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Determination of Hydrogen Cyanide in Cigarette Smoke by Continuous Flow Analysis Method Using Safer Chemistry *

by

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SUMMARY

A new safe and sensitive method to determine hydrogen cyanide (HCN) in cigarette smoke using continuous flow analyser (CFA) has been developed and validated. The use of highly toxic potassium cyanide (KCN) as a standard necessitates the development of a safer method for the determination of HCN in cigarette smoke. In this described method KCN is replaced by less toxic potassium tetracyanozincate (Lethal Dose LD_{50} oral is 7.49 mg/kg for KCN and 2000 mg/kg for potassium tetracyanozincate). Furthermore, the new method uses isonicotinic acid-barbituric acid (coupling reagent) instead of pyridine-pyrazolone as a reagent for the determination of HCN, and hence eliminates the use of pyridine. In this method HCN is trapped on both the Cambridge Filter Pad, then extracted with aqueous sodium hydroxide solution, and in an impinger containing the same solution. The solution thus extracted is oxidised to cyanogen chloride by Chloramine-T and treated with coupling reagent, the resulting stable chromophore was measured colorimetrically at 600 nm. The regression equation was linear in the range of 1 to 25 µg/mL for cyanide with a correlation coefficient (R^2) > 0.9998. The limit of detection (LOD) was 0.76 µg/cig and the overall relative standard deviation (RSD) of the method was less than 10%. Excellent recoveries of cyanide were obtained in the range from 92% to 112% and the HCN yields from the Kentucky Reference Cigarette 3R4F obtained from the newly developed method are in good agreement with those from the conventional KCN method. The proposed method is robust, reliable, selective and safer than any of the existing methods for determination of hydrogen cyanide in mainstream as well as in sidestream cigarette smoke. [Beitr. Tabakforsch. Int. 28 (2018) 191–202]

ZUSAMMENFASSUNG

Es wurde eine neue, sichere und empfindliche Methode zur Bestimmung von Cyanwasserstoff (HCN) im Zigarettenrauch unter Einsatz eines Continuous-Flow-Analysators (CFA) entwickelt und validiert. Der standardmäßige Einsatz des hochtoxischen Kaliumcyanids (KCN) macht die Entwicklung einer sichereren Methode zur Bestimmung von HCN im Zigarettenrauch erforderlich. In der hier beschriebenen Methode wird KCN durch das weniger toxische Kaliumtetracyanozinkat ersetzt (die letale Dosis LD_{50} oral liegt für KCN bei 7,49 mg/kg und für Kaliumtetracyanozinkat bei 2000 mg/kg). Bei der neuen Methode wird zudem Isonicotinsäure-Barbitursäure (Kupplungsreagenz) anstelle von Pyridin-Pyrazolon als Reagenz zur Bestimmung von HCN eingesetzt, sodass auf die Verwendung von Pyridin verzichtet werden kann. Bei dieser Methode erfolgt das Auffangen von HCN sowohl mithilfe des Cambridge Filter Pads und nachfolgender Extraktion mit wässriger Natriumhydroxidlösung als auch in einem Impinger mit der gleichen Lösung. Die so extrahierte Lösung wird mit Chloramin-T zu Cyanchlorid oxidiert und mit einem Kupplungsreagenz behandelt. Die farbmetrische Messung des hieraus resultierenden stabilen Chromophors erfolgte bei 600 nm. Die Regressionsgleichung für Cyanid war im Bereich von 1 bis 25 µg/mL

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linear, mit einem Korrelationskoeffizienten (R^2) > 0,9998. Die Nachweisgrenze lag bei 0,76 µg/cig und die relative Standardabweichung der Methode lag insgesamt bei unter 10%. Für Cyanid wurden ausgezeichnete Rückgewinnungsraten zwischen 92% und 112% erzielt und die mit der neu entwickelten Methode gewonnenen Ausbeuten an HCN aus der Kentucky Referenzzigarette 3R4F stimmen gut mit denen aus der herkömmlichen KCN-Methode überein. Die vorgeschlagene Methode ist robust, zuverlässig, selektiv und gefahrloser als alle existierenden Methoden zur Bestimmung von Cyanwasserstoff im Hauptstrom- und Nebenstromrauch von Zigaretten. [Beitr. Tabakforsch. Int. 28 (2018) 191–202]

