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Spectrophotometric Determination of Thiocyanate in Human Saliva by a Unique Iodine-Azide-Chromogenic Substrate Reaction and its Application in Distinguishing Tobacco Smokers from Non-Smokers†

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Abstract

A method to detect thiocyanate (SCN) in human saliva is presented. Thiocyanate concentrations appear to be diagnostic when classifying smokers or non-smokers, and in determining some clinical conditions. The method involves the reaction of SCN with excess iodine and azide, and spectroscopic detection of unreacted iodine by a chromogenic substrate, ABTS. The calibration was linear over the range of 12.5-150 µmol/L (slope = 0.0086 delta-Abs/SCN µmol/L, intercept = -0.0160 delta-Abs, R^2 0.9998). The method was applied to analyze 29 saliva specimens. The results were similar to those obtained from a gas chromatography-mass spectrometry method (slope = 0.9595, R^2 0.9790). Based on Grubbs equation applied to specimens from non-smoking subjects, a threshold concentration of 1100 µmol/L for SCN was determined to distinguish smokers from the non-smokers. The SCN concentrations in 18 out of 20 saliva specimens collected from 2 smokers were above this threshold. The specimens from smokers were also examined for nicotine and cotinine by a GCMS method. While nicotine concentrations were found to vary, the cotinine concentrations remained stable, 134±29 ng/mL. Generally, the presence of nicotine/cotinine in specimens only indicates exposure to tobacco products, but the presence of any of these compounds with elevated SCN, is an indication of smoking. The 18 out of 20 samples collected from the smokers met these criteria. The mechanism of iodine-azide-SCN reaction has also been elucidated. While SCN is irreversibly changed to sulfate and cyanide, it is the cyanide that is responsible for the catalytic conversion of iodine and azide to iodide and nitrogen. This method is useful when SCN in saliva needs to be accurately determined.

Keywords: *Saliva thiocyanate; Iodine-azide reaction; Tobacco smoking; Reaction mechanism*

Introduction

Thiocyanate (SCN) is the principal metabolic product of cyanide ingestion. It is present in serum, saliva, and urine in low concentrations [1-3]. While the major source of cyanide is the fruits and vegetables containing cyanogenic compounds [4], SCN itself is present in milk and cheese [5]. High concentrations of SCN may also be due to HCN ingestion from tobacco smoke [6] or from inhalation of smoke from fire [7]. Patients treated with sodium nitropruside due to impaired renal function or cardiovascular disease may show elevated SCN concentrations [8, 9]. In contrast, low levels of SCN in the plasma of tobacco smokers may confirm a toxic amblyopia [10, 11]. Therefore, measurement of SCN is important in determining some clinical conditions and in classifying patients as smokers or non-smokers.

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Several colorimetric methods are available for the determination of SCN in blood and urine [1, 2, 6, 12, 13]. Ion chromatography [14, 15], and gas chromatography-mass spectrometry (GCMS) [16] can also be used for this determination. SCN in saliva has also been studied by color reactions [3, 17, 18], high-performance liquid chromatography [19], infrared spectrometry [20], capillary zone electrophoresis [21, 22], and GCMS [23]. Some of the color reactions and chromatographic methods require proper derivatization before analysis. The overall objective of this study is to find a rapid screening method for determination of SCN in saliva by an iodine-azide redox reaction and use the method in differentiating smokers from non-smokers. The mechanism of this SCN-iodine-azide reaction is also discussed.

Materials and Methods

Chemicals, Reagents, and Supplies

Ammonium thiocyanate, sodium azide, potassium chromate, sodium cyanide, a mixed solution of iodine and potassium iodide (I2/I-0.95/2.1 N), and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salts (ABTS) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Phosphate buffer (0.5M, pH 4.3) was

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prepared by mixing 0.5M phosphoric acid and 0.5M Na_3PO_4 (40:42 mL), and by adjusting the pH with any of the two reagents. Azide and iodine are hazardous to environment. But the amounts left after testing 30 samples were relatively small (<0.7 and <3 mg, respectively). The test solutions were disposed according to local guidelines. Reagents and solvents are of analytical or HPLC grade. Deionized water was used throughout.

