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# Determination of Tobacco Specific Nitrosamines in Cigarette Mainstream Smoke: The CORESTA 2011 Collaborative Study *

*by*

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SUMMARY

A CORESTA Recommended Method (CRM 75) has been developed and published, applicable to the quantification of tobacco-specific nitrosamines (TSNAs), namely, *N*-nitrosonornicotine (NNN), *N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT) and 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) in cigarette mainstream smoke. The method involves smoke collection on a Cambridge filter pad under both ISO 3308 and the intense conditions adopted by Health Canada. An internal standard solution is added to the smoke collected on the pad and, after extraction, an aliquot is separated and quantitatively analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

CRM 63 involving gas chromatography coupled with a thermal energy analyser (GC-TEA) was previously developed by the CORESTA Special Analytes Group that had been set up to develop recommended methods on smoke components. However, by 2009 most laboratories had moved to similar LC-MS/MS methods for TSNA analysis and so this technique was chosen as the basis of a new CRM and to complement CRM 63. Initial joint experiments, specific experiments by single laboratories and ongoing discussions identified methodological aspects that needed to be 'standardised' before moving to a CRM.

A joint experiment by 15 laboratories was carried out in 2010–2011 that investigated and identified important methodological features that needed to be controlled or clarified. CRM 75 was produced through a final collaborative experiment involving 20 laboratories from 12 countries using both linear and rotary smoking machines. Some notes are included in the CRM to inform other laboratories that might wish to adopt the method, concerning aspects that need to be well controlled to provide data as robust as possible and to provide similar repeatability and reproducibility data.

Statistical evaluations were made according to ISO 5725 guidelines and are included. Under ISO smoking, the levels of reproducibility (R) expressed as a percentage of the mean of TSNA yields across laboratories are much greater than the levels found for "tar", nicotine and carbon monoxide and given in the relevant ISO standards. The R value was expressed as a percentage of the mean yield among-laboratories and across all of the studied products. Under ISO smoking R% values ranged from 25–60% for NNN; from 31–85% for NNK; from 47–58% for NAT and 40–99% for NAB. These levels are generally in line with those determined previously for TSNAs in CRM 63 and for other smoke analytes studied by the Special Analytes Group.

Under 'intense' smoking, R% values ranged from 30–88% for NNN; from 37–79% for NNK; from 47–83% for NAT and 42–111% for NAB. A plot of R against mean yields suggests that the 'intense' regime gives similar or slightly worse reproducibility than the ISO regime in spite of the higher yields generated. [Beitr. Tabakforsch. Int. 25 (2012) 507–519]

ZUSAMMENFASSUNG

Es wurde eine CORESTA Recommended Method (CRM 75) zur Anwendung bei der Quantifizierung von tabakspezifischen Nitrosaminen (TSNA), und zwar *N*-Nitrosonornikotin (NNN), *N*-Nitrosoanabasin (NAB), *N*-Nitrosoanatabin (NAT) und 4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanon (NNK), im Hauptstromrauch von Zigaretten entwickelt und veröffentlicht. Die Methode betrifft das Auffangen des Rauchs auf einem Cambridge-

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Filter sowohl gemäß ISO 3308 als auch gemäß den strengen Bedingungen von Health Canada. Eine interne Standardlösung wird zu dem auf dem Filter aufgefangenen Rauch hinzugefügt, und nach Extraktion wird ein Aliquot abgetrennt und durch Flüssigchromatographie-Tandem-Massenspektrometrie (LC-MS/MS) quantitativ analysiert. Die CORESTA Special Analytes Group, die eingerichtet wurde, um empfohlene Methoden für Rauchbestandteile zu entwickeln, hatte zuvor CRM 63 mit Gaschromatographie kombiniert mit einem Thermal Energy Analyser (GC-TEA) entwickelt. Bis 2009 waren jedoch die meisten Labore zu ähnlichen LC-MS/MS-Methoden für die TSNA-Analyse übergegangen, daher wurde dieses Verfahren als Grundlage einer neuen CRM und zur Ergänzung von CRM 63 ausgewählt. In ersten gemeinsamen Versuchen, spezifischen Versuchen einzelner Labore und fortlaufenden Gesprächen wurden methodische Aspekte identifiziert, die vor dem Übergang zu einer CRM ‚standardisiert' werden mussten. In einem 2010–2011 durchgeführten gemeinsamen Versuch von 15 Laboren wurden wichtige methodische Merkmale untersucht und identifiziert, die der Kontrolle und Klärung bedurften. CRM 75 entstand durch einen abschließenden Kooperationsversuch, an dem 20 Labore aus 12 Ländern unter Verwendung von linearen und rotierenden Rauchmaschinen beteiligt waren. In der CRM sind einige Hinweise für andere Labore enthalten, die die Methode verwenden möchten, im Hinblick auf Aspekte, die gut kontrolliert werden müssen, um eine größtmögliche Robustheit der Daten zu gewährleisten und ähnliche Daten zur Wiederholgenauigkeit und Reproduzierbarkeit hervorzubringen.
Statistische Auswertungen erfolgten gemäß den Richtlinien der ISO-Norm 5725 und sind enthalten. Unter ISO-Rauchbedingungen sind die als Prozentsatz des Mittelwerts der TSNA-Ausbeuten aller Labore ausgedrückten Werte der Reproduzierbarkeit (R) viel höher als die für Teer, Nikotin und Kohlenmonoxid festgestellten und in den betreffenden ISO-Normen angegebenen Werte. Der R-Wert wurde als Prozentsatz der mittleren Ausbeute zwischen den Laboren und bei allen untersuchten Produkten ausgedrückt. Unter ISO-Rauchbedingungen lagen die R%-Werte bei 25–60 % für NNN, 31–85 % für NNK, 47–58 % für NAT und 40–99 % für NAB. Im Allgemeinen stimmen diese Werte mit den zuvor in CRM 63 für TSNA bestimmten sowie mit den Werten für andere von der Special Analytes Group untersuchte Rauchanalyte überein.
Bei intensivem Rauchen lagen die R%-Werte bei 30–88 % für NNN, 37–79 % für NNK, 47–83 % für NAT und 42–111 % für NAB. Eine Darstellung von R gegenüber den mittleren Ausbeuten zeigte, dass die intensiven Rauchbedingungen trotz der erzielten höheren Ausbeuten zu einer ähnlichen oder wenig schlechteren Reproduzierbarkeit führen als die ISO-Bedingungen. [Beitr. Tabakforsch. Int. 25 (2012) 507–519]