RESUME

Une nouvelle méthode sensible et sûre fut mise au point et validée afin de déterminer le cyanure d'hydrogène (HCN) dans la fumée de cigarette à l'aide d'un analyseur à flux continu (AFC). L'utilisation de cyanure de potassium (KCN) hautement toxique en guise de norme nécessite l'élaboration d'une méthode plus sûre en vue de mesurer le HCN dans la fumée de cigarette. Dans la présente méthode, le KCN fut remplacé par une substance à la toxicité moins aigüe, à savoir du tétracyanozincate de potassium (La dose létale DL_{50} orale étant de 7,49 mg/kg pour le KCN alors qu'elle est de 2000 mg/kg pour le tétracyanozincate de potassium). En outre, la nouvelle méthode utilisa un réactif de couplage composé d'acide isonicotinique et d'acide barbiturique en remplacement de la combinaison pyridine-pyrazolone pour la détermination du HCN et fit ainsi l'impasse sur la pyridine. Grâce à cette méthode, le HCN fut piégé à la fois sur le tampon du disque filtrant Cambridge avant l'extraction à l'aide d'une solution d'hydroxyde de sodium sous forme aqueuse et dans l'épurateur contenant la même solution. La solution ainsi extraite fut oxydée en chlorure de cyanogène grâce la chloramine-T et traitée à l'aide du réactif de couplage; le chromophore stable obtenu fut mesuré par colorimétrie à 60 nm. L'équation de régression fut linéaire dans la plage allant de 1 à 25 µg/mL pour le cyanure, avec un coefficient de corrélation (R^2) supérieur à 0,9998. Le seuil de détection fut de 0,76 µg/cig et l'écart-type relatif général de la méthode fut inférieur à 10%. D'excellents rendements de cyanure furent obtenus dans une plage allant de 92% à 112% et les rendements de HCN obtenus, à partir de la cigarette de référence (3R4F, Université du Kentucky), grâce à la nouvelle méthode mise au point présentèrent une bonne concordance avec ceux obtenus par la méthode conventionnelle avec le KCN. La méthode proposée est solide, fiable et plus sûre que toute autre méthode existante pour déterminer le cyanure d'hydrogène dans la fumée principale ainsi que dans la fumée latérale de cigarette. [Beitr. Tabakforsch. Int. 28 (2018) 191–202]

1. INTRODUCTION

Hydrogen cyanide (HCN) in cigarette smoke is one of the constituents of concern listed by the World Health Organization - Framework Convention on Tobacco Control (WHO - FCTC) (1). HCN exists both in particulate phase as well as in gaseous phase and therefore, the methodology should be able to efficiently trap HCN in both phases. There are several methods reported for the determination of HCN in different matrices (2–12) and some specific methods for the determination of HCN in mainstream smoke and sidestream smoke of tobacco products by various techniques using colorimetry (13), spectrophotometry (14) gas chromatography (15–16), ion chromatography (17), polarography (18), liquid chromatography-tandem mass spectrometry (19) and continuous flow analysis (20–22). One widely used method for the analysis of HCN in cigarette mainstream smoke is according to Health Canada T-107 (23) by continuous flow analyser (CFA). Unfortunately, all these methods use potassium cyanide (KCN) as standard. Due to safety hazards and legislation the replacement of KCN as reference material for standard preparation is of great importance. Initially KCN replacement was tried with potassium thiocyanate (KSCN) and glycine. However, their use was not successful as they have led to non-stoichiometric reaction with the coloring reagents. The cyanide standard solution containing cyanide (II) complexed with zinc, nickel or cadmium (e.g., tetracyanozincate) preferably as an alkali metal compound can release the required cyanide ion. These compounds when used as standards are stable on storage and less toxic than alkali metal cyanides (such as KCN) which have been historically used. In view of the potential environmental impact of nickel and cadmium, we have identified potassium tetracyanozincate as the most suitable standard for the analysis of HCN. Cyanide from potassium tetracyanozincate is stable but easily releasable (24).

In summary, the new method, described herein, replaces KCN with potassium tetracyanozincate for the preparation of cyanide standard and quantifying HCN in cigarette smoke using CFA. The developed method eliminates the use of highly toxic KCN with safer standard potassium tetracyanozincate. The aim of the present investigation is not only to replace KCN but also to replace pyridine, which is harmful and has a very unpleasant odour. The present method also utilizes isonicotinic acid (25) for the formation of glutaconic aldehyde in place of pyridine which further substantiates a more environmentally friendly approach. The current study was performed with the Kentucky Reference Cigarette 3R4F and the equivalent of the HCN yields is compared with the existing method (23). The results from this study are in good agreement with the published data (26). The measurement of HCN is carried out using a modified Koenig's reaction, wherein the cyanogen chloride formed by the oxidation of cyanide released from potassium tetracyanozincate with Chloramine-T couples with isonicotinic acid and barbituric acid coloring reagents to form a stable chromophore. The reaction mechanism is shown in the scheme (Figure 1).

CFA is an automated wet chemical analysis. The CFA technique is simple, sensitive and facilitates faster through-put compared to other available techniques. In CFA, a sample is aspirated into a flowing carrier solution passing rapidly through small-bore tubing. The sample is mixed with a reagent in mixing coils, the resulting mixture reacts and develops a color and thus rendering determination of the analyte concentration.

