

CONFIDENTIAL

# UK SMOKE CONSTITUENTS STUDY

## ANNEX A

### Part 3 Method: Determination of nitrogen monoxide yields in the vapour phase of cigarette smoke

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*Setting standards  
in analytical science*

## **Determination of nitrogen monoxide (nitric oxide) in the vapour phase of mainstream cigarette smoke**

### **1 Principle**

- 1.1 The vapour phase of fresh smoke from each puff, of cigarettes smoked on a Borgwaldt rotary smoking machine, is analysed using an instrument which measures the chemiluminescence produced by the reaction of nitrogen monoxide, NO, (also known as nitric oxide) with ozone. This process is known as “puff by puff analysis”. Immediate analysis of the fresh smoke from each puff is necessary because of the rapid reaction of nitric oxide with other vapour phase constituents.
- 1.2 For the benchmark study, no correction is made for quenching or enhancement effects due to carbon dioxide and hydrocarbons present in the cigarette smoke. The two tend to balance each other out and so have only a small effect on the overall nitrogen monoxide yield.

### **2 Apparatus**

- 2.1 Smoking Machine: A Borgwaldt Model RM20 automatic rotary smoking machine having all modifications arising from the 1991 harmonisation process and housed in a draught free cabinet within the smoking environment. This machine is a restricted smoker and is adjusted to produce a bell-shaped puff profile with a puff volume of  $35 \pm 0.2$  mL, a puff duration of  $2.0 \pm 0.05$  sec and a puff frequency of  $60 \pm 1$  secs. It should be capable of sufficient compensation over the normal range of pressure drops encountered for different types of cigarettes and maintain the pre-set smoking conditions as the pressure drop of the cigarette changes during smoking. NB Full instructions for use of the Borgwaldt are detailed in another SOP<sup>i</sup>
- 2.2 During smoking the pneumatically operated extract hood should be in the down position and the doors to the cabinet housing should be closed. Before smoking, the air flow must be measured and adjusted to give an average value of  $200 \pm 30$  mm/sec (see HEA/B1-0003).
- 2.3 Cambridge Filter (CF) holders<sup>ii</sup>: 44 mm diameter holder for Borgwaldt smoking machine.
- 2.4 Cambridge Filter pads<sup>ii</sup>.
- 2.5 Old style Perspex 55 mm holder for additional protection of syringe & analyser. This is placed in line between the smoking machine and the pneumatic panel.
- 2.6 Cambridge Filter (CF) smoke trap: consisting of a CF holder (2.3) and pad (2.4) with the rough surface facing the oncoming smoke.
- 2.7 Labyrinth seals: plane/concave silicone rubber washer available in various sizes of orifice (and colour coded) to hold different diameter cigarettes.
- 2.8 Neoprene Washers: washers available with various sizes of orifice (size to use dependant on cigarette diameter) which fit into a well on the rear part of the labyrinth holder.
- 2.9 Labyrinth Holders: Manufactured from metal and comprising of two parts connected via an O-ring seal. One part is capable of holding 4 labyrinth seals. The concave faces of three of the washers are towards the cigarette. The fourth washer, the one farthest from the lit end of the cigarette faces in the opposite direction. The second part houses a neoprene washer.
- 2.10 A calibrated soap film flow meter, which will measure the puff volume to within  $\pm 0.1$  cm<sup>3</sup>.
- 2.11 Anemometer: Lambrecht anemometer model 642 (or equivalent)
- 2.12 A soap flow bubble meter (50 cm<sup>3</sup> maximum reading) for measuring gas flow rates.
- 2.13 Barometer