RESUME

Une méthode recommandée par CORESTA (CRM 75) a été élaborée et publiée; elle est applicable à la quantification des nitrosamines spécifiques du tabac (NAST), notamment la *N*-nitrosonornicotine (NNN), la *N*-nitrosoanabasine (NAB), la *N*-nitrosoanatabine (NAT) et le 4-(*N*-nitrosométhylamino)-1-(3-pyridyl)-1-butanone (NNK), dans le courant de fumée principal des cigarettes. La méthode implique la collecte de fumée sur un tampon de filtre Cambridge en respectant à la fois les critères de la norme ISO 3308 et les conditions de fumage intensif telles qu'adoptées par Health Canada. Une solution standard interne a été ajoutée à la fumée collectée sur le tampon et, après extraction, une aliquote a été prélevée et analysée quantitativement par chromatographie en phase liquide - spectrométrie de masse tandem (CPL-SM/SM).
La méthode CRM 63, qui implique la chromatographie en phase gazeuse couplée à un analyseur d'énergie thermique (CPG-AET), a été élaborée antérieurement par le Groupe spécial d'étude des analytes de CORESTA, qui avait été mis sur pied pour concevoir des méthodes recommandées sur des composants de la fumée. En 2009 cependant, la plupart des laboratoires étaient passés à des méthodes CPL-SM/SM similaires pour l'analyse des nitrosamines spécifiques au tabac et cette technique a donc été choisie pour servir de base à une nouvelle CRM (méthode recommandée par CORESTA) et compléter la méthode CRM 63. Des expériences initiales communes, des expériences spécifiques menées par des laboratoires individuels ainsi qu'un travail de réflexion continu ont permis d'identifier des aspects méthodologiques qui nécessitaient d'être " standardisés " avant de passer à une CRM.
Une expérience menée en commun par 15 laboratoires a été effectuée en 2010–2011 dans le but d'étudier et d'identifier les caractéristiques méthodologiques importantes qui nécessitaient un contrôle ou une clarification. La méthode CRM numéro 75 a été produite au moyen d'une expérience finale menée en collaboration par 20 laboratoires dans 12 pays et utilisant des machines à fumer aussi bien linéaires que rotatives. Des notes sont inclues dans la CRM pour informer d'autres laboratoires qui pourraient souhaiter adopter la méthode, concernant des aspects qui nécessitent d'être bien contrôlés pour fournir des données aussi fiables que possible et caractérisées par une répétabilité et une reproductibilité similaires.
Des évaluations statistiques ont été effectuées conformément aux lignes directrices de la norme ISO 5725 et sont jointes. Dans des conditions de fumage selon la norme ISO, les niveaux de reproductibilité (R) exprimés en pourcentage de la moyenne des rendements de NAST obtenus par l'ensemble des laboratoires sont bien plus élevés que les niveaux trouvés pour le goudron, la nicotine et le monoxyde de carbone et indiqués dans les normes ISO à ce sujet. La valeur R était exprimée en pourcentage du rendement moyen inter-laboratoires et pour tous les produits étudiés. Selon les conditions de fumage de la norme ISO, les valeurs R% se situaient entre 25–60% pour la NNN, entre 31–85% pour le NNK, entre 47–58% pour la NAT et entre 40–99% pour la NAB. Ces niveaux concordent généralement à ceux déterminés auparavant pour les NAST dans le cadre de la méthode CRM 63 et pour d'autres analytes de fumée étudiés par le Groupe d'étude spécial des analytes.
Dans des conditions de fumage " intensif ", les valeurs R% se situaient entre 30–88% pour la NNN, entre 37–79% pour le NNK, entre 47–83% pour la NAT et entre 42–111% pour la NAB. Une série de valeurs R en désaccord avec les

rendements moyens suggère que le régime de fumage "intensif " fournit des données dont la reproductibilité est similaire ou légèrement moins bonne que le régime ISO en dépit des rendements plus importants générés. [Beitr. Tabakforsch. Int. 25 (2012) 507–519]

## INTRODUCTION

Four tobacco specific nitrosamines (TSNAs) namely, NNN, NAB, NAT and NNK that are found in tobacco and cigarette smoke are of regulatory interest. For example, Health Canada mandates annual measurement of their levels in Canadian brands (1) and the World Health Organization (WHO) Study Group on Tobacco Product Regulation (TobReg) has identified NNN and NNK as 2 of the 9 priority smoke components (2) that they would like to include in future smoke measurements. The WHO is currently developing analytical methodology through the WHO Tobacco Laboratory Network (TobLabNet) in which several CORESTA members participate.

Between 1999 and 2005, the CORESTA Special Analytes Group studied the existing methodologies for the determination of TSNAs in cigarette mainstream smoke. Several analytical methodologies had been published for this determination and it was decided to develop a method involving gas chromatography coupled with a thermal energy analyser (GC-TEA), because this methodology was the most widely used in laboratories at that time (3–6). In 2005, after much work trying to reduce inter-laboratory variability and after carrying out a final collaborative study, CORESTA Recommended Method (CRM) 63 (7) was published.

New methodologies and equipment were becoming available as described in the literature (8–10); were being widely applied in many laboratories and were also being studied by TobLabNet for future use in WHO regulatory proposals (2). By 2009, it had been ascertained that many laboratories had changed their methodologies and applied the 'newer' liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique to measure smoke yields of TSNAs which were capable of higher sample throughput and did not require sample clean-up. The Special Analytes Group decided to investigate an LC-MS/MS method to complement the GC-TEA technique already available as CRM 63 (7).

A "Joint Experiment" was carried out using two reference cigarettes in which 14 laboratories participated, using their in-house LC-MS/MS methodologies and smoking under both the ISO (11) and the Canadian intense (CI) (12) smoking regimes. The applied LC-MS/MS methodology was very similar across laboratories and the reproducibility data compared well between the LC-MS/MS and the GC-TEA method. In general, cigarette mainstream smoke was collected on a Cambridge filter (CF) pad, an internal standard solution was added and after extraction, an aliquot was separated and quantitatively analysed by LC-MS/MS. Subsequently, a general methodology was agreed incorporating key learnings from the joint experiment.

CRM 75 (13) was produced through a final Collaborative Study involving 20 laboratories from 12 countries. The method includes some notes, to inform other laboratories that might wish to adopt it, about some of the main features that need to be well controlled to provide robust and consistent yields and similar repeatability and reproducibility data. Statistical evaluations were made according to ISO guidelines (14, 15).

## EXPERIMENTAL

### *Joint experiment protocol using in-house methodologies*

After conditioning (16), Kentucky reference cigarettes (KY3R4F and KY1R5F) were smoked under both the ISO and CI smoking regimes. Smoke was collected on a CF pad and TSNAs analysed using the in-house LC-MS/MS method that was regularly being used by each participating laboratory. In general, cigarette mainstream smoke was collected, an internal standard was added and after extraction, for example, with ammonium acetate solution, an aliquot was separated and quantitatively analysed by LC-MS/MS. Five replicates for each reference cigarette were generated.

### *Collaborative study using the Recommended Method*

The test samples were chosen to cover both flue-cured Virginia and US blended styles and representing three different “tar” yields (high, medium and low smoking under ISO 3308 (11) for each style. The intention was to choose products at each “tar” level with two different “tar”/nicotine ratios so that the effect of different smoke ratios on reproducibility could also be investigated. The chosen test samples are given in Table 1.

The study protocol was based on an existing methodology used in regular practice by Japan Tobacco with refinements suggested from the learnings during the joint experiment and further guidance notes based on the Sub Group discussions before and after the collaborative study, all of which are now reflected in CRM 75 (13). Five replicates for each reference cigarette were generated. Laboratories were also requested to supply nicotine-free dry particulate matter (NFDPM), nicotine and carbon monoxide (CO) yield data for each of the products.

Some typical LC-MS/MS chromatograms are given in Figure 1.

## RESULTS

### *1. Joint experiment using in-house methodologies*

Participating laboratories provided information on their methodologies by answering a questionnaire. Within their responses, some differences in their methodologies became apparent and were taken into consideration when writing the CRM and helped to provide either clarification or guidance notes in the final version.