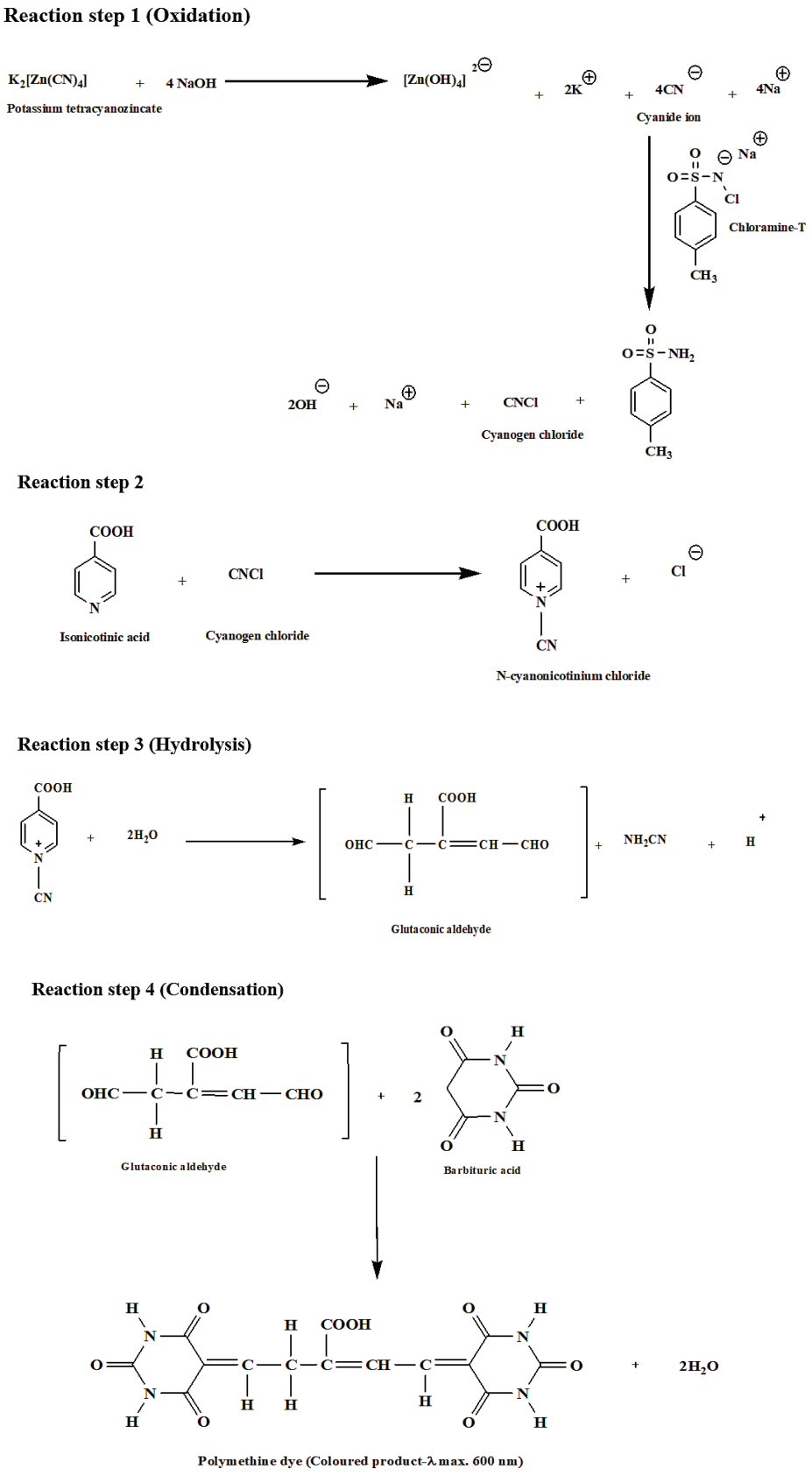

Figure 1. Scheme of the reaction mechanism during the measurement of HCN.

2. EXPERIMENTAL

2.1. Reagents

Potassium tetracyanozincate $K_2[Zn(CN)_4]$ in water containing 1000 mg/L CN, sodium hydroxide (NaOH), potassium di-hydrogen phosphate (KH_2PO_4), polyethylene glycol dodecyl ether (Brij-35), and di-sodium hydrogen phosphate (Na_2HPO_4), 20 mM silver nitrate solution ($AgNO_3$), *p*-dimethylaminobenzylidene rhodanine ($C_{12}H_{12}N_2OS_2$), ferrous sulphate ($FeSO_4.7H_2O$), citric acid ($C_6H_8O_7.H_2O$), and sodium carbonate (Na_2CO_3) were procured from Merck (Bengaluru, India).
Chloramine-T ($C_7H_7ClNNaO_2S.3H_2O$), iso-nicotinic acid ($C_5H_4NCO_2H$), and barbituric acid ($C_4H_4N_2O_3$) were procured from Sigma Aldrich (Bengaluru, India). All reagents were prepared using deionized water (18.2 mΩ/cm).

