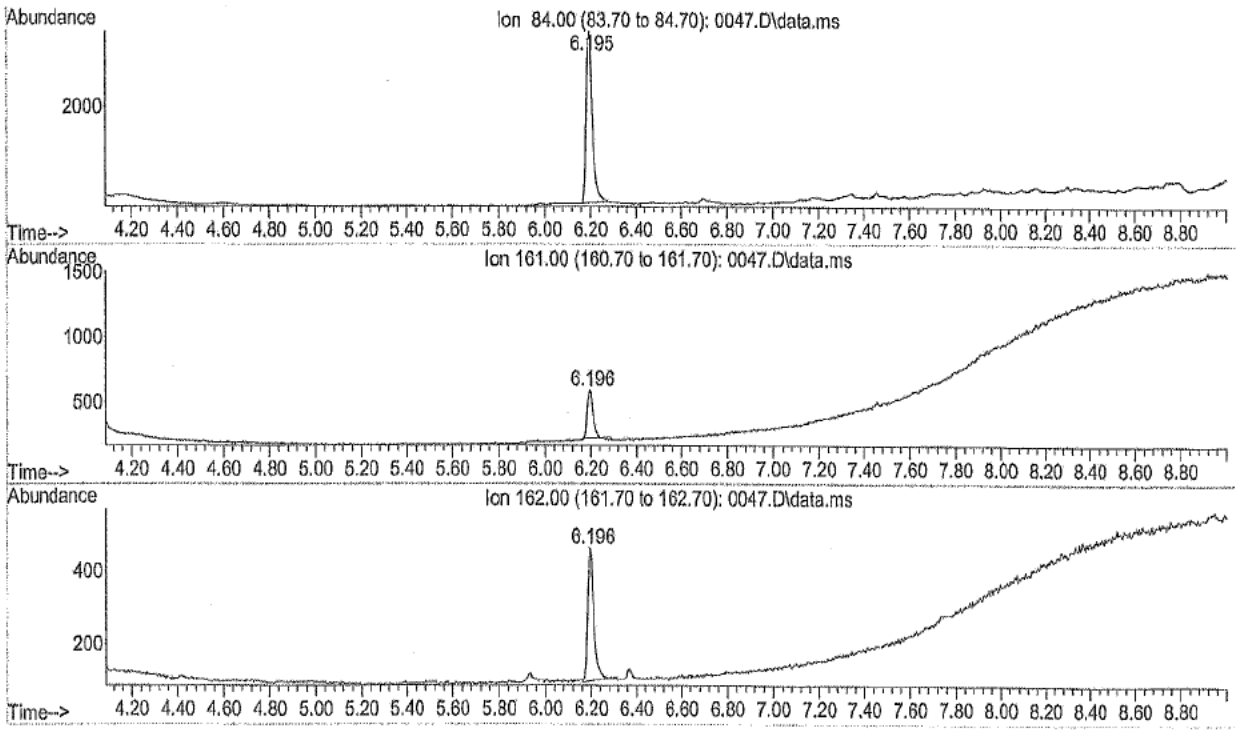
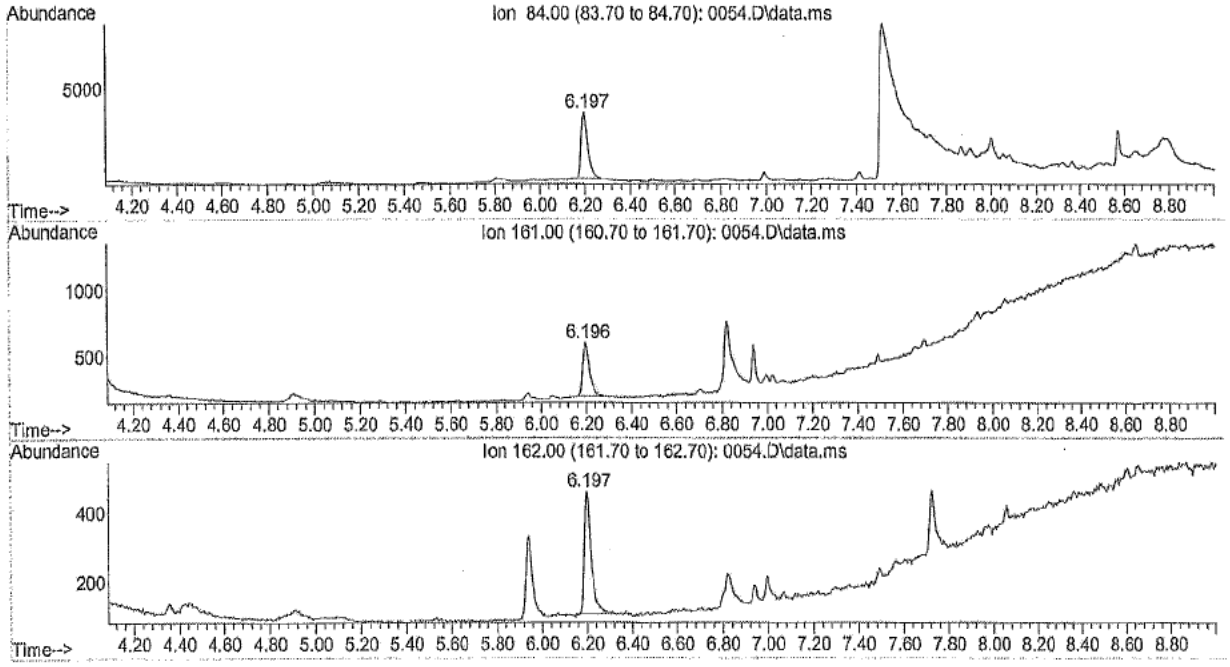