- Different numbers of cigarettes were smoked in different laboratories under the ISO smoking regime (5, 10 or 20 per replicate) and under the CI smoking regime (2, 3, 4, 5 or 10 cigarettes per replicate).
- There was an even split between those laboratories using linear and those using rotary smoking machines

**Table 1. Cigarette test samples.**

| Blend style | NFDPM / nicotine ratio | ISO NFDPM and nicotine yields (mg/cig) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Low [a] | | | Medium | | | High | | |
| | | NFDPM | Nicotine | T/N | NFDPM | Nicotine | T/N | NFDPM | Nicotine | T/N |
| US blended | High | 1.7 | *KY1R5F* 0.16 | 10.6 | 8.0 | *Sample 2* 0.61 | 13.3 | 9.5 | *Sample 1* 0.58 | 16.4 |
| US blended | Low | 1.3 | *Sample 7* 0.14 | 9.1 | 6.4 | *Sample 3* 0.62 | 10.4 | 8.2 | *KY3R4F* 0.72 | 11.4 |
| Virginia | High | 1.8 | *Sample 5* 0.18 | 10.0 | | | | 9.7 | *Sample 6* 0.71 | 13.6 |
| Virginia | Low | 3.6 | *Sample 4* 0.40 | 9.2 | | | | 14.2 | *CM6* 1.38 | 10.3 |

[a] T/N = NFDPM / Nicotine ratio.

NOTE: Mean yields determined in this study are given rather than specification data. It can be noted that, in spite of members having good knowledge of European, Canadian, American, Japanese and Chinese products, it was not possible to easily find conventional products with a wider range of T/N ratios than those chosen.

and sometimes laboratories swapped between machine type for the different smoking regimes.

- CF pads were analysed immediately by some laboratories. In other cases, they were stored overnight at 5 °C or for up to one week at room temperature. The volume of solvent to extract TSNAs from the CF pads ranged from 10–100 mL, depending on the number of cigarettes smoked and the diameter of the CF pads. The extraction time ranged from 30 min to overnight. The extraction device was either a wrist action shaker; an orbital shaker or an ultrasonic bath.
- The C18 HPLC column ranged from 2.5–5 µm particle size; 2.0–4.6 mm internal diameter and 50–150 mm length. The mobile phase was either ammonium acetate or acetic acid in water and methanol. Laboratories used individual programming depending on column dimensions with a flow rate of 0.2–1.0 mL depending on the particle size. The column temperature ranged from room temperature to 700 °C.
- For the joint experiment, the ionising mode was electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI). Chromatography separations espe-

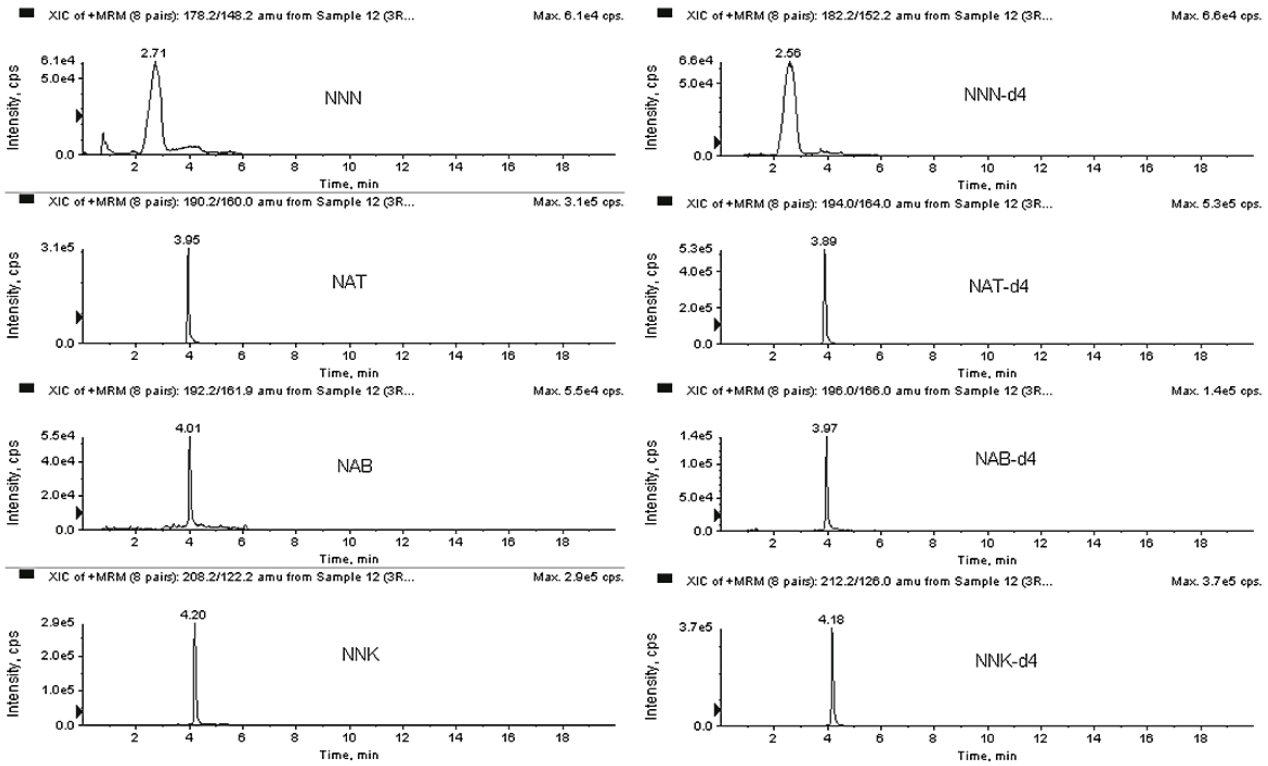


**Figure 1. Chromatograms of TSNAs in mainstream cigarette smoke extract for KY3R4F.**

cially on the first broad NNN peak may require changes in mobile phase from those recommended. For example, one laboratory reported that the peak shape of deuterated NNN is improved by using aqueous ammonium acetate, depending on the column and when applying the APCI ionisation system.
- Some laboratories used four deuterated internal standards (NNN-d4; NNK-d4; NAT-d4 and NAB-d4) whereas others only used two (NNN-d4 and NNK-d4). No laboratory forced the calibration through zero.
- Calibration standard stock solutions were stored for 5 months to one year in a freezer (< 0 °C) or refrigerator (< 4 °C). Working solutions were stored from one week to six months in a refrigerator.
- The majority of laboratories added the internal standard solution to the smoked CF pad and then added the extraction solvent at the start of the extraction process.

It is not the intention to report the full analyses from the joint experiment but to concentrate discussions on the collaborative study. A summary of the TSNA yield data from 14 laboratories participating in the joint experiment is given in Table 2. The 2005 KY2R4F reference cigarette data have been included to enable a comparison between the performance of CRM 63 and CRM 75. It is not ideal since the KY2R4F reference is no longer available but shows that LC-MS/MS methodology provided yields with similar levels of data variability between laboratories to that given by GC-TEA (7, 13). TSNA yields for the KY3R4F reference cigarette appear somewhat lower than the yields from the KY2R4F reference cigarettes and are probably due to blend changes.

*Collaborative study using the Recommended Method*

The method involves smoke collection on a CF pad. An internal standard solution is added to the smoke collected on the pad and, after extraction, an aliquot is separated and quantitatively analysed by liquid chromatography - tandem mass spectrometry (LC-MS/MS). In contrast to CRM 63, by applying the LC-MS/MS methodology, no sample clean-up and concentration step is needed to provide the necessary measurement sensitivity.