2.2. Instrumentation

The linear smoking machines used were the Cerulean SM 450H and Cerulean SM 405 SV. Continuous Flow Analyser (Skalar San++) (CFA) consisting of sampler, peristaltic pump, chemistry module, colorimeter equipped with 10 mm flow cell, and 600 nm filter, computer/data handling system. Further information can be found in Appendix 1.

2.3. Preparation of reagents

All the reagents were prepared from analytical grade chemicals (unless otherwise specified) and dissolved in deionized water. Chloramine-T solution (0.4% *w/v*) was prepared in water daily and stored in an amber-colored reagent bottle. The color reagent was prepared by dissolving 12.8 g of barbituric acid and 13.6 g of iso-nicotinic acid in 1000 mL water. This reagent was stable for 3 months if stored at 4 °C.
The buffer solution was prepared by dissolving 13.6 g of potassium dihydrogen phosphate and 0.28 g of disodium hydrogen phosphate in 1000 mL water. Brij-35 solution (0.5 mL) was added to the buffer solution and mixed thoroughly. The buffer solution was stored in an amber colored bottle.

2.4. Standard preparation

The primary stock solution of 500 µg/mL CN was prepared from 1000 µg/mL of potassium tetracyanozincate $K_2[Zn(CN)_4]$ by diluting an appropriate amount of the standard, based on the purity of cyanide standard, with 0.2 N NaOH. The cyanide standard purity was determined by titrating with 20 mM silver nitrate solution using *p*-dimethylaminobenzylidene rhodanine indicator (27). Calibration solutions were prepared by diluting the primary stock solution in 0.1 N NaOH to obtain the following concentration of HCN (Table 1). Standard stock solution and calibration solutions were stable for one month when stored at 4 °C.

2.5. Sample collection and extraction

The Kentucky Reference Cigarettes 3R4F were sampled according to ISO 8243:2013 (28) and conditioned according to ISO 3402:2000 (29).

2.5.1 Mainstream smoke

The conditioned cigarette samples were smoked according to ISO 4387:2000 (30) and the Health Canada Intense (HCI) regime (31) with the impinger containing 0.1 N NaOH (30 mL for ISO and 40 mL for HCI) and connected to the smoking machine to collect the vapour phase HCN. The 44-mm Cambridge filter pad, containing trapped mainstream HCN from 5 cigarettes for the ISO and 3 cigarettes for the HCI regime was extracted with 40 mL 0.1 N NaOH by shaking at 180 rpm for 30 min. An aliquot of the extract was filtered and analysed for HCN by CFA method. The HCN impinger trappings were also filtered and analysed by CFA.
Laboratory Fortified Matrix (LFM) samples were analysed to evaluate the potential matrix effect. 5 mL of control cigarette particulate extract was made up to 10 mL using 0.1 N NaOH. Similarly, 5 mL of control cigarette particulate phase extract was fortified with 0.1 mL 500 µg/mL CN standard and made up to 10 mL with 0.1 N NaOH. Laboratory Fortified Blank (LFB) samples were analysed by fortifying the conditioned filter pad with 0.4 mL of 500 µg/mL CN standard and with 39.6 mL of 0.1 N NaOH then extracted. Laboratory Reagent Blank

Table 1. Calibration standards.

Standard volume (mL)	Dilution (mL)	HCN standard concentration (µg/mL)	HCN (µg/cig) Mainstream smoke ISO with 5 cigarettes	HCN (µg/cig) Mainstream smoke HCI with 3 cigarettes	HCN (µg/cig) Sidestream smoke ISO with 3 cigarettes
0.1	50	1.04	14.5	27.7	41.6
0.3	50	3.12	43.6	83.1	124.7
0.5	50	5.20	72.7	138.5	207.8
0.7	50	7.27	101.8	193.9	290.9
1.0	50	10.39	145.5	277.1	415.6
1.5	50	15.59	218.2	415.6	623.4
2.0	50	20.78	290.9	554.1	831.2
2.5	50	25.98	363.7	692.7	1039.0

(LRB) samples were analysed by extracting conditioned filter pads with 40 mL of 0.1 N NaOH.
LRBs, LFBs and LFMs were processed through the sample preparation procedure described above. Sample solutions were stable for 12 h and the samples needed to be analysed within 12 h after trapping. Results can be found in Appendix 2.

2.5.2 Sidestream smoke

The conditioned cigarette samples were smoked according to ISO 20773:2013 (32) with impingers, containing 60 mL of 0.1 N NaOH, connected to the smoking machine to collect the vapour phase HCN and extracted by following the Health Canada method T-205 (33).
The 44-mm Cambridge filter pad containing trapped sidestream HCN from 3 cigarettes was placed into a 125 mL Erlenmeyer flask and 30 mL of 0.1 N NaOH was added to the flask. The inner wall of the fish tail chimney was rinsed with 2 × 15 mL 0.1 N NaOH and transferred to the flask to determine HCN. The HCN from the pad was extracted by shaking at 180 rpm for 30 min. An aliquot of the extract was filtered and analysed for HCN by the CFA method. HCN trapped in the impingers was filtered and analysed by CFA.