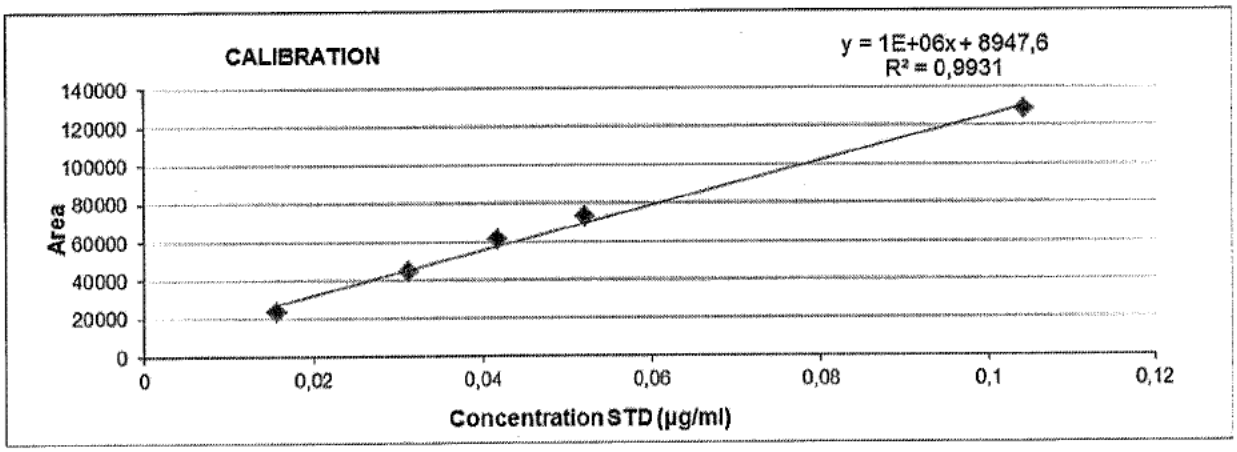
Figure 5: Enriched sample chromatogram (LOQ addition)



Linearity

Method Linearity was tested on the Nicotine reference standard on 5 different concentration levels from 30% 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	0.9931
Slope	1168127.2195
Intercept	8947.6376

Confidence interval at 95% for the intercept	[-0.0088;0.0088]

[redacted]

Accuracy

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				
	Recovery %	**Average Recovery %**	**95% confidence interval lower limit**	**95% confidence interval upper limit**
Reconstituted sample at 40%	99.02	102.82	98.76	106.89
Reconstituted sample at 100%	102.49			
Reconstituted sample at 200%	106.97			

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:

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

where

$\bar{x}$ = 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 ($t_{cal} \leq t_{tab}$). 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 $_{\text{active ingredient}}$ = 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 (see Annex#3: excel sheet). The experimental RSD% respects the acceptance criteria.

Analyte	Assay (% w/w)	Acceptance criteria	RSD%
Nicotine content	0.0000072	RSD% $_{\text{NICOTINE}} \leq 15.2\%^{(*)}$	1.3

$^{(*)}$ = 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:

$\% \ RSD_R = 2^{(1-0.5\log C)}$

[redacted]

Repeatability
Repeatability was obtained injecting a sample 6 times and is expressed as RSD% of the test results. Results are reported below.
The method proved to be repeatable (see Annex#3: excel sheet).

Analyte in the preparation S1	Assay (% w/w)	RSD%
Nicotine content	0.0000060	1.7

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 ≈ 30 and fall in the linearity and accuracy range (see Annex#6: excel sheets).

ANOMALY TEST (DIXON, GRUBBS):
The precision data set were subjected to statistical analysis to verify the presence of values statistically abnormal.
On results, originated from the same sample or from similar samples, it is necessary to verify the adequacy of the data to be statistically processed by appropriate tests that allow us to discard outliers.
In this regard we have carried out the Dixon and Grubbs tests, formulating the null hypothesis H0 as: the data is not to be excluded from the calculations.
For none of the tests the null hypothesis was rejected (see Annex#7: excel sheets).

Statistical consideration
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.
$\sigma_R = 0.02^* C^{0.8495}$ 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 $1/2\ \sigma_R \leq Sr \leq 2/3\ \sigma_R$.
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 (σR x 0.5).