- 2.14 Nitrogen Monoxide analyser : A Chemlab chemiluminescence analyser designed for the measurement of nitric oxide in gases and vapour. Ozone is generated by passing oxygen between a central electrode at a potential of 7kV and an outer electrode which is grounded. The ozone enriched oxygen flow is passed into a reaction cell where it is diluted with nitrogen. The analyser has a facility for direct introduction of a vapour phase into the reaction cell via a gas sampling valve with a 1 cm<sup>3</sup> sampling loop. This valve can be used manually, for calibration, or for automatic sampling which is achieved by a simple logic system in which the timing sequence is initiated by a 24 V DC signal from the smoking machine. The radiation from the exothermic reaction of nitrogen monoxide with ozone (to produce nitrogen dioxide) is measured between 650 and 950 nm by a photomultiplier. Broad band exothermic transmission from the ozone-oxygen mixture with the other cigarette smoke constituents is excluded by using a red transmission filter, type 2030, to remove radiation below 650 nm. Nitrogen is used to equalise the quenching reaction when sampling standards and cigarette smoke.
- 2.15 3-way valve. This allows connection between the analyser and either the smoking machine or standard gas mixture cylinders. Connections should be of PTFE or stainless steel. The connecting tubing from the smoking machine to the analyser should be as short as possible.
- 2.16 Male/female quick release connectors – male connectors are fitted to the cylinder – female connector to the PTFE tubing attached to the three way valve
- 2.17 Integrator/Recorder - Shimadzu Model CR3A (or equivalent).

### 3 Reagents

- 3.1 Nitrogen - oxygen free grade (from laboratory piped system)
- 3.2 Oxygen
- 3.3 Calibration gas mixtures :

Certified standard gas mixtures containing approximately 100, 400, 700, and 1500 volumes per million (vpm) of nitrogen monoxide in nitrogen. The gas mixtures are supplied in aluminium cylinders with a certificate of analysis giving the exact composition.

### 4 Precautions

- 4.1 Adequate ventilation is essential both from the point of view of excess nitric oxide calibration gases and perhaps more importantly the ozone generated in situ. Whenever the analyser EHT is switched on, the external extract fan must be running.

### 5 Setting up the nitrogen monoxide analyser

- 5.1.1 Connect the nitrogen supply, regulator pressure 60 psi<sup>i</sup> (4.2 bar), with copper/white plastic tubing to the GREEN gas entry port. Adjust the analyser nitrogen pressure control to 55-57 psi (4 bar). Connect a suitable flow meter to the tubing from the gas exit vent at the rear of the analyser. Set the nitrogen flow-rate to 120 ± 3 mL per minute (25 mL in 12.5 seconds) using the analyser nitrogen flow valve.
- 5.1.2 Connect the oxygen supply, regulator pressure 13 psi (0.9 bar), with copper/black plastic tubing to the BLUE gas entry port of the analyser. Adjust the oxygen pressure control to read 10 psi (0.7 bar). Set the combined flow-rate to 150 ± 3 mL per minute (25 mL in 10 seconds) using the analyser oxygen flow valve.

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<sup>i</sup> Psi = pounds per square inch

- 5.1.3 Disconnect the flow meter and re-position the end of exit vent tubing so that any gas is extracted up the chimney.
- 5.1.4 Ensure the delay switch at the rear of the analyser is 'off', the analyser gain is set to approximately 950, the "test" switch is at the zero position, the sensitivity switch at the rear is on "medium" and the "range" on the front of the instrument to 1.
- 5.1.5 Switch on the "mains" and the "ozone fan" and leave for 20 minutes to stabilise. Check that the 'slider valve' functions in the automatic mode by switching the "test" switch to the "run" position. The valve should operate without the alarm sounding. Also visually check that there has been no drift in the supply pressures for the nitrogen and oxygen and adjust if necessary.

## 6 Setting up the Integrator

- 6.1.1 Connect the integrator (2.17) to the analyser using a suitable cable and switch on the mains.
- 6.1.2 The following parameters have been found satisfactory :-

Width	1	Slope	828
Drift	0*	Min Area	50
T.Dbl	8*	Stop Time	20
Atten	1	Speed	10
Methods	1207	Format\$	2001
Spl. Wt.	100*	Is Wt	1*

- 6.2 A printout of the current parameter settings can be obtained by pressing "Shift down and List" and then "width". Parameters marked with an asterisk should not be changed. If necessary, the remaining parameters can be adjusted to improve data handling.
- 6.3 If the system has been disconnected and re-assembled the 'noise' level needs to be determined. Press "Shift Down" and then "S Test" and compare the result with the 'slope' value in the parameters. If the 'noise' is much greater than the 'slope' then spurious peaks will be integrated as if they were sample peaks. Increasing the slope value can eliminate this. However, care must be taken because setting the 'slope' too high may result in the 'loss' of small genuine sample peaks.