*2.1. Observations on internal validation*

The LC-MS/MS method is stable between different operators, fast and robust. It is necessary to validate all aspects of the method internally. The method allows scope for the use of different technical equipment and other aspects such as chromatographic separation conditions to be optimised within each participating laboratory during their validation procedure. Examples of operating conditions that work well in practice in many laboratories are given in the CRM along with notes on some of the parts of the method where caution is required. In this regard, some laboratories observed that:
- Mixing the internal standard with the extraction solution gives less variable data between replicates. The extraction volume may need to be adjusted when using different filter pad sizes.The calibration curve should be extended to the highest measured yields.
- Chromatography retention time may be different depending on column specification and performance.
- The first ion source in the detectors requires frequent cleaning. For example, one laboratory reported that the chromatography profile may be less clean for the 5$^{th}$ replicate on the 5$^{th}$ day of the defined protocol. Another laboratory reported that the type of LC pumps may affect the chromatography.
- Different laboratories use different methods to derive their limits of detection (LOD) and limits of quantification (LOQ). Any conclusions on such values should be made cautiously. The LOQ/LOD could be based on signal to noise ratio/first transition but laboratories may use either the first or the second transition. Calculation of the LOQ/LOD could also be based on the value of the standard deviation of the lowest standard. Furthermore, differences in conditions and sensitivity of

**Table 2. Summary of joint experiment data in ng/cig.** Data from 2005 study using 2R4F are also included for comparison.

| Smoke analyte | KY1R5F | | | | KY3R4F | | | |
|---|---|---|---|---|---|---|---|---|
| | ISO regime | | CI regime | | ISO regime | | CI regime | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| | *Data is given from 14 laboratories for TSNAs by LC-MS/MS analysis* | | | | | | | |
| NNN | 45.1 | 3.7 | 241.3 | 22.4 | 115.8 | 7.7 | 285.4 | 20.5 |
| NNK | 22.3 | 3.3 | 114.4 | 15.4 | 97.8 | 10.2 | 245.4 | 24.2 |
| NAT | 44.3 | 8.2 | 225.0 | 36.9 | 114.6 | 18.6 | 268.6 | 41.0 |
| NAB | 6.1 | 0.8 | 26.4 | 4.0 | 12.5 | 1.7 | 28.8 | 3.8 |
| | *Data is given from 3 laboratories for TSNAs by GC-TEA analysis.* | | | | | | | |
| NNN | 43.1 | 6.1 | 208.2 | 29.9 | 105.8 | 16.9 | 259.2 | 39.0 |
| NNK | 21.9 | 0.9 | 97.9 | 7.2 | 93.3 | 12.1 | 206.1 | 13.4 |
| NAT | 38.5 | 8.8 | 173.8 | 32.2 | 92.1 | 15.3 | 223.2 | 37.9 |
| NAB | 6.1 | 1.8 | 21.8 | 5.2 | 10.5 | 1.9 | 30.6 | 5.9 |
| | | | | | *KY2R4F (2005 data)* | | | |
| NNN | — | — | — | — | 146.0 | 18.2 | — | — |
| NNK | — | — | — | — | 141.4 | 26.2 | — | — |
| NAT | — | — | — | — | 143.4 | 25.6 | — | — |
| NAB | — | — | — | — | 16.6 | 3.3 | — | — |

technical equipment could influence individual LOD and LOQ values. However, laboratories should ensure that the LOQ should significantly exceed the level of the lowest calibration standard.

*2.2. Data analysis for collaborative study*

Twenty laboratories reported mainstream TSNA yields after smoke collection under both the ISO and CI regimes.

*2.2.1. Outlier analysis*

Statistical calculations were based on ISO guidelines and applied to determine estimates of repeatability (r) and reproducibility (R) of smoke yields where all of the participating laboratories used the same analytical method (14, 15). As a preliminary step, the subsets of data were analysed for the presence of outliers using the Mandel's *k* and *h* graphical consistency techniques. However, decisions regarding actual removal of data from subsets were made based on the Cochran's and Grubbs' numerical outlier tests prior to the estimation of the repeatability (r) and reproducibility (R) limits, with outliers being removed and stragglers retained. The data set, after removal of outliers identified in Table 3, was used to provide estimates of means, repeatability and reproducibility. It can be observed that there were slightly fewer outliers for the intense smoking regime possibly due to the higher variability of that regime as discussed below.

It was clear when assessing the raw data that Laboratory 16 was a fairly consistent outlier among all test samples under both mainstream ISO and CI smoking regimes. Consequently all data for this laboratory were removed prior to performing any of the previously described statistical analyses.

**Table 3. Summary of laboratory codes with variance outliers (Cochran) and mean outliers (Grubbs).**

| Sample | TSNA | ISO smoking regime | | TSNA | Intense smoking regime | |
|---|---|---|---|---|---|---|
| | | Variance outliers [a] | Mean outliers | | Variance outliers | Mean outliers |
| 1 | NNN | 8, 12 | 7 | NNN | 8 | |
| | NAT | 8 (4), 12 (2) | | NAT | 15 (2) | |
| | NAB | 12 (2) | | NAB | | |
| | NNK | | 7 | NNK | | |
| 2 | NNN | | | NNN | | |
| | NAT | 9 (2) | | NAT | 19 (4) | |
| | NAB | 19 (4) | | NAB | | |
| | NNK | 1 | | NNK | 17 (single rep), 19 | |
| 3 | NNN | | 14 | NNN | | |
| | NAT | | | NAT | 6 (2+4) | |
| | NAB | 19 (2+4) | | NAB | | |
| | NNK | 10 (single replicate) | | NNK | | |
| 4 | NNN | | | NNN | | |
| | NAT | 19 (2), 19 (4) | | NAT | | |
| | NAB | 21 (4) | | NAB | 9 (4), 17 (4), 19 (2+4) | |
| | NNK | | | NNK | 19, 21 | |
| 5 | NNN | | | NNN | 17, 19 | |
| | NAT | | | NAT | | |
| | NAB | 9 (2+4), 12 (4), 19 (2+4) | | NAB | | |
| | NNK | | 15 | NNK | | |
| 6 | NNN | 10 | 14 | NNN | | |
| | NAT | | 8 (2) | NAT | 19 (2+4) | |
| | NAB | 12 (4), 21 (4) | | NAB | 12 (4), 19 (4) | |
| | NNK | 4 and 11 (single reps) | | NNK | 19, 21 | |
| 7 | NNN | 9 | | NNN | 1 | 11 |
| | NAT | | | NAT | | 8 (2) |
| | NAB | 19 (2) | | NAB | 19 (2+4) | |
| | NNK | 9, 21 | | NNK | | |
| CM6 | NNN | | 14 | NNN | | 14 |
| | NAT | | 8 (2) | NAT | 17 (2) | |
| | NAB | 9 (2+4), 10 (2), 19 (2+4), 21 (2), 21 (4, single rep) | | NAB | 9 (2+4), 21 (4) | |
| | NNK | | | NNK | | |
| KY1R5F | NNN | | | NNN | | 11 |
| | NAT | | | NAT | 6 (4) | |
| | NAB | | 7 (2) | NAB | 9 (2), 19 (2+4) | |
| | NNK | | | NNK | | |
| KY3R4F | NNN | 19 | | NNN | | |
| | NAT | 15 (2), 6 (4) | | NAT | | |
| | NAB | 10 (4) | | NAB | | |
| | NNK | | | NNK | | |