2.6. Sample analysis

The sample analysis was performed using CFA. The auto sampler was operated at 1:2 sample-to-wash ratio, wherein the sample time was 60 sec and wash time was 120 sec. Calibration standards were run to construct the calibration curve relating to µg/mL of HCN according to peak height. 0.1 N NaOH solution was run through the instrument at regular interval to allow the baseline correction. It can be noted that treatment of unreacted cyanide is described in Appendix 3.

3. RESULTS AND DISCUSSION

3.1. Calibration curves, limit of detection (LOD) and limit of quantification (LOQ)

The calibration curve for HCN across a range of concentrations (Table 2), as mentioned in section 2.4, was plotted by linear regression of the peak response. HCN showed excellent linearity with correlation coefficient (R^2) higher than 0.9998 (Figure 2).

Table 2. Linearity of the HCN calibration curve.

Level	Standard concentration (µg/mL)	Peak response (µg/mL)
1	1.04	1.02
2	3.12	3.23
3	5.20	5.24
4	7.27	7.28
5	10.39	10.29
6	15.59	15.84
7	20.78	20.84
8	25.98	25.86

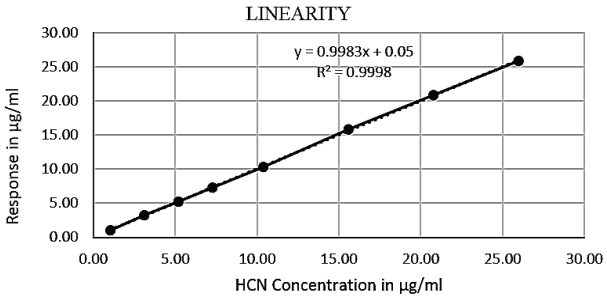

Figure 2. Linearity graph of HCN.

Table 3. Limit of detection (LOD) and limit of quantification (LOQ).

Replicate No.	Response	Mainstream smoke		Sidestream smoke
		ISO smoking regime response	HCI smoking regime response	ISO smoking regime response
	HCN (µg/mL)	HCN (µg/cig)		
1	1.03	14.4	27.5	41.2
2	1.00	14.0	26.7	40.1
3	1.03	14.4	27.4	41.1
4	1.05	14.6	27.9	41.8
5	1.05	14.7	28.0	42.0
6	1.04	14.5	27.7	41.6
7	1.05	14.7	28.1	42.1
8	1.03	14.4	27.4	41.1
9	1.01	14.1	26.8	40.2
10	1.05	14.7	27.9	41.9
Mean	1.03	14.5	27.5	41.3
SD	0.018	0.254	0.483	0.725
LOD	0.05	0.76	1.45	2.18
LOQ	0.18	2.54	4.83	7.25

Table 4. Recovery studies.

Vapour phase solution recovery

Sl. No	Level	Standard spiked concentration (µg/mL)	Spiked concentration to vapour phase (µg/cig)	Control vapour phase matrix (µg/mL)	Spiked + control vapour phase (µg/cig)	Recovered concentration (µg/mL)	Recovered concentration (µg/cig)	Vapour phase extract Recovery (%)	Average recovery (%)
1	LOQ Level	0.2	12	—	—	0.21	1.27	106.0	106.0
2						0.21	1.26	106.0	
3						0.21	1.26	106.0	
1	Low	0.1	6	4.81	28.86	5.85	35.11	104.2	109.2
2						5.93	35.58	112.0	
3						5.93	35.55	111.5	
4	Mid	7.5	45	4.81	28.86	12.79	76.76	106.5	102.9
5						11.97	71.79	95.4	
6						12.82	76.93	106.8	
7	High	15	90	4.81	28.86	19.45	116.71	97.6	99.1
8						19.89	119.33	100.5	
9						19.69	118.16	99.2	

Particulate phase extract recovery

Sl. No	Level	Standard spiked concentration (µg/mL)	Spiked concentration to particulate phase (µg/cig)	Control particulate phase matrix (µg/mL)	Spiked + control particulate phase (µg/cig)	Recovered concentration (µg/mL)	Recovered concentration (µg/cig)	Particulate phase extract recovery (%)	Average recovery (%)
1	LOQ Level	0.2	1.6	—	—	0.21	1.68	105.0	105.0
2						0.22	1.76	105.0	
3						0.21	1.70	105.0	
1	Low	1	8	2.67	21.36	3.73	29.87	106.4	105.2
2						3.75	30.03	108.4	
3						3.68	29.42	100.8	
4	Mid	7.5	60	2.67	21.36	10.55	84.38	105.0	106.7
5						10.68	85.44	106.8	
6						10.79	86.34	108.3	
7	High	15	120	2.67	21.36	18.54	148.28	105.8	101.8
8						16.59	132.71	92.8	
9						18.71	149.67	106.9	
LFM [a]		20	0.00	0.00	0.00	21.13	169.03	105.6	106.0
		20	0.00	0.00	0.00	21.51	172.11	107.6	
		20	0.00	0.00	0.00	20.93	167.45	104.7	