## 7 Calibration of nitrogen monoxide analyser

- 7.1 Clip on the push-seal connector from the highest standard gas mixture. Set the calibration gas supply to 5 psi (0.3 bar).
- 7.2 Turn the "test" switch of the analyser to "sample", set the integrator to "run" and open the on/off tap of the gas regulator.
- 7.3 Turn the 3-way valve to connect the analyser to the calibration standard and allow 5-10 seconds for the gas line and sampling loop to be thoroughly purged with the calibration standard.
- 7.4 Turn the 3-way valve to 'off', wait 1-2 seconds for the gas in the line/sampling loop to equalise to atmospheric pressure and then introduce the gas sample into the reaction cell by pressing the sample valve 'in' followed by withdrawal to the 'out' position.
- 7.5 Repeat steps 7.3 & 7.4 until at least 6 readings have been obtained.
- 7.6 Stop the integrator recording. A print out of the results will then be produced.
- 7.7 It is normal practice to set the gain of the analyser so that the mean of the individual peak areas for the top standard is within 5% of the calibration value. If this is not so adjust the gain, up or

down, and repeat the readings. NB Consult a senior officer for advice if a large change to the gain is required. The range of integrator readings should not be more than 4% of the mean. Discard any data outside this range and make further readings.

- 7.8 Once the criteria has been achieved obtain readings for the other standard gas mixtures without any further adjustment of the analyser controls but applying the same repeatability limits to the mean.
- 7.9 Turn off all the standard gas cylinders after use.
- 7.10 This calibration should be made at the commencement and end of each smoking period or three times during a complete day's smoking.

## 8 Setting up the smoking machine

- 8.1 NB: Full instructions on the operation of the Borgwaldt smoking machine are given in another SOP<sup>i</sup>.
- 8.2 Switch on the "mains" switch of the Borgwaldt smoking machine control box (BCB) and set the puff duration to 2.0 seconds (or its calibrated equivalent) and the intermission time to 30 seconds. To 'warm up' the smoking machine, push in the "volume control" switch and leave to cycle automatically for 20 minutes and then release the "volume control".
- 8.3 Check the white sealing pad on the sealing segment - it should extend out of the groove in the segment. If it has been compressed it should be replaced. Lightly oil the sealing pad and place in position. Lightly oil the sealing ring and fit into position with the metal stop to the inside right. Tighten the sealing segment. Activation of the "continuous drive" can help move/tighten the sealing ring into place.
- 8.4 Insert Neoprene washers of appropriate size into each of the holders and push into position. Attach the other halves of the labyrinth holders each containing 4 labyrinth seals of size appropriate to the brand to be smoked. The concave side of three of the seals should face the cigarette and the fourth should be reversed. Fit a 44 mm CF holder and pad (2.6) to the smoking machine and connect to the pneumatic panel control box (PP) using PTFE tubing. NB a second protective trap (55 mm diameter) is used to make the connection to the PP.
- 8.5 Turn the 3-way valve so that the pointed arrow is in line with the connection to the smoking machine. Set the switch on the analyser to "run" and check both instruments are in sequence by pressing the 'volume control' on the BCB. The syringe in the PP should operate in conjunction with the sampler valve of the analyser. Take several puffs to purge the sampling loop. Re-set the switch to the "zero" position.
- 8.6 Using the anemometer (2.11), calibrate the airflow to  $200 \pm 30$  mm per second<sup>i</sup>. Measurements are made, before smoking, on alternative days when the machine is in continuous use. If measurements were not made on the previous day then airflow must be measured before use.
- 8.7 Check that the puff duration is equivalent to  $2.00 \pm 0.05$  seconds by inserting the jack leads from the time counter into the "puff duration" sockets on the front of the PP and pressing the 'volume control' switch on the BCB. The piston speed should be adjusted if necessary. Replace the jack leads in the "intermission" sockets and set the intermission time to 4.00 seconds.
- 8.8 Cigarettes are smoked on the odd ports and therefore fit short (less than butt length of brand to be smoked) blank cigarette filters into the even ports. The "10 cigarette" switch on the BCB needs to be turned on.
- 8.9 Check the puff volume with the flow meter (2.12). If the puff volume is outside  $35 \pm 0.2$  mL, set to within these limits using the piston adjustment facility. After adjustment, ensure the slipper clutch is re-tightened and if necessary adjust the dead volume of the piston to 1 - 2 mL.