[a] Figures in brackets denote the use of either 2 or 4 calibration standards

**Table 4. Mean yields, repeatability and reproducibility data obtained using CORESTA Recommended Method 75**.

| Sample | | NNN (ng/cig) | | | | NNK (ng/cig) | | | | NAT (ng/cig) | | | | NAB (ng/cig) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N [a] | M | r | R | N | M | r | R | N | M | r | R | N | M | r | R |
| *Yields from smoking under the ISO regime* | | | | | | | | | | | | | | | | |
| 1 | 16 | 277 | 47 | 70 | 18 | 133 | 33 | 41 | 16 | 145 | 27 | 74 | 17 | 20.0 | 5.2 | 9.0 |
| 2 | 18 | 37.3 | 8.8 | 12.4 | 17 | 24.5 | 7.8 | 10.2 | 16 | 40.5 | 8.2 | 22.5 | 14 | 5.3 | 1.4 | 2.7 |
| 3 | 17 | 24.0 | 6.7 | 7.8 | 18 | 17.9 | 5.3 | 9.0 | 16 | 27.9 | 6.7 | 16.2 | 14 | 3.7 | 1.5 | 2.1 |
| 4 | 18 | 9.56 | 4.2 | 5.8 | 14 | 3.6 | 1.7 | 3.0 | 15 | 11.0 | 3.4 | 6.2 | 13 | 1.5 | 0.6 | 0.9 |
| 5 | 18 | 12.1 | 3.6 | 5.6 | 13 | 3.3 | 1.8 | 2.1 | 16 | 12.8 | 3.5 | 7.2 | 11 | 1.8 | 0.5 | 0.9 |
| 6 | 16 | 22.7 | 10.8 | 11.9 | 15 | 7.2 | 2.7 | 5.7 | 16 | 27.9 | 8.5 | 16.1 | 13 | 3.6 | 1.3 | 3.5 |
| 7 | 17 | 10.5 | 3.0 | 4.8 | 15 | 7.4 | 1.9 | 3.1 | 16 | 14.4 | 3.5 | 6.8 | 14 | 1.8 | 0.6 | 0.9 |
| CM 6 | 18 | 20.0 | 5.1 | 8.1 | 17 | 26.5 | 6.5 | 8.6 | 17 | 33.7 | 8.5 | 18.6 | 13 | 3.7 | 0.9 | 2.7 |
| KY1R5F | 19 | 44.4 | 8.6 | 16.7 | 19 | 21.8 | 3.9 | 8.0 | 17 | 45.8 | 9.2 | 23.4 | 16 | 6.5 | 1.5 | 2.9 |
| KY3R4F | 18 | 115 | 18 | 34 | 19 | 97.1 | 14.6 | 30.5 | 16 | 113 | 14 | 55 | 16 | 13.0 | 2.3 | 5.2 |
| *Yields from smoking under the CI regime* | | | | | | | | | | | | | | | | |
| 1 | 18 | 603 | 122 | 225 | 18 | 297 | 71 | 144 | 17 | 322 | 64 | 214 | 17 | 42.9 | 10.8 | 21.4 |
| 2 | 18 | 87.5 | 27.9 | 35.9 | 17 | 55.5 | 15.7 | 21.2 | 15 | 91.2 | 20.5 | 58.9 | 16 | 11.8 | 3.6 | 7.3 |
| 3 | 18 | 68.6 | 19.7 | 29.9 | 18 | 49.9 | 16.3 | 19.5 | 15 | 76.8 | 19.7 | 47.7 | 16 | 10.1 | 4.1 | 7.2 |
| 4 | 18 | 34.9 | 16.8 | 28.6 | 13 | 12.1 | 3.2 | 9.3 | 16 | 39.4 | 9.9 | 25.2 | 14 | 5.5 | 2.9 | 6.2 |
| 5 | 16 | 51.2 | 14.0 | 42.4 | 17 | 15.0 | 7.2 | 11.9 | 16 | 54.0 | 15.8 | 44.7 | 15 | 6.7 | 2.5 | 5.0 |
| 6 | 18 | 48.0 | 23.5 | 32.8 | 15 | 14.3 | 4.2 | 8.4 | 15 | 54.0 | 16.7 | 33.0 | 14 | 7.5 | 2.7 | 6.2 |
| 7 | 16 | 63.6 | 17.6 | 23.3 | 18 | 46.6 | 13.9 | 23.2 | 16 | 83.0 | 16.7 | 49.1 | 15 | 8.7 | 1.9 | 4.7 |
| CM 6 | 18 | 37.9 | 14.4 | 21.3 | 19 | 50.8 | 16.5 | 27.4 | 17 | 64.8 | 17.1 | 40.3 | 15 | 7.5 | 2.2 | 6.4 |
| KY1R5F | 18 | 237 | 49 | 72 | 19 | 121 | 20 | 52 | 16 | 230 | 37 | 138 | 16 | 27.8 | 5.3 | 12.2 |
| KY3R4F | 19 | 297 | 73 | 88 | 19 | 252 | 58 | 92 | 17 | 279 | 63 | 132 | 17 | 31.2 | 7.7 | 13.2 |

[a] N = number of laboratories (outliers removed); M = mean yield; r = repeatability and R = reproducibility at 95% confidence interval. Data have been rounded to one decimal point.

*2.2.2. Determination of inter-laboratory repeatability and reproducibility variance*

For each of the test samples the estimates of the mean, repeatability and reproducibility were determined according to ISO 5725-2 section 7.4.4 (15) using the data that remained following the removal of outliers. These are given in Table 4.

Levels of repeatability are similar to those found in other studies (7, 18). The reproducibility (R) value was expressed as a percentage of the mean yield among-laboratories and across all of the studied products. In this way, a comparison of the reproducibility of the four TSNAs relative to each other and relative to NFDPM, nicotine and CO yields obtained from the same samples can be made as shown in Table 5.

Under ISO smoking, the levels of reproducibility of TSNA yields among laboratories are much greater than the levels given in ISO 4387 (17) for NFDPM and nicotine. Under ISO smoking R% values ranged from 25–60% for NNN; from 31–85% for NNK; from 47–58% for NAT and 40–99% for NAB. These levels are generally in line with those determined for selected volatiles (18). Interestingly for TSNAs, the lowest "tar" yielding products (< 2mg) did not necessarily give the most variable data. Under 'intense' smoking, R% values ranged from 30–88% for NNN; from 37–79% for NNK; from 47–83% for NAT and 42–111% for NAB.

Graphical plots of yield reproducibility 'R' against mean yields from this work are shown for total particulate matter (TPM) in Figure 2 and for the four TSNAs in Figures 3 to 6. All five analytes were measured on the same samples.