[a] Laboratory Fortified Matrix

Method sensitivity was evaluated by analysing the lowest calibration standard (1.04 µg/mL) a minimum of 10 times as an unknown. The results are summarized in Table 3. The LOD and LOQ were calculated as 3 times and 10 times the standard deviation respectively. The calculated LOD and LOQ were 0.05 µg/mL and 0.18 µg/mL which were equivalent to 0.76 µg/cig, 2.54 µg/cig, 1.45 µg/cig, 4.83 µg/cig, 2.18 µg/cig and 7.25 µg/cig for mainstream ISO, mainstream HCI and sidestream ISO smoking regimes respectively. The method gave a range of testing of 2.54 µg/cig to 1039.0 µg/cig.

3.2. Performance of the method

The accuracy of the method was evaluated by fortifying the control sample (LFMs) which were prepared according to the procedure mentioned in section 2.5. The results are shown in Table 4, the recoveries of particulate and vapour phase ranged from 92% to 112%.

The precision of the method was evaluated by measuring HCN yields in mainstream smoke of the 3R4F cigarette generated under the ISO regime for 19 times. As shown in Table 5, the value ranged from 82.8 to 96.0 µg/cig with %RSD of 4.5. The repeatability and reproducibility of the method within one laboratory was evaluated by measuring HCN yields in mainstream smoke of the 3R4F cigarette generated under the ISO regime by different analysts on 6 different days. As shown in Table 6 and Table 7, the results indicate the accuracy (reproducibility standard deviation SR) of HCN in mainstream smoke is ± 4, reproducibility limit (R) is ± 9, and the precision of HCN in mainstream smoke measurement is 5%, which is very good. The means of different analysts are well within the reproducibility limit.

3.3. Comparative evaluation of the method

The new method was compared with the existing KCN method (Health Canada T-107) by analysing HCN in 3R4F

Table 5. Precision of the measurement of HCN in the mainstream smoke of Kentucky 3R4F cigarette.

Replicate	HCN (µg/cig)		
	Vapour phase	Particulate phase	Total
1	49.9	44.1	94.0
2	46.4	36.7	83.1
3	48.9	39.7	88.6
4	50.9	38.6	89.5
5	52.9	40.0	92.9
6	53.4	36.5	89.9
7	48.4	41.1	89.5
8	47.0	36.8	83.9
9	44.7	38.0	82.8
10	51.5	44.0	95.5
11	51.2	42.0	93.2
12	51.7	41.3	93.0
13	49.4	43.5	93.0
14	47.5	41.7	89.2
15	49.2	41.8	91.0
16	49.1	35.9	85.1
17	51.6	44.4	96.0
18	45.8	40.6	86.4
19	49.6	42.0	91.6
Average	49.4	40.5	89.9
SD	2.32	2.65	4.07
%RSD	4.7	6.6	4.5

Table 6. Repeatability (r) and Reproducibility (R) within one laboratory.

Day No.	3R4F HCN (µg/cig)		
	Analyst-1	Analyst-2	Analyst-3
1	93.6	89.5	92.7
2	82.8	83.9	88.9
3	88.2	82.8	90.7
4	89.1	95.5	84.7
5	92.4	93.2	95.8
6	89.5	93.0	86.0
Average	89.3	89.6	89.8
SD	3.79	5.26	4.17

cigarette smoke for 19 times (Table 8) and subjected to statistical evaluation. As shown in Table 9, the t-test reveals that, since p value is >0.5 both the means of KCN and the newly developed method are found to be equal, there is no significant difference between average mean value and standard deviation of two methods. The results of the study are in good agreement with published data of the Kentucky Reference Cigarette 3R4F (26). Hence the developed method is accurate and precise when compared to this existing KCN method.

Table 8. Comparison of results between the Health Canada T-107 and the new method for measuring HCN in mainstream smoke of the Kentucky 3R4F cigarette generated under the ISO smoking regime.

Replication	KCN method (Health Canada T-107) HCN (µg/cig)	New method HCN (µg/cig)
1	93.9	94.0
2	83.0	83.1
3	88.5	88.6
4	89.4	89.5
5	92.8	92.8
6	89.8	89.9
7	89.4	89.5
8	83.8	83.9
9	82.6	82.8
10	95.4	95.5
11	93.1	93.2
12	92.9	93.0
13	92.9	93.0
14	89.1	89.2
15	90.9	91.0
16	85.0	85.1
17	96.0	96.0
18	86.3	86.4
19	91.5	91.6
Average	89.8	89.9
SD	4.09	4.07
%RSD	4.6	4.5

Table 9. Statistical evaluation of both methods (Data from Table 8) by t-test with two-sample-assuming equal variances.

