An automated workflow for high-throughput nicotine quantitation using high-performance liquid chromatography coupled to high-resolution accurate mass spectrometry for prototype testing

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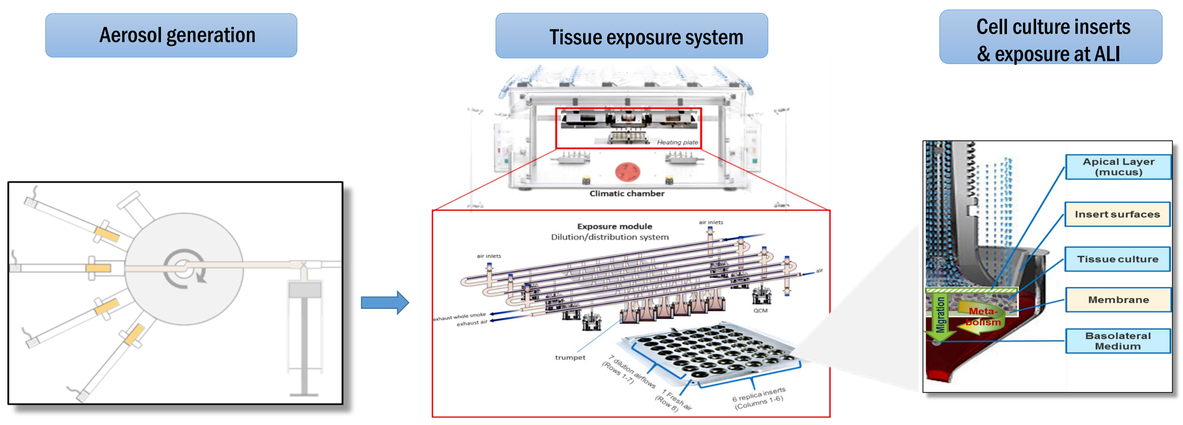
Introduction and objectives

Aerosol exposure of human three-dimensional (3D) organotypic airway epithelial tissue cultures growing at the air-liquid interface (ALI) is a well-established *in vitro* model enabling systems biology-based assessment of Reduced-Risk Products (RRPs) [1]. This model [2] is routinely used to assess and compare the biological impact upon exposure to aerosol from a heat-not-burn tobacco product, the Tobacco Heating System (THS) 2.2, with that of smoke from a 3R4F reference cigarette at the ALI using the Vitrocell® system. Nicotine, one of the major constituents of tobacco, can be used as marker of exposure. Therefore, an automated workflow for high-throughput nicotine quantitation was designed, enabling analysis of a large number of samples within a short time.

Aerosol generated from conventional and heat-not-burn products was deposited into a phosphate-buffered saline (PBS) solution serving as a surrogate matrix to measure deposition of compounds in the Vitrocell® system. The procedure was automated using a protocol developed in Pipeline Pilot (BIOVIA) from acquisition to reporting, and the method was validated by assessing selectivity, linearity, accuracy, limit of detection (LOD), and stability.

Methods

Aerosol generation and exposure of organotypic tissue cultures at the air-liquid interface (ALI)



Smoking machine: SM2000, Philip Morris International
Puff profile: Health Canada regimen (2 puffs/min. of 55 mL and 2 sec. aspiration and 8 sec. exhaust).

Exposure system: Vitrocell® 24/48 system can be used to simultaneously expose 48 cultures to up to 7 different smoke concentrations, with 6 replicates per concentration.

Organotypic tissue culture:
- Bronchial (human)

Surrogate matrix:
- PBS

Figure 1: Aerosol generation and exposure of tissue at the air-liquid interface.

Analytical method

An automated workflow was established to enable analysis of large number of samples.

A protocol developed in Pipeline Pilot BIOVIA® was used in pre-processing to automatically create a sequence table for analysis and vial print list. This is also used in a post-processing step for reporting. Vials were prepared using Virtuoso Vial Identification System (Thermo Fisher Scientific).

Data acquisition was performed using an Agilent 1290 Infinity® ultra-high-performance liquid chromatograph (UHPLC) coupled to a micrOTOF QII quadrupole time-of-flight mass spectrometer from Bruker®.

A high-throughput method in full-scan positive electrospray ionization mode using reverse-phase liquid chromatography was used to perform the analysis within three minutes of cycle time per analysis.