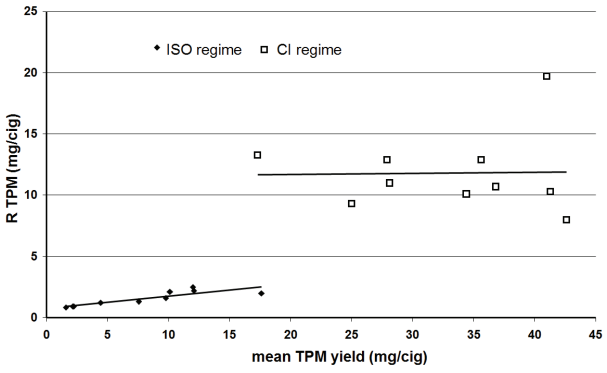


**Figure 2. Relationship of TPM reproducibility (R) to TPM yield.**

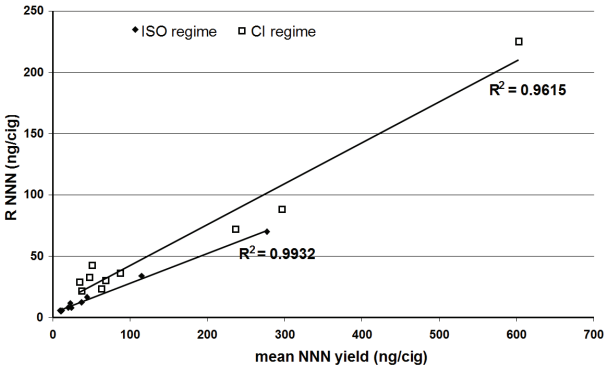


**Figure 3. Relationship of NNN reproducibility (R) to NNN yield.**

**Table 5. Comparison of reproducibility of TNCO and TSNA yields.**

| Sample | NFDPM | | Nicotine | | CO | | NNN | | NNK | | NAT | | NAB | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (mg/cig) | R (%) | Mean (mg/cig) | R (%) | Mean (mg/cig) | R (%) | Mean (ng/cig) | R (%) | Mean (ng/cig) | R (%) | Mean (ng/cig) | R (%) | Mean (ng/cig) | R (%) |
| *ISO smoking regime* | | | | | | | | | | | | | | |
| 1 | 9.5 | 19 | 0.58 | 15 | 9.7 | 26 | 277 | 25 | 133 | 31 | 145 | 51 | 20.0 | 45 |
| 2 | 8.0 | 16 | 0.61 | 11 | 9.2 | 22 | 37.3 | 33 | 24.5 | 42 | 40.5 | 56 | 5.3 | 51 |
| 3 | 6.4 | 12 | 0.62 | 23 | 7.5 | 22 | 24.0 | 33 | 17.9 | 50 | 27.9 | 58 | 3.7 | 56 |
| 4 | 3.6 | 25 | 0.40 | 28 | 4.4 | 36 | 9.6 | 60 | 3.6 | 85 | 11.0 | 56 | 1.5 | 57 |
| 5 | 1.8 | 46 | 0.18 | 25 | 2.4 | 41 | 12.1 | 46 | 3.3 | 62 | 12.8 | 56 | 1.6 | 49 |
| 6 | 9.7 | 20 | 0.71 | 14 | 12.0 | 22 | 22.7 | 52 | 7.2 | 79 | 27.9 | 58 | 3.6 | 99 |
| 7 | 1.3 | 62 | 0.14 | 30 | 2.0 | 39 | 10.5 | 46 | 7.4 | 41 | 14.4 | 47 | 1.8 | 53 |
| CM 6 | 14.2 | 14 | 1.38 | 15 | 14.2 | 17 | 20.0 | 41 | 26.5 | 32 | 33.7 | 55 | 3.7 | 71 |
| KY1R5F | 1.7 | 54 | 0.16 | 37 | 3.2 | 35 | 44.4 | 38 | 21.8 | 37 | 45.8 | 51 | 6.5 | 45 |
| KY3R4F | 8.2 | 13 | 0.72 | 19 | 11.1 | 21 | 115 | 30 | 97.1 | 31 | 113 | 49 | 13.0 | 40 |
| *CI smoking regime* | | | | | | | | | | | | | | |
| 1 | 25.25 | 43 | 1.26 | 33. | 24.5 | 17 | 603 | 37 | 295 | 48 | 322 | 66 | 42.9 | 50 |
| 2 | 23.56 | 23 | 1.52 | 28 | 24.7 | 13 | 87.5 | 41 | 55.5 | 38 | 91.2 | 65 | 11.8 | 62 |
| 3 | 23.16 | 23 | 1.75 | 14 | 24.6 | 16 | 68.6 | 44 | 49.9 | 39 | 76.8 | 62 | 10.1 | 71 |
| 4 | 18.37 | 30 | 1.63 | 29 | 23.5 | 21 | 34.9 | 82 | 12.1 | 77 | 39.4 | 64 | 5.5 | 111 |
| 5 | 12.46 | 71 | 0.91 | 46 | 19.3 | 68 | 51.2 | 83 | 15.0 | 79 | 54 | 83 | 6.7 | 75 |
| 6 | 22.89 | 26 | 1.68 | 31 | 23.9 | 16 | 48.0 | 68 | 14.3 | 59 | 54 | 61 | 7.5 | 84 |
| 7 | 15.67 | 32 | 1.10 | 22 | 22.5 | 18 | 63.6 | 36 | 46.6 | 50 | 83 | 59 | 8.7 | 53 |
| CM 6 | 29.14 | 15 | 2.75 | 24 | 26.1 | 15 | 37.9 | 56 | 50.8 | 54 | 64.8 | 62 | 7.5 | 86 |
| KY1R5F | 17.64 | 31 | 1.04 | 23 | 28.6 | 21.8 | 237 | 30 | 121 | 43 | 229.8 | 60 | 27.8 | 44 |
| KY3R4F | 25.87 | 22 | 1.96 | 22 | 30.9 | 18.6 | 297 | 30 | 252 | 37 | 279 | 47 | 31.2 | 42 |

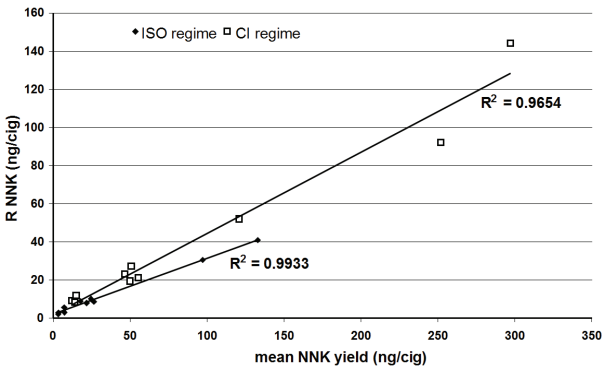


**Figure 4. Relationship of NNK reproducibility (R) to NNK yield.**

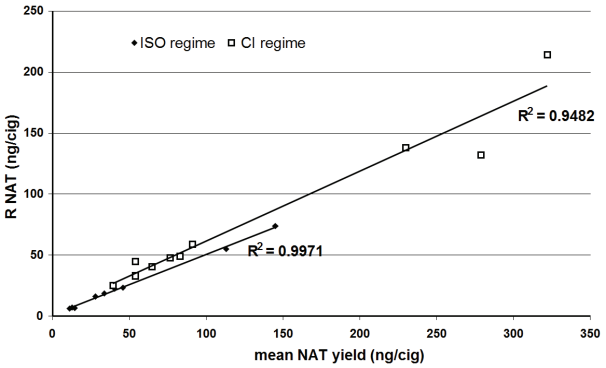


**Figure 5. Relationship of NAT reproducibility (R) to NAT yield.**

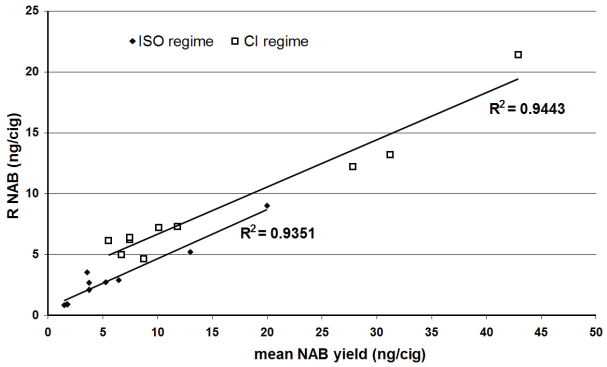


**Figure 6. Relationship of NAB reproducibility (R) to NAB yield.**

These findings suggest that the 'intense' regime gives similar or even slightly worse yield reproducibility than the ISO regime for TSNAs in spite of the higher yields generated. This has been found previously in CORESTA and ISO collaborative studies for TPM, NFDPM and water yields to a greater extent and for nicotine yields to a much lesser extent whereas CO yields appeared unaffected (19, 20). This may have implications in determining TSNA measurement tolerances for regulatory purposes.