% w/w	*Nicotine*
Xmedium	0.0000072
*Ue = K * σR*	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 ± t*Sr/RADQ(n)

% w/w	*Nicotine*
Xmedium	0.0000072
*± t*Sr/RADQ(n)*	± 0.0000001

DETERMINATION OF (-)-NICOTINE CONTENT IN FIVE BATCHES OF THE TEST ITEM "VX1"

Sample ID	***Batch***	***(-)-Nicotine (*)***
ACE-2016-00134916	A	0,069 µg/ml
ACE-2016-00134917	B	0,073 µg/ml
ACE-2016-00134918	C	0,072 µg/ml
ACE-2016-00134919	D	0,080 µg/ml
ACE-2016-00134920	E	0,082 µg/ml
	Average (µg/ml)	*0,075*
	SD (µg/ml)	*0,005*
	RSD%	*7.2*

(*) Nicotine LOQ = 0.04 µg/ml = 0,000004 % w/w, according to Validated method S-2016-03411AM.
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 average content in five production batches of test item was 0.075 µg/ml with a deviation standard equal to 0.005 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
N.7	ANOMALY TEST – EXCEL SHEET

ANNEX#1: NICOTINE - REFERENCE STANDARD CoA

[redacted]

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
CAS-No.: [54-11-5]
Usage : Insecticide

Molar mass: 162.23 g/Mole
Recomm. storage temp.: roomtemp.

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)	complying
Assay (GC)	99.1 area %
Refractive index (n 20/D)	1.5278
Date of Analysis	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 aliqout 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 adulteration of the substance.
- We herewith confirm that the delivery is affected 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.

[redacted]

1/3
23/09/16 UF

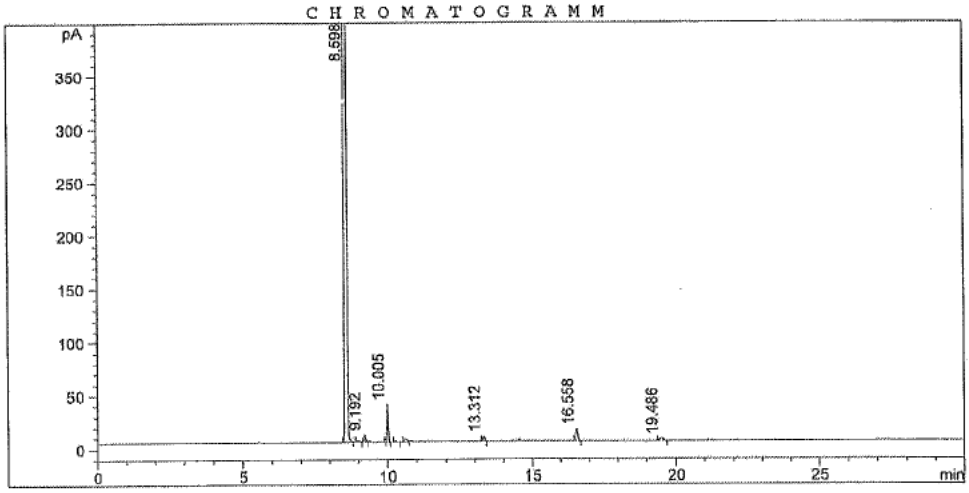
This document was produced electronically and is valid without a signature

[redacted]

GLC-Method Analytical Department

Article : (-)-Nicotine
Article-No : 36733
Batch : SZBE205XV
Column : SP-1701, 30m, 0,32mm i.D., 1.0µm Film
Inj.-Temp. : 280°C
Det.-Temp. : 280°C - FID
Oven-Temp. : 150°C to 250°C (10°C/min) hold 20min
Split : 1:100
Flow : 1ml He/min
Inj.v. : 0,2µl
Evaluation : uncorrected
Operator : Schulz

C H R O M A T O G R A M M



Area Percent Report

#	Meas. Re	Height	Area	Area %
1	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	10.61	2.5	13.7	0.04
8	13.31	5.0	19.2	0.06
9	16.56	11.1	61.8	0.20
10	19.49	2.3	16.1	0.05

2/3
23/09/16 VF

[redacted]

[redacted]

[redacted]