Data processing was performed with Bruker® Quant analysis 2.2 and exported to BIOVIA Pipeline Pilot for automated reporting.

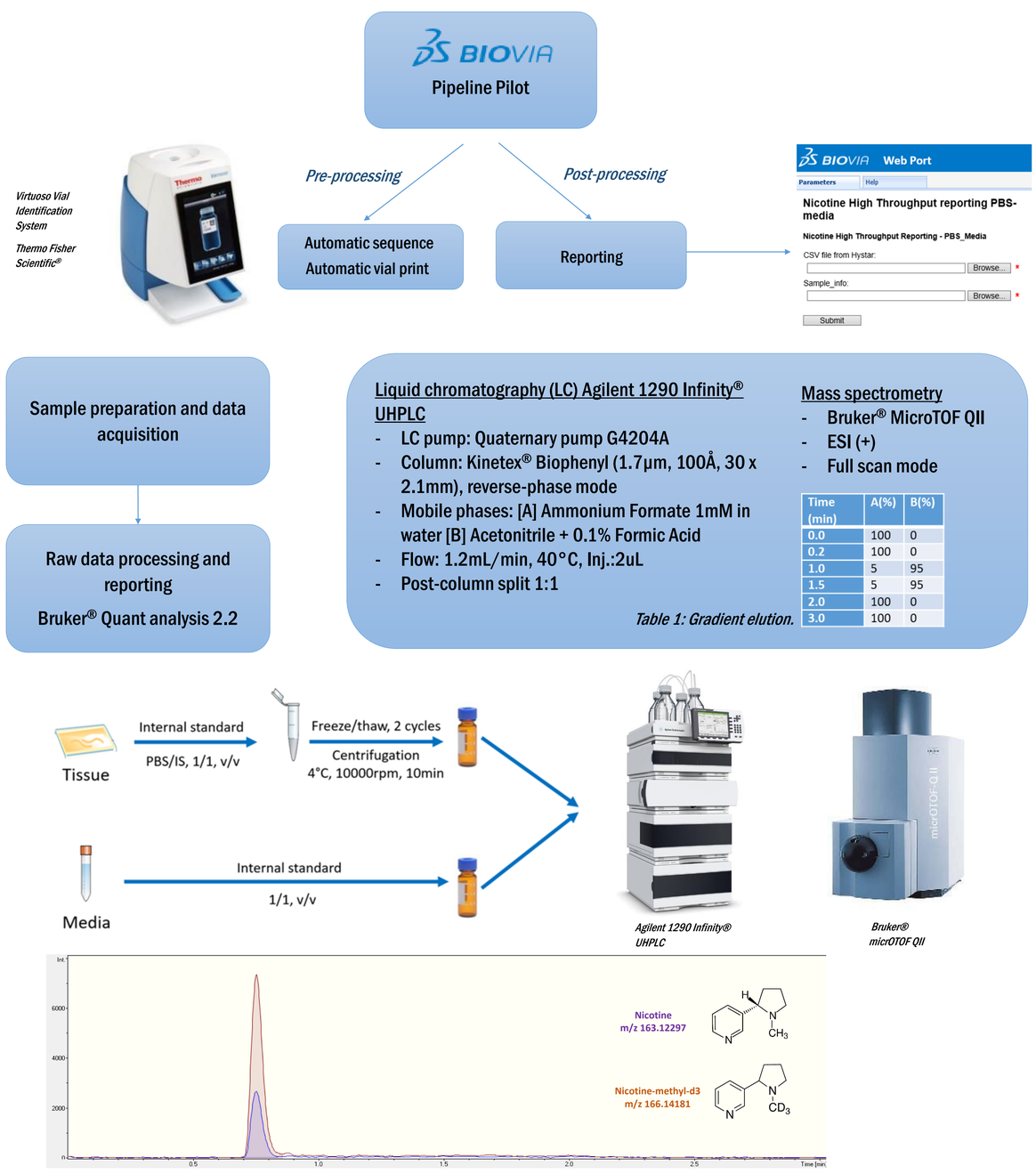


Table 1: Gradient elution.

Nicotine and its labeled homologue internal standard (nicotine-methyl-d3) were monitored by extracting compound-specific ions with accurate mass detection ± 5ppm. Quantification was performed by isotope dilution technique by plotting the area ratio between the analyte of interest and the internal standard.

Figure 2: An automated workflow for nicotine quantitation.