- 8.10 At the beginning of the day, check each port for leaks. Move the ring to connect each port in turn to the PP. Connect the leak detector to the port and take a puff. The liquid in the leak detector should rise up to between the minimum and maximum marks and remain steady. If the liquid starts to move, take the holder apart, clean and/or replace the seals, reassemble and check for leaks.

## 9 Smoking

- 9.1 Cigarettes are conditioned<sup>iii</sup> at a temperature of  $22 \pm 1^\circ\text{C}$  and  $60 \pm 2\%$  relative humidity for a minimum of 48 hours but not exceeding 10 days. Ten cigarettes are required for each determination.
- 9.2 Butt marking is to ISO butt length specifications<sup>iv</sup>. Filtered cigarettes are smoked to a measured butt length equal to either the tipping paper + 3 mm or filter length + 8 mm whichever is longer. The minimum butt length is 23 mm and this is also used for non filter brands. All smoking is conducted in an environment of temperature  $22 \pm 2^\circ\text{C}$  and  $60 \pm 5\%$  relative humidity<sup>iii</sup>.
- 9.3 ISO conditions<sup>ii</sup> for smoking cigarettes apply. The smoking machine puffing parameters are  $35 \pm 0.2\text{ cm}^3$  puff volume with  $2.0 \pm 0.02$  second puff duration once every  $60.0 \pm 0.5$  seconds.
- 9.4 A minimum of five determinations are performed for each brand. The smoking of the cigarette brands are randomised so that samples from the same brand are smoked on different days.
- 9.5 Press “motor” and load the conditioned cigarettes, pushing each firmly against the Neoprene washer. Place one cigarette in each port, the first five consecutive ports being used for the butt marked samples. Ensure the window of the butt length detector (BLD) is cleaned of tar and adjust the BLC so that the inner edge of the shield is in line with the butt mark. Check the position with all five marked cigarettes.
- 9.6 Switch the nitrogen monoxide analyser to “run” and set the integrator also to “run”. Ensure that the three-way valve is set to connect to the piston exhaust.
- 9.7 Lower the pneumatic hood over the cigarettes and rotate the ring to position 17. NB This allows sufficient time to start the machine and warm up the lighter.
- 9.8 Press “start” on the BCB, hold the switch in whilst carousel turns at least one position. NB This resets the puff counter on the pneumatic panel to zero.
- 9.9 Switch on the lighter (warm up time ca 10 seconds).
- 9.10 When cigarette position 1 reaches the CF holder, light the cigarette avoiding touching the cigarette with the lighter element. Continue until all the cigarettes have been lit noting any clearing puffs where cigarettes have not lit at the first attempt.
- 9.11 Switch off the lighter and switch on the “BLC” on the BCB.
- 9.12 During the smoking run record the barometric pressure, temperature and relative humidity.
- 9.13 Allow the cigarettes to smoke and when each cigarette reaches the butt length cut off the burning coal when two positions past the CF port, so as not to accidentally trigger the BLD. These are then automatically swept into a receptacle, which should contain water.
- 9.14 When all the cigarettes have been extinguished, stop the smoking machine by cancelling the “BLD” and “motor” buttons on the BCB. Record the number of lit puffs registered by the PP on the smoke analysis sheet. On completion of smoking clear the gas lines by taking 5 clearing puffs using the “volume control”. Note the total number of puffs. Switch the integrator to “stop” and allow it to printout the stored record of peaks.
- 9.15 Replace the CF trap with a clean one (2.6), and check the puff volume is within limits - adjust if necessary.