**Table 6. Effect of NFDPM / nicotine ratio on reproducibility (R) expressed as percentage of mean.**

| T / N ratio | Analyte | Low ISO "tar" | | Medium ISO "tar" | | High ISO "tar" | |
|---|---|---|---|---|---|---|---|
| | Smoking regime | ISO | CI | ISO | CI | ISO | CI |
| | | *T/N = 10.6* | | *T/N = 13.3* | | *T/N = 16.4* | |
| High | NNN | 38 | 30 | 33 | 41 | 25 | 37 |
| | NNK | 37 | 43 | 42 | 38 | 31 | 48 |
| | NAT | 51 | 60 | 56 | 65 | 51 | 66 |
| | NAB | 45 | 44 | 51 | 62 | 45 | 50 |
| | | *T/N = 9.1* | | *T/N = 10.4* | | *T/N = 11.4* | |
| Low | NNN | 46 | 36 | 33 | 44 | 30 | 30 |
| | NNK | 41 | 50 | 50 | 39 | 31 | 37 |
| | NAT | 47 | 59 | 58 | 62 | 49 | 47 |
| | NAB | 53 | 53 | 56 | 71 | 40 | 42 |
| | | *T/N = 10.0* | | | | *T/N = 13.6* | |
| High | NNN | 46 | 83 | | | 52 | 68 |
| | NNK | 62 | 79 | | | 79 | 59 |
| | NAT | 56 | 83 | | | 58 | 61 |
| | NAB | 49 | 75 | | | 99 | 84 |
| | | *T/N = 9.2* | | | | *T/N = 10.3* | |
| Low | NNN | 60 | 82 | | | 41 | 56 |
| | NNK | 85 | 77 | | | 32 | 54 |
| | NAT | 56 | 64 | | | 55 | 62 |
| | NAB | 57 | 111 | | | 71 | 86 |

*2.2.3. Effect of the NFDPM/nicotine ratio on TSNA reproducibility*

Table 6 shows the reproducibility data expressed as a percentage of the mean and grouped according to the NFDPM/nicotine ratio. There are some random changes but no clear trends and so it is important not to over-interpret the gathered data. For TSNAs, it appears that neither the ISO "tar" yield of the studied products nor the different NFDPM/ nicotine ratio significantly affect the reproducibility data when expressed as a percentage of the mean.

*2.2.4. Effect of number of calibration standards on reproducibility*

Since NNN and NNK calibration standards were used by all participating laboratories, the evaluation obtained with these 2 internal standards is only useful for NAT and NAB results. Thirteen of the 19 laboratories provided data for calibration procedures involving 2 deuterated standards (NNN-d4 and NNK-d4) and 4 deuterated standards (NNN-d4, NNK-d4, NAT-d4 and NAB-d4).
Of these 13 laboratories, 9 used exactly the same set of test samples (paired) in applying either 2 or 4 internal standards in their calibration procedures. This was evidenced by the fact that the reported NNN and NNK results with either 2 or 4 internal standards were identical. For the laboratories that did not appear to use paired test samples, the results for both NNN and NNK with either 2 or 4 internal standards were different for all test samples. These labs were excluded from the proceeding statistical analysis on NAT and NAB data.
Based on the comparisons in both the ISO and CI data sets, there was no evidence to suggest that there exists a similar systematic bias across all laboratories but, rather, a number of labs do show evidence of unique bias within their own laboratories when applying 2 calibration standards as compared to 4 calibration standards. For that reason the data are not broken down in this report and it is recommended to apply four internal standards. The guidance note in the recommended method states that when laboratories either have problems obtaining 4 internal standards or have either checked and/or validated that using 2 internal standards gives comparable data then the method can be run using NNK-d4 as a substitute for the NAT and NAB standards.
For samples that have low NAT yields, such as some Chinese blends, it was recommended to use 4 standards. Four standards may also make data more robust, for example if analysis is intended for regulatory purposes.

*2.2.5. Effect of smoking machine type on reproducibility*

The mean yields, repeatability and reproducibility values, separated by smoking machine type (linear and rotary), under both smoking regimes are shown in Tables 7 and 8 for NNN and NNK respectively.
Various designs and manufacturers of smoking machines have been grouped together and some conclusions have already been drawn. In the following analysis machines have been grouped as either linear or rotary machines. For the ISO smoking, 11 laboratories used linear and 9 used rotary smoking machines. For the CI smoking, 12 used linear and 8 used rotary smoking machines.
Under the CI regime, the mean NNN and NNK yields from linear machines tend to be slightly higher than from rotary machines although this is not consistent and not significant. There appears to be a slight tendency for the within-lab NNK repeatability variance from linear machines to be greater than or equal to that from rotary machines. Overall, no significant differences in yield reproducibility of TSNAs were found between machine types when smoking under either the ISO or CI regimes.

**Table 7. NNN mean yield, comparisons of repeatability and reproducibility [a] standard deviations between linear and rotary smoking machines.**

| Sample | NNN mean yield (ng/cig) | | | | NNN repeatability - SD (ng/cig) | | | | NNN reproducibility- SD (ng/cig) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Linear (L) | Rotary (R) | *p* (*t*-test) | Result | Linear (L) | Rotary (R) | *p* (*F*-test) | Result | Linear (L) | Rotary (R) | *p* (F-test) | Result |
| *ISO smoking regime - 11 laboratories used linear and 9 used rotary smoking machines* | | | | | | | | | | | | |
| 1 | 283 | 272 | 0.043 | L > R | 18 | 15 | 0.164 | | 28 | 21 | 0.171 | |
| 2 | 36.7 | 38.0 | 0.185 | | 3.5 | 2.5 | 0.034 | L > R | 4.2 | 4.6 | 0.172 | |
| 3 | 24.3 | 23.4 | 0.139 | | 2.5 | 2.2 | 0.287 | | 2.9 | 2.6 | 0.464 | |
| 4 | 9.5 | 9.6 | 0.949 | | 1.5 | 1.5 | 0.471 | | 1.9 | 2.3 | 0.170 | |
| 5 | 11.6 | 12.7 | 0.009 | L < R | 1.4 | 1.1 | 0.097 | | 2.0 | 1.9 | 0.474 | |
| 6 | 22.0 | 23.7 | 0.079 | | 3.8 | 3.9 | 0.419 | | 4.5 | 3.9 | <0.001 | L > R |
| 7 | 10.1 | 11.0 | 0.013 | L < R | 1.1 | 1.1 | 0.468 | | 1.9 | 1.4 | 0.089 | |
| CM 6 | 20.8 | 19.0 | 0.002 | L > R | 1.9 | 1.6 | 0.162 | | 3.3 | 1.9 | 0.016 | L > R |
| KY1R5F | 42.4 | 46.6 | <0.001 | L < R | 3.3 | 2.8 | 0.185 | | 6.6 | 4.2 | 0.064 | |
| KY3R4F | 112 | 118 | 0.026 | L < R | 6.0 | 6.0 | 0.379 | | 14 | 9.4 | 0.065 | |
| *CI smoking regime - 12 laboratories used linear and 8 used rotary smoking machines* | | | | | | | | | | | | |
| 1 | 634 | 564 | <0.001 | L > R | 44 | 43 | 0.472 | | 80 | 61 | 0.159 | |
| 2 | 88.0 | 86.9 | 0.682 | | 10.5 | 9.0 | 0.187 | | 13 | 13 | 0.245 | |
| 3 | 70.1 | 66.7 | 0.118 | | 6.3 | 7.7 | 0.103 | | 8.1 | 13 | 0.038 | L < R |
| 4 | 37.5 | 31.7 | 0.005 | L > R | 7.1 | 4.0 | 0.001 | L > R | 11 | 7.9 | 0.243 | |
| 5 | 57.9 | 44.4 | <0.001 | L > R | 4.6 | 5.3 | 0.225 | | 13 | 14 | 0.432 | |
| 6 | 48.7 | 47.2 | 0.538 | | 7.3 | 9.4 | 0.065 | | 8.0 | 15 | 0.004 | L < R |
| 7 | 62.6 | 64.8 | 0.254 | | 6.0 | 6.5 | 0.350 | | 7.4 | 9.5 | 0.145 | |
| CM 6 | 39.6 | 35.2 | 0.006 | L > R | 4.7 | 5.7 | 0.118 | | 7.8 | 6.4 | 0.099 | |
| KY1R5F | 241 | 234 | 0.194 | | 21 | 10 | <0.001 | L > R | 30 | 19 | 0.205 | |
| KY3R4F | 305 | 286 | 0.004 | L > R | 29 | 20 | 0.009 | L > R | 32 | 26 | 0.424 | |