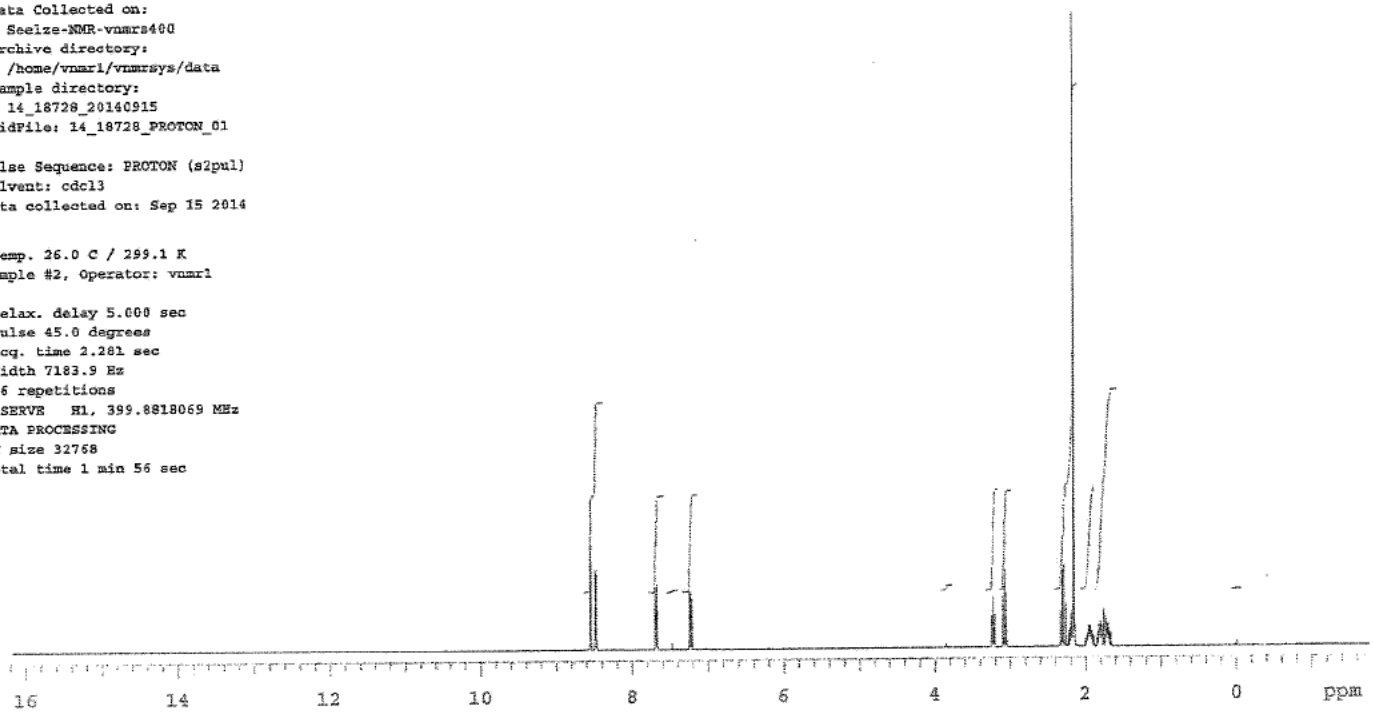
(-)-Nicotin
PESTANAL
#36733 Ch.:SZBE205XV

Sample Name:
14_18728
Data Collected on:
Seelze-NMR-vnmrs400
Archive directory:
/home/vnmrl/vnmrsys/data
Sample directory:
14_18728_20140915
FidFile: 14_18728_PROTON_01

Pulse Sequence: PROTON (s2pul)
Solvent: cdcl3
Data collected on: Sep 15 2014

Temp. 26.0 C / 299.1 K
Sample #2, Operator: vnmrl

Relax. delay 5.000 sec
Pulse 45.0 degrees
Acq. time 2.281 sec
Width 7183.9 Hz
16 repetitions
OBSERVE H1, 399.8818069 MHz
DATA PROCESSING
FT size 32768
Total time 1 min 56 sec



3/3
23/09/16 VR

[redacted]

ANNEX#2 : LINEARITY - EXCEL SHEET

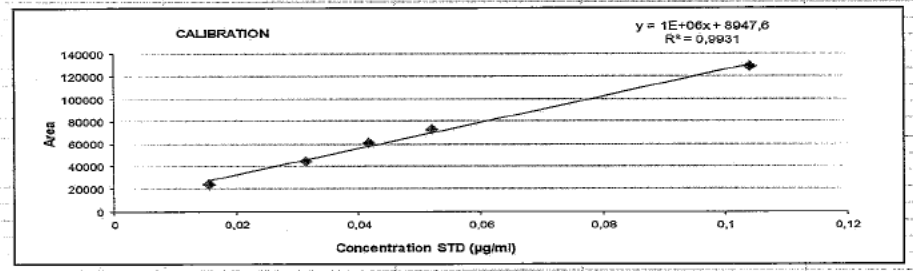
STUDY NUMBER	S-2016-03411AM
DATE	23-Sep-16
PARAMETER TO VALIDATE	LINEARITY
PRODUCT	VX-1

STANDARD MOTHER SOLUTIONS:

Preparation	Name	Weight (g)	Assay (%)	Dilution (ml)	Concentration 1 (mg/ml)	Sampling (ml)	Dilution (ml)	Concentration 2 (mg/ml)
STD	Nicotine	0,1051	99,1	50,0	2,0831	0,1	20,0	0,0104

STANDARD JOB SOLUTIONS:

STD Level	STD Volume taken (µl)	Dilution (ml)	STD Concentration (µg/ml)	STD Area (Rt= 6.20 min, m/z 84)	STD Area (average)	RF ([STD]/Area)	RF (average)	Assesment % (between injections)
40% (LOQ)	30,0	20,0	0,016	23060 23763	23412	6,77E-07 6,57E-07	6,67E-07	3,00
60%	60,0	20,0	0,031	43678 45744	44711	7,15E-07 6,83E-07	6,99E-07	4,62
80%	80,0	20,0	0,042	62267 59938	61103	6,69E-07 6,95E-07	6,82E-07	3,81
100%	100,0	20,0	0,052	72889 73232	73061	7,14E-07 7,11E-07	7,13E-07	0,47
200%	200,0	20,0	0,104	128310 128422	128366	8,12E-07 8,11E-07	8,11E-07	0,09
							7,15E-07	*Average*
							7,9	*RSD%*