- 9.16 Reset the puff counter and check that the gas pressure readouts have not changed, adjust if necessary.
- 9.17 Commence the testing of the next brand (9.5 to 9.16).

## 10 Calculation

- 10.1 Use an Excel spreadsheet to calculate the linear regression for the standard concentrations vs. analyser values. Do not force the line through the origin. It has been found that differences between separate sets of calibration data obtained on the same day are minimal ( $< \pm 2\%$ ). Therefore, unless there is good reason to think differently, all calibration data obtained on a single day can be pooled to produce a single regression line that can then be applied to all samples smoked on that day.
- 10.2 Inspect the integrator printout and identify any minor spurious data which sometimes are produced. These can normally be identified from an unexpected retention time (puffs and therefore peaks should occur at intervals of 0.1 minute). Subtract the sum of any such peaks from the total integrated sum of the chromatogram.
- 10.3 Divide the corrected integrated sum by the total number of lit puffs to determine the mean value (i.e. per puff). Use the regression equation line to calculate the average nitrogen monoxide concentration ( $\text{vpm puff}^{-1}$ ) in the vapour phase.
- 10.4 The nitrogen monoxide yield (as  $\mu\text{g}$  per cigarette) is obtained from

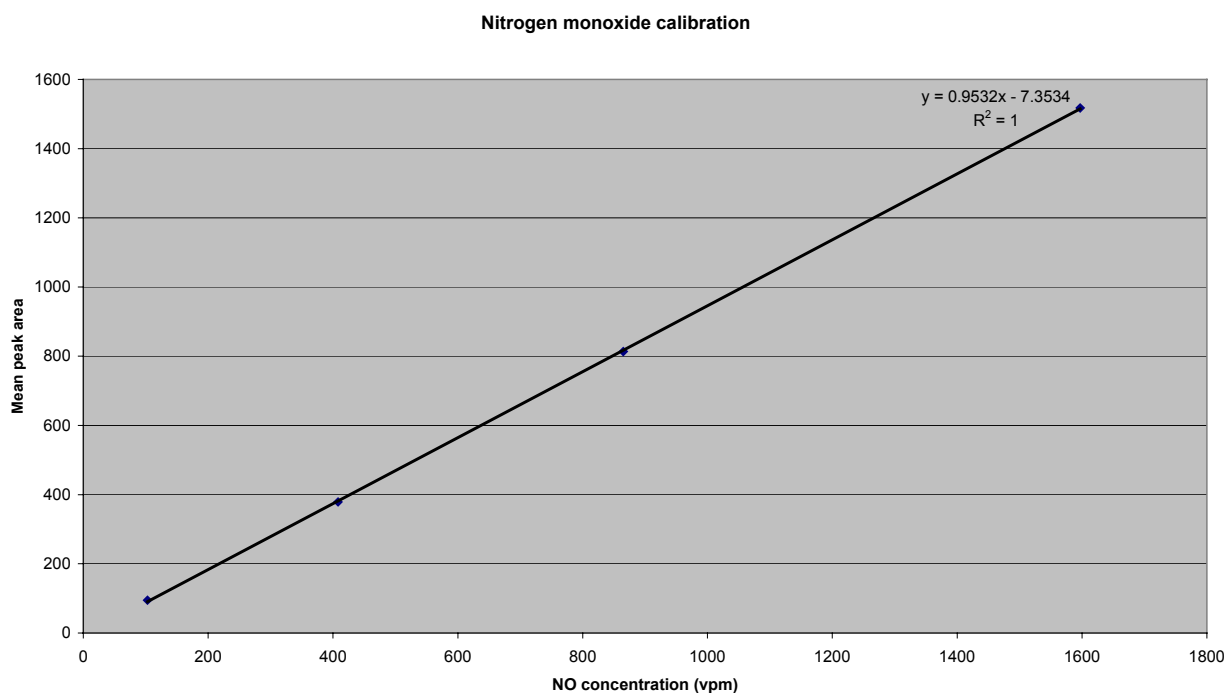
$$\text{NO yield}(\mu\text{g cig}^{-1}) = \frac{\text{NO conc}(\text{vpm puff}^{-1}) \times N(\text{puff}) \times P(\text{mm Hg}) \times 273.2(^{\circ}\text{C}) \times V(\text{mL}) \times 30.01(\text{g mol}^{-1})}{S(\text{cig}) \times (273.2 + T)^{\circ}\text{C} \times 22414(\text{mL mol}^{-1}) \times 760(\text{mm Hg})}$$