Parameters	KCN method	New method
Mean	89.79242	89.89306
Variance	16.7031	16.57779
Observations	19	19
Pooled variance	16.64045	
Hypothesized mean difference	0	
df	36	
t Stat	-0.07604	
P(T<=t) one-tail	0.469903	
t critical one-tail	1.688298	
P(T<=t) two-tail	0.939805	
t critical two-tail	2.028094	

Apart from the above, the analysis of variance (ANOVA) for both methods have been carried out (Table 10), and the inference is at 95% confidence limit, the F statistical value is lower than the F critical value for between-group (KCN *vs.* Zincate) variations. And furthermore F statistic value is lower than F critical value for within-group variations. This indicates that there is no significant variability within each method and between them.

Table 7. Evaluation of the within-lab repeatability and reproducibility standard deviation and limits.

Sample ID	HCN (µg/cig)	Repeatability standard deviation (S_r)	Reproducibility standard deviation (S_R)	Repeatability limit (r)	Reproducibility limit (R)	RSDr	RSDR
3R4F	89.6	4.45	4.45	8.90	8.90	4.97	4.97

Table 10. Statistical evaluation of both methods by ANOVA (single factor).

Groups	Count	Sum	Average	Variance		
KCN method	19	1706.056	89.79242	16.7031047		
New method	19	1707.968	89.89306	16.57779252		
Source of variation	SS	df	MS	F	P-value	F crit
Between groups	0.096226	1	0.096226	0.005782654	0.939805	4.113165
Within groups	599.0562	36	16.64045	1.027612713	0.466833	1.47
Total	599.1524	37	16.19331			

The results of HCN (Table 11) under the HCI regime also demonstrate that the method can be used effectively for both ISO and HCI regime. The results of the study are in good agreement with published data of the Kentucky Reference Cigarette 3R4F (26).
Furthermore, the above results were validated by recovery studies with a recovery of more than 99% at 5 µg/mL. The data can be found in Appendix 2.

3.4. Sidestream smoke results

The method was extended for the analysis of HCN in sidestream smoke. The results (Table 12) established that the method is versatile.

Table 11. HCN yields in Kentucky 3R4F mainstream smoke under HCI smoking regime.

Replication	Vapour phase	Particulate phase	Vapour phase	Particulate phase	Total
	HCN (µg/mL)		HCN (µg/cig)		
1	19.61	9.17	261.4	122.2	383.6
2	20.62	9.50	274.9	126.6	401.6
3	20.64	9.82	275.2	130.9	406.1
4	21.99	9.08	293.2	121.0	414.2
5	20.18	9.84	269.1	131.2	400.3
6	18.57	8.69	247.6	115.9	363.5
Average	20.3	9.3	270.2	124.7	394.9
SD	1.14	0.45	15.26	6.03	18.34
%RSD	5.6	4.8	5.6	4.8	4.6

Table 12. HCN yields in Kentucky 3R4F sidestream smoke under ISO smoking regime.

Replication	Vapour phase	Particulate phase	Vapour phase	Particulate phase	Total
	HCN (µg/mL)		HCN (µg/cig)		
1	3.13	1.51	62.5	30.1	92.6
2	2.98	1.45	59.5	29.1	88.6
3	3.24	1.35	64.9	26.9	91.8
4	2.71	1.51	54.3	30.1	84.4
5	4.35	1.25	87.0	24.9	111.9
6	3.23	1.45	64.6	29.0	93.6
Average	3.3	1.4	65.5	28.4	93.8
SD	0.56	0.10	11.26	2.04	9.48
%RSD	17.2	7.2	17.2	7.2	10.1

4. CONCLUSIONS

A new method has been developed and validated for the analysis of HCN in mainstream smoke (under both ISO and HCI smoking regimes) as well as in sidestream smoke (ISO regime) by replacing toxic KCN with safer potassium tetracyanozincate $K_2[Zn(CN)_4]$ in the Health Canada method (T-107). The validation experiments indicated that the method is selective, accurate, precise and reliable. Replacement of KCN and pyridine in the analysis of HCN by this method has found that the method is safer compared to the published colorimetric methods. Statistical evaluation of the existing method and the newly developed method for HCN analysis in mainstream smoke using Kentucky Reference Cigarette 3R4F reveals yield equivalence. Thus, the newly developed method for determination of HCN in mainstream smoke using potassium tetracyanozincate $K_2[Zn(CN)_4]$ can be adopted in suitable laboratories to avoid the potential safety threat posed by potassium cyanide.