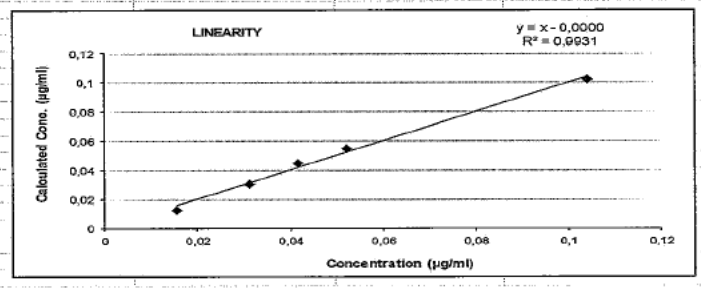
Slope	1168127,2195
Intercept	8947,6376
Correlation (R)	0,9966
R^2	0,9931

ACCEPTABILITY CRITERIA		
R >	0,99	VALID
Confidence limits b (95%)	contain the zero	VALID

Concentration	Signal	Calculated Conc.	% Calculated Conc. vs Conc.
0,016	23411,500	0,012	79,3
0,031	44711,000	0,031	98,0
0,042	61102,500	0,045	107,2
0,052	73060,500	0,055	105,4
0,104	128366,000	0,102	98,2

Slope (a)	1,0000	1,39E-17	Intercept (b)
st. dev. of a	0,048	0,003	st. dev. of b
r2	0,9931	0,003	st. dev. of y
F	433,1	3,00	df
SSreg	0,00	0,000031	SSresid
		3,182	t 95% (n-2 degrees of freedom)

Confidence limit b (95%)	
L+	0,0088
L-	-0,0088



[redacted]

ANNEX#3 : PRECISION-REPEATABILITY - EXCEL SHEET

STUDY NUMBER	S-2016-03411AM
DATE	24-Sep-16
PARAMETER TO VALIDATE	PRECISION AND REPEATABILITY
PRODUCT	VX-1

STANDARD MOTHER SOLUTIONS:

Reference	Weigh (g)	Assay (%)	Dilution (ml)	Conc. 1 (mg/ml)
Nicotine	0,1051	99,1	50	2,083
Nicotine	0,1160	99,1	50	2,299

Reference	Sampling (ml)	Dilution (ml)	Conc. 2 (mg/ml)
STD 1	0,1	20,0	0,010
STD 2	0,1	20,0	0,011

STANDARD JOB SOLUTIONS:

STD Level	Sampling (µl)	Dilution (ml)	STD Concentration (µg/ml)	STD Area (Rt= 6.20 min, m/z 84)	STD Area (average)	RF ([STD]/Area)	RF (average)	Assesment % (between injections)
Level 40%	30,0	20,0	0,016	23060 23763	23411,5	6,77E-07 6,57E-07	6,67E-07	3,00
Level 60%	60,0	20,0	0,031	43678 45744	44711,0	7,15E-07 6,83E-07	6,99E-07	4,62
Level 80%	80,0	20,0	0,042	62267 59938	61102,5	6,69E-07 6,95E-07	6,82E-07	3,81
Level 100%	100,0	20,0	0,052	72889 73232	73060,5	7,14E-07 7,11E-07	7,13E-07	0,47
Level 200%	200,0	20,0	0,104	128310 128422	128366,0	8,12E-07 8,11E-07	8,11E-07	0,09
							7,15E-07	*Average*
							7,9	*RSD%*

Slope	1168127,2195
Intercept	8947,6376
Correlation (R)	0,9966
R^2	0,9931

ACCEPTABILITY CRITERIA	
limit	5
Agreement % of STD1-STD2	VALID
Agreement % of STD1-STD1check	VALID

PRECISION

Sample preparations	Weight (g)	Area (Rt= 6.20 min, m/z 84)	Sample Dilution Volume (ml)	Nicotine conc. (µg/ml)	Nicotine %	
1	1,0081	50938	2,0	0,072	0,0000071	
2	1,0110	51578		0,073	0,0000072	
3	1,0100	52157		0,074	0,0000073	
4	1,0076	51063		0,072	0,0000072	
5	1,0088	51194		0,072	0,0000072	
6	1,0087	52314		0,074	0,0000074	
		51541		*0,073*	*0,0000072*	*Average*
		1,1		*1,4*	*1,3*	*RSD%*

STANDARD SOLUTION 2

Standard Solution	Sampling (µl)	Volumetric flask (ml)	Conc. (mg/ml)	Area (Rt= 6.20 min, m/z 84)	Fr (Conc/Area)	Fr (Average)	Assessment -inj (%)	Assessment STD1 - STD2
STD 2	100,0	20,0	0,057	81452 78563	7,06E-07 7,32E-07	7,19E-07	3,61	**0,82**

[redacted]