Equipment

For SCN analysis, a Perkin-Elmer UV/Vis spectrophotometer, model Lambda 35, with a tungsten lamp was used. Cuvettes were made of glass suitable for absorption spectra in the range of 334 to 2500 nm. The optical path and the cell volume were 10 mm and 1.4 mL, respectively. For nicotine and cotinine analysis, a GCMS system, consisting of a 6890N GC and 5975 MSD from Agilent Technologies (Palo Alto, CA) was used. Helium was used as carrier gas. The head pressure on the capillary column (15 m x 0.25-mm i.d. x 0.20 μm, 5% phenyl polysiloxane, J&W Scientific, Rancho Cordova, CA) was 8-10 psi. The instrument was operated in pulse-split and temperature program mode. The split ratio, pulse pressure, and pulse time were 10:1, 30 psi, and 0.50 min, respectively. The oven temperature was increased from 80 ºC (held for 0.5 min) to 120 ºC at 15 ºC/min and then to 220 ºC at 40 ºC/min and held for 1.33 min. The Selected Ion Monitoring (SIM) was used to monitor ions m/z 161 (M^+-1) and 162 (M^+) for nicotine and m/z 165 (M^+-1) and 166 (M^+) for nicotine-D_4, m/z 175 (M^+-1) and 176 (M^+) for cotinine, and m/z 101 (M^+-pyridine) and 179 (M^+) for cotinine-D_3. The MS detector was operated at 300-400 v above autotune.

Preparation and stability of ABTS solution (2.5 mmol/L)

ABTS (206 mg, FW 549) was dissolved in 150 mL of phosphate buffer (0.2 M, pH 5.3). The solution was stable for at least 3 months at 3-5 ºC.

Preparation and stability of iodine, azide, SCN, and cyanide solutions

Iodine/Iodide (I_2/I^-, 3/6.6 mmol/L in water) was prepared by diluting 0.157 mL of commercially available 0.95/2.1 N (I_2/I^-) to 50 mL with water. The solution was stable for at least 2 months. Sodium azide (50 mmol/L), ammonium thiocyanate (20 mmol/L), sodium cyanide (10 mmol/L), and potassium chromate (2.0 mmol/L) stock solutions were prepared by dissolving appropriate amount of the reagent in water. Thiocyanate and chromate solutions were stable for at least 2 years, whereas, cyanide and azide solutions were stable for 1 and 2 month, respectively. Working solutions were prepared by diluting the stock solutions. All solutions were stored at 3-5 ºC but brought to room temperature before analysis.

Preparation of saliva samples

Unstimulated saliva samples (oral fluids) were collected from smoking and non-smoking individuals directly into a polypropylene collection tubes. Sp1 was collected from one of the authors (BDP). Other specimens were collected anonymously. In our laboratory, anonymous collection of specimens that are used to prepare quality control materials and in method development is exempt from IRB review. A re-collection time of at least one day was instituted when collecting additional samples from the same subject (Sp1 and Sp4). Two smokers (Sm1 and Sm2) collected samples after smoking a cigarette. After a period of 1-2 days, they collected samples again after smoking another cigarette. Individual collection times were provided by the smokers. Approximately 3 mL of saliva was centrifuged at RCF 7300 x g for 30 min, and 0.5 mL of the clear oral solution was diluted to 5 mL with water (10x dilution), prior to analysis. Samples were diluted further if measured concentrations were above the limit of linearity.

Specimen analysis for SCN by iodine-azide reaction

Fifty microliter of SCN standards (12.5, 25, 50, 100, and 150 μmol/L in water) and saliva specimens (10x dilution) were added separately to a set of 10-mL polypropylene tubes containing 0.05 mL of phosphate buffer (0.5 M, pH 4.3), 0.06 mL of iodine solution (I_2/I^- 3/6.6 mmol/L in water), and 0.05 mL of azide (50 mmol/L in water). The pH of the final solutions was pH 5.2 $\pm$ 0.1. Buffer helped to get the pH in a narrow range, so that oxidation condition remained the same for all samples. A water-blank was prepared by mixing 0.110 mL of water with 0.05 mL of phosphate buffer and 0.05 mL of azide. An iodine-blank was also prepared by mixing 0.06 mL of iodine with 0.05 mL of water, 0.05 mL of phosphate buffer, and 0.05 mL of azide. The total volume in the tubes was 0.210 mL. After 5 min of reaction at room temperature, a solution of 2 mL of ABTS (2.5 mmol/L) was added to the tubes. The test solutions immediately turned green. After an additional 3 min of reaction time at room temperature, the absorption readings were recorded at 420 nm. The water-blank was set to zero before sample readings.