Results

Method validation

The method was validated testing selectivity, linearity, LOD, lower limit of quantification (LLOQ), accuracy, and stability. Accuracy measurements were within the acceptance criterion (± 15%). Quantitation was performed by isotopic dilution technique using stable isotope-labelled nicotine as internal standard.

1. Selectivity

Selectivity of chromatographic separation was proven for nicotine and its internal standard (nicotine-methyl-d3), separated from other interferences, and was assessed in blank samples, 3R4F smoke samples, and THS 2.2 aerosol matrix samples.

Nicotine:
- Spike level 1 = 150ng/mL
- Spike level 2 = 800ng/mL
- Spike level 3 = 1500ng/mL

Nicotine-methyl-d3 (Internal Standard):
- 850ng/mL

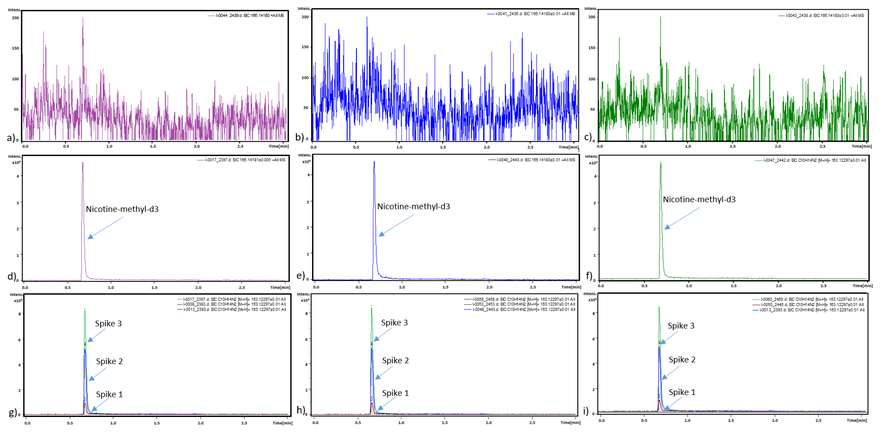


Figure 3: Chromatograms of: a) blank PBS , b) blank matrix 3R4F, c) blank matrix THS 2.2, d) PBS with internal standard, e) matrix 3R4F with internal standard, f) matrix THS 2.2 with internal standard, g) PBS spiked with nicotine h) matrix 3R4F spiked with nicotine, i) matrix THS 2.2 spiked with nicotine.

2. Linearity

Working range:
- 50ng/mL–2000ng/mL
- 8 calibration points, N=3
- Origin excluded

Linear regression:
- 1/x weighting

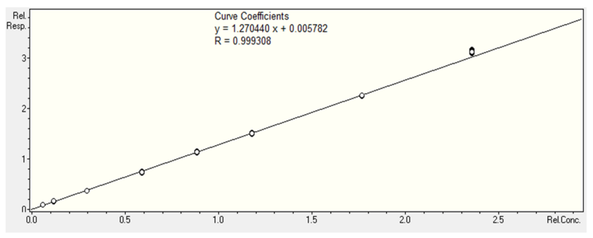


Figure 4: Linearity of calibration curve for nicotine with a correlation coefficient 0.999.

3. Accuracy

Accuracy was tested by spiking known concentrations of analytes at three different levels (3*LLOQ, 40% ULOQ, and 75% ULOQ, N=5).

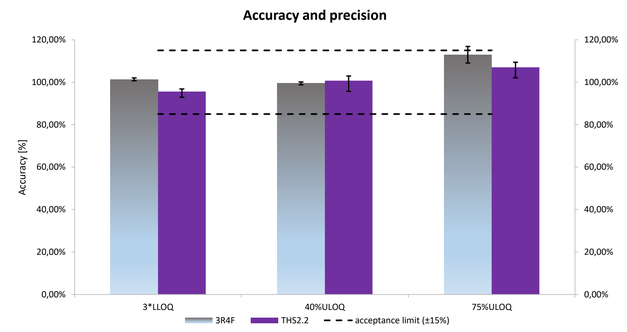


Figure 5: Accuracy profile of nicotine measurement.

4. Limit of detection (LOD), lower limit of quantitation (LLOQ)

$$LOD = \frac{3.3\sigma}{s} \qquad LLOQ = \frac{10\sigma}{s}$$

Figure 6: Equation used to calculate LOD and LLOQ.