REPEATABILITY

Sample preparations	Weight (g)	Area (Rt= 6.20 min, m/z 84)	Sample Diluition Volume (ml)	Nicotine conc. (µg/ml)	Nicotine %	
1	1,0163	44273	2,0	0,060	0,00000595	
2		43519		0,059	0,00000582	
3		44944		0,062	0,00000606	
4		44061		0,060	0,00000592	
5		45188		0,062	0,00000611	
6		44697		0,061	0,00000602	
		44447		***0,061***	***0,0000060***	***Average***
		1,4		***1,7***	***1,7***	***RSD%***

STANDARD SOLUTION 1 check

Standard Solution	Sampling (µl)	Volumetric flask (ml)	Conc. (mg/ml)	Area (Rt= 6.20 min, m/z 84)	Fr (Conc/Area)	Fr (Average)	Assessment -inj (%)	Assessment STD1 - STD1check
STD 1 check	100,0	20,0	0,052	73858	7,05E-07	7,18E-07	3,53	**0,69**
				71298	7,30E-07			

[redacted]

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

STUDY NUMBER	S-2016-03411AM
DATE	24-Sep-16
PARAMETER TO VALIDATE	ACCURACY
PRODUCT	VX-1

STANDARD MOTHER SOLUTIONS:

Reference	Weigh (g)	Assay (%)	Dilution (ml)	Conc. 1 (mg/ml)
Nicotine	0,1040	99,1	50	2,061
Nicotine	0,1160	99,1	50	2,299

Reference	Sampling (ml)	Dilution (ml)	Conc. 2 (mg/ml)
STD 1	0,1	20,0	0,010
STD 2	0,1	20,0	0,011

STANDARD JOB SOLUTIONS:

STD Level	Sampling (µl)	Dilution (ml)	STD Concentration (µg/ml)	STD Area (Rt= 6.20 min, m/z 84)	STD Area (average)	RF ([STD]/Area)	RF (average)	Assesment % (between injections)
Level 40%	40,0	20,0	0,021	20826 20399	20612,5	9,90E-07 1,01E-06	1,00E-06	2,07
Level 60%	60,0	20,0	0,031	37936 35439	36687,5	8,15E-07 8,72E-07	8,44E-07	6,81
Level 80%	80,0	20,0	0,041	49030 48066	48548,0	8,41E-07 8,58E-07	8,49E-07	1,99
Level 100%	100,0	20,0	0,052	59741 59531	59636,0	8,63E-07 8,66E-07	8,64E-07	0,35
Level 200%	200,0	20,0	0,103	103030 103578	103304,0	1,00E-06 9,95E-07	9,95E-07	0,53
							9,10E-07	*Average*
							8,8	*RSD%*

Slope	966562,6143
Intercept	5941,0515
Correlation (R)	0,9928
R^2	0,9856

Value to subtract (Area)	44447

ENRICHED SOLUTION

Sample preparations	Weight (g)	Theoretical conc. (µg/ml)	Sample Diluition Volume (ml)	Area (Rt= 6.20 min, m/z 84)	Correct Area (Rt= 6.20 min, m/z 84)	Analyte Assay conc. (µg/ml)	Recovery (%)	Recovery/Level (average)	Assesment % (between preparations)
LOQ-1	1,0082	0,021	2,0	64398	19951	0,020	96,80	99,02	4,48
LOQ-2	1,0083	0,021		65313	20866	0,021	101,24		
100%-1	1,0081	0,052		105199	60752	0,052	101,87	102,49	1,20
100%-2	1,0067	0,052		106934	61487	0,053	103,10		
200%-1	1,0083	0,103		155929	111482	0,111	107,63	106,97	1,24
200%-2	1,0082	0,103		154550	110103	0,110	106,30		

GLOBAL RECOVERY	
% w/w average	102,82
Standard deviation	3,87
global RSD%	3,77

Confidence interval:	$t_{0,025}$ (n=6-1; 95%)	RADQ(n)	µ (-)	µ (+)
µ = Xaverage ± t (s/n½)	2,571	2,45	98,76	106,89

t-Tests for bias:	|Xaverage - µ|	sd*√n	t calc	tcalc ≤ ttab
t calc = |Xaverage - µ|/(sd*√n) ≤ ttab	2,82	9,48	0,30	VALID

ACCEPTABILITY CRITERIA	
SYSTEM SUITABILITY (≤)	5
Agreement % of STD1-STD2	VALID
Agreement % of STD1-STD1check	VALID
Agreement % between recovery level	VALID

[redacted]

FIVE BATCHES ANALYSIS

Sample preparations	Weight (g)	Area (Rt=6.20 min, m/z 84)	Sample Dilution Volume (ml)	Analyte Assay conc. (µg/ml)	Analyte Assay %	
VC-1 lot A	1,0084	39493	2,0	0,069	0,0000069	
VC-1 lot B	1,0097	41167		0,073	0,0000072	
VC-1 lot C	1,0074	40759		0,072	0,0000072	
VC-1 lot D	1,0085	44395		0,080	0,0000079	
VC-1 lot E	1,0094	45658		0,082	0,0000081	
				0,075	***0,0000075***	***Average***
				0,005	***0,0000005***	***SD***
				7,2	***7,1***	***RSD%***