Specimen analysis for nicotine and cotinine

Fifty microliters of a nicotine-D_4/cotinine-D_3 as internal standard (200/200 ng/mL in methanol), 0.5 mL of carbonate buffer (1.5M, pH 9.5) and 1 mL of methylene chloride were added separately to 0.5 mL of saliva specimens and nicotine/cotinine standards (0, 2.5, 5, 10, 25, 100 ng/mL in saliva) in 10-mL centrifuge-tubes. The mixtures were shaken (100 cycles/min) for 20 min and then centrifuged to get a clear organic layer. The organic solutions were transferred to separate tubes and evaporated to dryness at 50 ºC under nitrogen. It was reconstituted with 0.04 mL of ethyl acetate and analyzed by GCMS.

Experiments to determine the mechanism of the iodine-azide-SCN reaction

Iodine-cyanide reaction (without azide)

Fifty microliters of cyanide standards (200, 500, 1000, and 2000 μmol/L in water) were added separately to a set of 10-mL tubes containing 0.05 mL of phosphate buffer (0.5 M, pH 4.3) and 0.06 mL of iodine solution (I_2/I^- 3/6.6 mmol/L in water). A water-blank was prepared by mixing 0.110 mL of water with 0.05 mL of phosphate buffer. Similarly, an iodine-blank was prepared by mixing 0.06 mL of iodine with 0.05 mL of water, and 0.05 mL of phosphate buffer. The final volume of the standard and blank solutions was 0.16 mL.

In another experiment conducted simultaneously with the iodine-cyanide reaction, 0.05 mL of chromate standards (250, 500, 1000, 1500, and 3000 μnormal/L, μN) in water (same as 83.3, 166.7, 333.3, 500, and 1000 μmol/L for Cr^{6+} to Cr^{3+}) were added separately to a set of tubes containing 0.05 mL of 1 M HCl. An acid-blank was prepared by mixing 0.05 mL of 1M HCl with equal amount of water. Sixty microliters of water was added to all tubes to bring the total volume to 0.16 mL. Both cyanide and chromate solutions were allowed to stand for 5 min at room temperature. After this period, 2 mL

of ABTS (2.5 mmol/L) was added to all tubes. After allowing another 3 min for the I-CN-ABTS and Cr-ABTS reactions, the absorption readings were recorded at 420 nm. The water blank was set to zero before the cyanide readings and the acid-blank was set to zero before the chromate readings. The readings were stable for at least 40 min.

Iodine-cyanide reaction with azide

This experimental analysis was similar to that of iodine-cyanide reaction, with the exception of adding 0.05 mL of azide (50 mmol/L in water) to the water-blank, iodine-blank, and iodine-cyanide tubes. To ensure equal volumes, 0.05 mL of water was added to all chromate and acid-blank tubes. The total volume in all tubes was 0.21 mL. After addition of ABTS, the absorption readings were recorded as mentioned before.