For Tables 7 and 8, differences in means were evaluated on the raw data with outliers removed using a two-sided *t*-test (*p* values given). Differences in repeatability variance (*i.e.,* within-lab variance) were evaluated using the *F*-test ratio of repeatability variances (i.e. $(s_r)^2$) with degrees of freedom = *pc* x (*n*-1) where *pc* is the number of machines in the class and *n* = 5 is the number of replicates. Differences in reproducibility variance (*i.e.,* lab-to-lab variance) were evaluated using an *F*-test ratio of a component, $(s_d)^2$, of the between-laboratory variance statistic $(s_L)^2$ with degrees of freedom = *pc* - 1 where *pc* is the number of machines in the class.

[a] The comparisons of reproducibility were not done on the actual reproducibility variances, but rather on the between-laboratory variance statistical component $(s_d)^2$.

**Table 8. NNK mean yield, comparisons of repeatability and reproducibility [a] standard deviations between linear and rotary smoking machines.**

| Sample | NNK mean yield (ng/cig) | | | | NNK repeatability SD (ng/cig) | | | | NNK reproducibility SD (ng/cig) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Linear (L) | Rotary (R) | *p* (t-test) | Result | Linear (L) | Rotary (R) | *p* (*F*-test) | Result | Linear (L) | Rotary (R) | *p* (F-test) | Result |
| *ISO smoking regime - 11 laboratories used linear and 9 used rotary smoking machines* | | | | | | | | | | | | |
| 1 | 137 | 128 | 0.004 | L > R | 14 | 8.0 | 0.001 | L > R | 15 | 12 | 0.508 | |
| 2 | 25.0 | 23.8 | 0.127 | | 3.0 | 2.4 | 0.117 | | 3.6 | 3.5 | 0.345 | |
| 3 | 19.1 | 16.4 | <0.001 | L > R | 1.6 | 2.2 | 0.032 | L < R | 2.9 | 2.9 | 0.361 | |
| 4 | 3.8 | 3.11 | 0.007 | L > R | 0.71 | 0.34 | 0.001 | L > R | 1.0 | 1.1 | 0.283 | |
| 5 | 3.44 | 3.19 | 0.170 | | 0.69 | 0.53 | 0.103 | | 0.75 | 0.70 | 0.319 | |
| 6 | 7.35 | 7.0 | 0.468 | | 0.96 | 0.91 | 0.390 | | 1.6 | 2.7 | 0.040 | L < R |
| 7 | 7.77 | 6.79 | <0.001 | L > R | 0.75 | 0.52 | 0.028 | L > R | 1.1 | 0.85 | 0.403 | |
| CM 6 | 27.0 | 26.0 | 0.144 | | 2.6 | 1.98 | 0.044 | L > R | 3.1 | 3.0 | 0.353 | |
| KY1R5F | 22.1 | 21.5 | 0.288 | | 1.5 | 1.2 | 0.064 | | 3.4 | 2.2 | 0.091 | |
| KY3R4F | 96.5 | 97.8 | 0.580 | | 5.7 | 4.5 | 0.068 | | 11 | 11 | 0.421 | |
| *CI smoking regime - 12 laboratories used linear and 8 used rotary smoking machines* | | | | | | | | | | | | |
| 1 | 320 | 260 | <0.001 | L > R | 26 | 25 | 0.423 | | 47 | 32 | 0.095 | |
| 2 | 55.9 | 55.2 | 0.655 | | 6.0 | 5.1 | 0.184 | | 8.1 | 7.0 | 0.384 | |
| 3 | 52.1 | 47.3 | 0.001 | L > R | 5.3 | 6.3 | 0.154 | | 6.2 | 6.8 | 0.486 | |
| 4 | 13.3 | 10.1 | <0.001 | L > R | 1.1 | 1.2 | 0.343 | | 3.0 | 2.9 | 0.510 | |
| 5 | 16.9 | 12.3 | <0.001 | L > R | 2.6 | 2.4 | 0.338 | | 3.9 | 3.0 | 0.189 | |
| 6 | 15.6 | 12.4 | <0.001 | L > R | 1.8 | 1.0 | 0.003 | L > R | 2.7 | 2.3 | 0.466 | |
| 7 | 48.0 | 45.0 | 0.083 | | 4.5 | 5.3 | 0.158 | | 8.5 | 7.9 | 0.329 | |
| CM 6 | 53.5 | 47.1 | 0.001 | L > R | 5.8 | 5.8 | 0.484 | | 11 | 7.1 | 0.051 | |
| KY1R5F | 127 | 114 | <0.001 | L > R | 8.0 | 6.0 | 0.020 | L > R | 22 | 9.3 | 0.008 | L > R |
| KY3R4F | 260 | 241 | 0.005 | L > R | 22 | 17 | 0.063 | | 30 | 35 | 0.154 | |

[a] The comparisons of reproducibility were not done on the actual reproducibility variances, but rather on the between-laboratory variance statistical component $(s_d)^2$.

## CONCLUSIONS

The CORESTA Special Analytes Sub Group has developed a CRM to determine levels of four TSNAs (NNN, NNK, NAT and NAB) in mainstream smoke by LC-MS/MS. The CRM optimises key components of the methodology that contribute to variability of test results.

Levels of variability from reference products under ISO smoking compared well between the LC-MS/MS and the GC-TEA methods.

There was similar or worse yield reproducibility under the CI regime than the ISO regime for all TSNAs. The relative levels of reproducibility among laboratories for the studied analytes are much greater than the levels found for "tar", nicotine and carbon monoxide given in ISO 4387 (17). Their levels were in line with those previously evaluated during work on CRM 58 for benzo[*a*]pyrene (21) and the previously developed CRM for TSNAs (7).

The evaluation obtained with 2 versus 4 internal standards is useful for NAT and NAB results. A number of laboratories showed evidence of unique bias within their own laboratories when applying 2 as compared to 4 calibration standards. Therefore, the CRM recommends applying four internal standards with caveats included in the guidance notes.

In some laboratories, the CI regime tends to give higher yields from the linear smoking machines than from rotary machines. However, these differences are inconsistent and are not statistically significant.


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