STANDARD SOLUTION 2

Standard Solution	Sampling (µl)	Volumetric flask (ml)	Conc. (mg/ml)	Area (Rt=6.20 min, m/z 84)	Fr (Conc/Area)	Fr (Average)	Assessment -inj (%)	Assessment STD1 - STD2
STD 2	100,0	20,0	0,057	69494	8,27E-07	8,57E-07	6,98	0,83
				64808	8,87E-07			

[redacted]

ANNEX#5 : STATISTIC - EXCEL SHEET

STUDY NUMBER	S-2016-03411 AM
DATE	23 September 2016
PARAMETER TO VALIDATE	HORWITZ
PRODUCT	VX-1

Sample ID	Unit	Set data
ACE-2016-00123817	% w/w	0,0000071
		0,0000072
		0,0000073
		0,0000072
		0,0000072
		0,0000074
	number of tests	6

t student (two sided):

n-1	95%	99%
2	4,303	9,925
3	3,182	5,841
4	2,776	4,604
5	2,571	4,032
6	2,447	3,707
7	2,365	3,499
8	2,306	3,355
9	2,262	3,250
10	2,228	3,169
11	2,201	3,106

t value (95%, n-1)		2,571	
Media	Xmedium	0,0000072	% w/w
Standard deviation (Sr)	$\sqrt{[\Sigma(Xi-Xmedium)^2/(n-1)]}$	9,47E-08	% w/w
Variance (Sr^2)	$[\Sigma(Xi-Xmedium)^2/(n-1)]$	8,98E-15	% w/w
Limit of repeatability r	$\sqrt{2} * Sr * t_{(0,95,\ n-1)}$	0,0000003	% w/w
RSD%	Sr/media*100	1,31	
theoretical value		0,00001	% w/w
Definition of C (mass/mass)		0,00000	mg/mg
Horwitz equation ($\%RSD_R$):	$\%RSD_R = 2^{(1-0.5*\log C)}$	22,63	
standard deviation according to Horwitz (S_R):	$S_R = 0.02 * C^{0.8495}$	0,00000	
σ_R (%):	σ_R (%) = S_R * 100	0,00000	%
Horwitz corrected (%RSDr) :	$\%RSDr = \%RSD_R*0.67$	15,16	

Acceptability of the value of RSD%:

RSD% ≤ %RSDr	VALID

Significance repeatability 1/2 σR ≤ Sr ≤ 2/3 σR:

1/2 σR ≤	Sr	≤ 2/3 σR
0,00000	0,000	0,00000

Expanded uncertainty:

U_E =	K * σR =	Not applicable

RESULTS:	*X = Xmedium ± U_E*	*0,0000072*	±	*Not applicable*
	*X = Xmedium ± t*Sr/RADQ(n)*	*0,0000072*	±	*0,0000001*

[redacted]

ANNEX#6 : LOQ - EXCEL SHEET

STUDY NUMBER	S-2016-03411AM
DATE	24-Sep-16
PARAMETER TO VALIDATE	LOQ
PRODUCT	VX-1

STANDARD MOTHER SOLUTIONS:

Reference	Weigh (g)	Assay (%)	Dilution (ml)	Conc. 1 (mg/ml)
Nicotine	0,1160	99,1	50	2,299
Nicotine	0,1080	99,1	50	2,141
Nicotine	0,1065	99,1	50	2,111

Reference	Sampling (ml)	Dilution (ml)	Conc. 2 (mg/ml)
STD 1	0,1	20,0	0,011
STD 2	0,1	20,0	0,011
STD 3	0,1	20,0	0,011

STANDARD JOB SOLUTIONS:

STD Level	Sampling (µl)	Dilution (ml)	STD Concentration (µg/ml)	STD Area (Rt= 6.20 min, m/z 84)	STD Area (average)	RF ([STD]/Area)	RF (average)
40% (LOQ) n.1	40,0	20,0	0,023	22528	22528,0	1,02E-06	1,02E-06
40% (LOQ) n.2	40,0	20,0	0,021	21764	21764,0	9,84E-07	9,84E-07
40% (LOQ) n.3	40,0	20,0	0,021	20672	20672,0	1,02E-06	1,02E-06
						1,01E-06	***Average***
						2,1	***RSD%***

[redacted]

ANNEX#7 : ANOMALY TEST - EXCEL SHEET

1) Shapiro-Wilk Test

STUDY NUMBER	S-2016-03411 AM
DATE	24/09/2016
PARAMETER TO VALIDATE	ANOMALY TEST
PRODUCT	VX-1

NORMALITY TEST - SHAPIRO-WILK (UNICHIM n.179/2)