where

NO yield	Nitrogen monoxide yield
NO conc	Nitrogen monoxide concentration (volume/volume expressed in parts per million – ‘vpm’)
N	Total number of lit puffs
P	Pressure in mm Hg during the smoking
30	Molar mass of nitrogen monoxide <sup>ii</sup>
V	Puff volume (normally 35 mL)
S	Number of cigarettes smoked (normally 10)
T	Temperature in °Celsius during smoking
22414	Volume of 1 mole of gas

<sup>ii</sup> source [www.nist.gov](http://www.nist.gov)

## 11 Method validation



**Figure 1 Calibration curve for nitrogen monoxide.**

11.1 *Limit of detection:* The analyser should be set up so that a blank sample (air) gives zero response, i.e. nitrogen monoxide is not detected (see 6.3)..

11.2 *Precision:* The following values were obtained for 1R4F and 1R5F.

<b>Cigarette Brand</b>	<b>Nitrogen monoxide yield</b> µg per cigarette	<b>Comments</b>
1R4F	254 ± 23.9	(five determinations - same day)
1R4F	276 ± 14.9	(five determinations – different days)
1R5F	90 ± 10.0	(five determinations – same day)
1R5F	98.7 ± 4.4	(five determinations – different days)

11.3 Normal laboratory QC procedures should be in place as summarised in the table below:

Aim	How achieved
To show smoking is to ISO conditions	<ul style="list-style-type: none"> <li>• Check puff volumes</li> <li>• Check puff profiles</li> <li>• Establish correct butt mark lengths</li> </ul>
To show calibration is satisfactory	<ul style="list-style-type: none"> <li>• Check standard deviation of each standard</li> <li>• Print graph and 'look at curve' - check for linearity</li> <li>• Check R<sup>2</sup> (&gt;0.99), intercept close to Zero</li> <li>• Monitor slope and intercept throughout the day</li> </ul>
To show analytical instrument is calibrated and operating satisfactorily	<ul style="list-style-type: none"> <li>• Use certified standards which are in date</li> <li>• Check 'trace' for sensible pattern- e.g. peak areas increase during the smoking of a sample – blank puffs do not give a response</li> </ul>
To show standard laboratory equipment is functioning satisfactorily	<ul style="list-style-type: none"> <li>• Conditioning cabinet – temperature and relative humidity</li> <li>• Smoking environment – temperature and relative humidity</li> <li>• Air flows – within specification</li> </ul>

<sup>i</sup> HEA/B1-0003 Tar, nicotine and carbon monoxide yields of cigarettes using a rotary Borgwaldt smoking machine.

<sup>ii</sup> ISO 3308:2000 – Routine analytical cigarette smoking machine – Part 1: Definitions and standard conditions

<sup>iii</sup> ISO 3402: 2000 - Tobacco and tobacco products – atmosphere for conditioning and testing

<sup>iv</sup> ISO :4387: 2000 - Methods for chemical analysis of tobacco and tobacco products – Part 14: Determination of total and nicotine- free dry particulate matter using a routine analytical smoking machine