The LLOQ was determined at 50ng/mL using the lowest calibration level as a standard. The upper limit of quantification (ULOQ) was defined by the highest calibration standard (2000ng/mL) of the linearity of the curve.

Compound	LOD [ng/mL]	LLOQ [ng/mL]	Linearity range [ng/mL]	Correlation Coeficient	Slope (s)	STD (σ)
Nicotine	12,85	49,5	50-2000	0,999308	1,27044	4,95

5. Stability

Stability was assessed by comparing the aged stock solutions with freshly prepared solutions (N=5), diluted at three different concentrations (Level 1=150ng/mL, Level 2=1000ng/mL, Level 3=1350ng/mL) stored at –20°C.

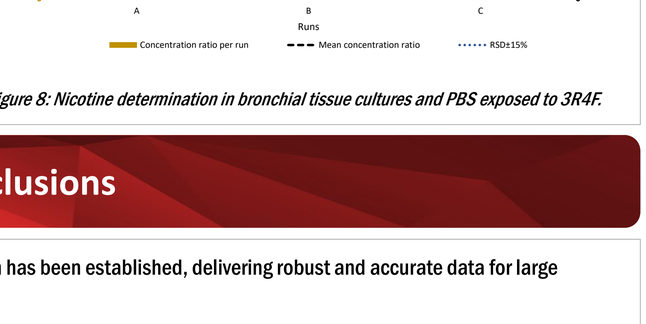


Figure 7: Stability of solutions within 6 months.

Determination of nicotine in tissue culture and PBS samples exposed to aerosol

Samples were generated by exposure human organotypic bronchial cultures (MucilAir™, Epithelix, Geneva, Switzerland) and PBS to 3R4F cigarette smoke diluted at 13% for 28 minutes in the Vitrocell® 24/48 exposure system.

- Tissue culture and PBS exposed to 3R4F (13%, 28 min)
- Three separated exposure runs (A, B & C) performed (9 replicates each)
- Tissue cultures and PBS were placed alternately in every other well within one row of the Vitrocell® module
- Concentrations in tissue cultures normalized by PBS values
- Good intra/inter run reproducibility (RSD<15%)

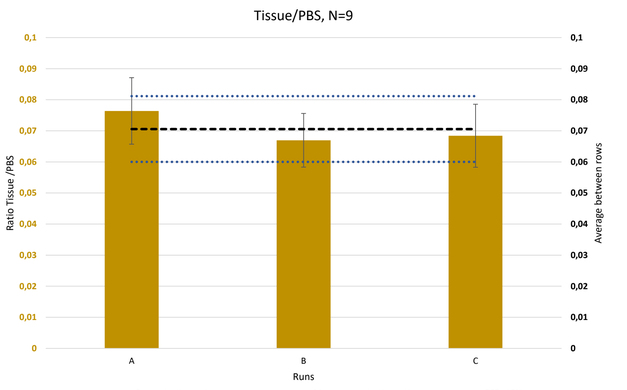


Figure 8: Nicotine determination in bronchial tissue cultures and PBS exposed to 3R4F.

Conclusions

- An automated workflow for high-throughput nicotine determination has been established, delivering robust and accurate data for large numbers of samples within a short time.
- The method is suitable for routine-based application to accurately determine the concentration of nicotine in PBS and tissues from samples exposed to cigarette smoke and aerosol from heat-not-burn tobacco products.
- The method has been validated for accuracy, linearity, selectivity, and stability, matching acceptance criteria according to standard regulation [3, 4].

References

1. Reduced-Risk Products ("RRPs") is the term we use to refer to products that present, are likely to present, or have the potential to present less risk of harm to smokers who switch to these products versus continued smoking. We have a range of RRPs in various stages of development, scientific assessment, and commercialization. Because our RRPs do not burn tobacco, they produce far lower quantities of harmful and potentially harmful compounds than found in cigarette smoke.
2. Iskandar, A. R. et al. 3-D nasal cultures: Systems toxicological assessment of a candidate modified-risk tobacco product, *ALTEX-Alternatives to animal experimentation*, 34(1), 23-48, 2017
3. Committee for Medicinal Products for Human Use, *European Medicines Agency Science Medicines Health*, 2011
4. Kalakuntla Rao R., Santosh Kumar K., *Journal of Pharmaceutical Sciences and Research*, Vol.1(3), 1-10, 2009

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Competing Financial Interest

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