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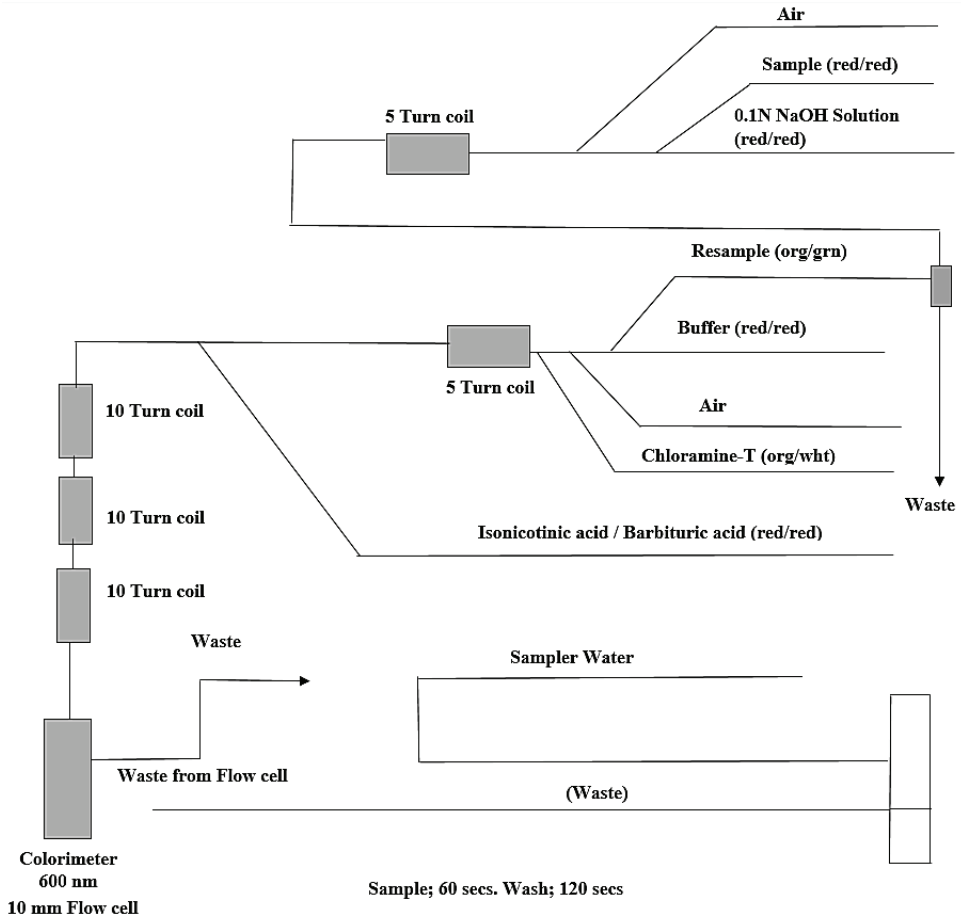
Appendix 1. Flow diagram of continuous flow analyzer.



Appendix 2. Results of the quality control.

	HCN content (µg/mL)	Spiked concentration	% Recovery [a]	Preparation
LRB[b]	0.00 0.00	Theoretical HCN content = 5.195 µg/mL	—	One conditioned pad + 40 mL of 0.1N NaOH
LFB[c]	5.03 5.30	HCN content recovered (b-a) = 5.165 µg/mL	99.4	One conditioned pad + 0.4 mL of standard stock (500 µg/mL) + 39.6 mL of 0.1N NaOH
LFM-A[d]	4.50 4.48	Theoretical HCN content = 5.195 µg/mL	—	5 mL of LFM made up to 10 mL with 0.1N NaOH
LFM-B[d]	9.77 9.86	HCN content recovered (B-A) = 5.325 µg/mL	102.5	5 mL of LFM + 0.1 mL of standard stock (500 µg/mL) made up to 10 mL with 0.1N NaOH

[a] % Recovery = [(HCN content recovered/theoretical HCN content) × 100]
[b] LRB: Laboratory Reagent Blank
[c] LFB: Laboratory Fortified Blank
[d] LFM: Laboratory Fortified Matrix

Appendix 3.

Cyanide waste treatment

Cyanide in the form of cyanogen chloride waste solution from the Auto analyser alongside solutions of Antidote-A (a mixture of ferrous sulphate 10 g/L and citric acid 1 g/L) and Antidote-B (sodium carbonate solution 10g/L) are run into the waste receptacle and a Buchner flask, which is constantly kept stirred. The stirred content overflows into a collection bottle. The collection bottle when three quarters full is removed, stoppered and kept in a fume cupboard overnight. The waste solution is then tested for the presence of any active cyanide (Prussian blue test). The contents are then drained into the sink with copious amount of tap water.

Prussian blue test for cyanide and cyanogen chloride waste solutions

In a fume cupboard transfer 2.5 mL of waste solution to a test tube. Make alkaline with 1 mL 5M sodium hydroxide solution and 3–4 drops of freshly prepared saturated ferrous sulphate solution. Heat with care, cool then, acidify with 1 mL 5M hydrochloric acid. Stand for 5–10 min until the solution is clear. Add 3–4 drops of 1% ferric chloride solution. A deep blue solution or a precipitate indicates that cyanide is still present in the solution and that further treatment with Antidote-A and Antidote-B is required before disposal of the solution.