Id. number	set	copy	copy/2 (fine)	pairs of work		differences	factors	difference for factor
ACE-2016-00123817	0,0000071	0,0000071	0,0000074	0,00	0,00	0,00	0,6431	0,0000
	0,0000072	0,0000072	0,0000073	0,00	0,00	0,00	0,2806	0,0000
	0,0000073	0,0000072	0,0000072	0,00	0,00	0,00	0,0875	0,0000
	0,0000072	0,0000072						
	0,0000072	0,0000073						
	0,0000074	0,0000074						

deviance SQ	0,000	kp: 5%	kp: 1%	ḱp	$W=b^2/SQ$	b
media	0,000	-1,645	-2,330	-0,6515	0,8828	0,000
num.data n	6	H0 not rejected *normal*	H0 not rejected *normal*			

	n = 3	n = 4	n = 5	n = 6	n = 7	n = 8	n = 9	n = 10	n = 11	n = 12
	0,7071	0,6872	0,6646	0,6431	0,6233	0,6052	0,5888	0,5739	0,5601	0,5475
		0,1677	0,2413	0,2806	0,3031	0,3164	0,3244	0,3291	0,3315	0,3325
				0,0875	0,1401	0,1743	0,1976	0,2141	0,2260	0,2347
						0,0561	0,0947	0,1224	0,1429	0,1586
								0,0399	0,0695	0,0922
										0,0303

	γ	η	ε
3	-0,625	0,386	0,7500
4	-1,107	0,714	0,6297
5	-1,530	0,935	0,5521
6	-2,010	1,138	0,4963
7	-2,356	1,245	0,4533
8	-2,696	1,333	0,4186
9	-2,968	1,400	0,3900
10	-3,262	1,471	0,3660
11	-3,485	1,515	0,3451
12	-3,731	1,571	0,3270

[redacted]

2) Grubbs Test

STUDY NUMBER	S-2016-03411 AM
DATE	24/09/2016
PARAMETER TO VALIDATE	ANOMALY TEST
PRODUCT	VX-1

ANOMALY TEST - GRUBBS (UNICHIM n.179/2)

Id. number	set	copy
ACE-2016-00123817	0,0000071	0,0000071
	0,0000072	0,0000072
	0,0000073	0,0000072
	0,0000072	0,0000072
	0,0000072	0,0000073
	0,0000074	0,0000074
	n	6
	s	0,000
	Media	0,000

number of suspicious values at each end: 1

$G_{1,n}$ (Largest)	1,411	Correct value
$G_{1,1}$ (Smallest)	1,009	Correct value
Critical values G_2	5%	1%
Sk,Lk	1,887	1,973

Significance levels:

L,S	1%	5%
4	1,496	1,481
5	1,764	1,715
6	1,973	1,887
7	2,139	2,020
8	2,274	2,126
9	2,387	2,215
10	2,482	2,290
11	2,564	2,355
12	2,636	2,412

NOTE: *The extreme value (min/max) can be considered:*
- "Correct", and then accepted as consistent with the other data, if the criterion is lower than the significance level of 5%.
- "Dispersed", if the criterion met the significance level of 1%.
- "Anomalous", if the criterion does not met any significance level.

[redacted]

3) Dixon Test

STUDY NUMBER	S-2016-03411 AM
DATE	24/09/2016
PARAMETER TO VALIDATE	ANOMALY TEST
PRODUCT	VX-1

ANOMALY TEST - DIXON (UNICHIM n.179/2)

Id. number	set	copy							
ACE-2016-00123817	0,0000071	0,0000071			0,00000023	$x_n - x_1$			
	0,0000072	0,0000072			0,00000020	$x_n - x_2$			
	0,0000073	0,0000072			0,00000002	$x_2 - x_1$			
	0,0000072	0,0000072			0,00000004	$x_3 - x_1$			
	0,0000072	0,0000073			0,00000019	$x_{n-1} - x_1$			
	0,0000074	0,0000074			0,00000004	$x_n - x_{n-1}$			
					0,00000014	$x_n - x_{n-2}$			
					Critical values 95%				
			number: 3 to 7						
			R_{min} $[(x_2 - x_1)/(x_n - x_1)]$	0,108	0,628	val.min.correct	**correct**	min.	
			R_{max} $[(x_n - x_{n-1})/(x_n - x_1)]$	0,157	0,628	val.max.correct	**correct**	max.	
			number: 8 to 12						
			R_{min} $[(x_2 - x_1)/(x_{n-1} - x_1)]$					min.	
			R_{max} $[(x_n - x_{n-1})/(x_n - x_2)]$					max.	
			number: 13 to 18						
			R_{min} $[(x_3 - x_1)/(x_{n-1} - x_1)]$					min.	
			R_{max} $[(x_n - x_{n-2})/(x_n - x_2)]$					max.	
		6	**n**						

Significance levels:

L,S	5%	1%
3	0,970	0,994
4	0,829	0,926
5	0,710	0,821
6	0,628	0,740
7	0,569	0,680
8	0,608	0,717
9	0,564	0,672
10	0,530	0,635
11	0,502	0,605
12	0,479	0,579
13	0,611	0,697
14	0,584	0,670
15	0,565	0,647
16	0,546	0,627
17	0,529	0,610
18	0,514	0,594

NOTE: *The extreme value (min/max) can be considered:*
- "Correct", and then accepted as consistent with the other data, if the criterion is lower than the significance level of 5%.
- "Dispersed", if the criterion met the significance level of 1%.
- "Anomalous", if the criterion does not met any significance level.

[redacted]