Results and discussion

Method validation

The Iodine-azide reaction is known to progress slowly, but reaction rate can be accelerated by adding a small amount of divalent sulfur compounds [24-28]. In our experiment, the iodine-azide reaction was monitored and the result compared with that of an iodine-blank with no azide. When a chromogenic compound, ABTS, was allowed to react with the unreacted iodine [28, 29] and the absorption readings were examined, the difference in absorption between the iodine-azide and iodine-blank reactions appeared to be small, indicating very weak reaction (1.179 $\pm$ 0.010 Abs vs. 1.215 $\pm$ 0.008 Abs, *N*=4). However, when SCN was added to the reaction mixture, the reaction started almost immediately. In the iodine-azide-SCN reaction, ABTS was selected as the chromogenic substrate to determine the unreacted iodine. The selection was based on a strong molar absorptivity (λ_{max} 415 nm, ϵ 2.304 x 10^4 L.mole^{-1}.cm$^{-1)}$ and stability (at least 3 months at 3-5 ºC) of ABTS [29]. In the iodine-azide-SCN experiments the absorption readings of the oxidized-ABTS (chromogen) were taken at 420 nm instead of 415 nm. No significant difference in absorptions at the apex of the peak was observed [29]. The linearity of the SCN responses was based on the consumption of iodine by SCN. The simplified I-azide-SCN (1a, unbalanced) and the I(leftover)-ABTS (1b) reactions are explained in [Figure 1]. The details of the reactions are explained later. When the absorbance due to iodine consumed (delta-Abs) were plotted against the SCN concentrations, the amount of SCN was found to be linear over concentrations ranging from 12.5 to 150 µmol/L (0.725-8.70 µg/mL). The linearity, precision, and accuracy are presented in [Table 1]. In this method, the limit of detection (LOD) is considered as the lowest limit of quantitation of SCN (LOQ, 12.5 µmol/L). The absorption readings of the final solutions were stable for at least 40 min, allowing testing of a large number of specimens in a batch analysis. The validated method appeared appropriate for testing saliva specimens for SCN.

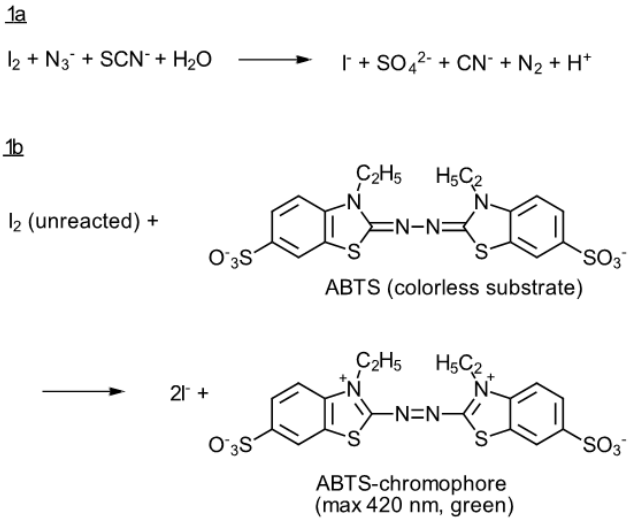

Figure 1: Initial iodine-azide-SCN reaction (1a, unbalanced) followed by a reaction of unreacted iodine with a chromogenic substrate, ABTS (1b)

Table 1: Validation of SCN Concentrations Based on Iodine-Azide-SCN Reaction Using ABTS as Chromogenic Substrate

SCN, µmol/L Theory	ABTS Abs λ 420 nm	I^0 Consumed delta-Abs[a]	SCN, µmol/L Found[b]
Linearity			
0	1.456		
12.5	1.367	0.09	12.2
25	1.254	0.2	25.4
50	1.05	0.41	49.1
75	0.825	0.63	75.3
100	0.602	0.85	101
150	0.189	1.27	149

SCN, µmol/L Theory	SCN,µmol/L Found Av $\pm$ CV %	Within theory, %
Precision and accuracy (N=5)		
12.5	12.3 $\pm$ 4.5	98
25	25.1 $\pm$ 3.3	100
50	51.1 $\pm$ 6.0	100
75	74.3 $\pm$ 4.2	99
100	107.9 $\pm$ 2.2	108
150	146.7 $\pm$ 1.1	98

[a]Abs due to iodine(I^0) consumed by (SCN^- + N_3^-) reaction. Example ($Abs_{SCN\ 0}$ – Abs_{sample})

[b]Calculated from slope = 0.0086 delta-Abs/SCN, µmol/L and intercept = -0.0160 delta-Abs; R^2 0.9998
At 95% confidence, slope interval (0.0084, 0.0088) delta-Abs/SCN, µmol/L and intercept interval (-0.0215, -0.0105) delta-Abs

Thiocyanate in saliva specimen

The iodine-azide-SCN colorimetric method was applied to test SCN in 29 saliva specimens and the results were compared with that of a GCMS method [23] [Table 2]. The values between the two methods compared favorably for all specimens (slope = 0.9595, R^2 0.9790). Two smokers (Sm1, Sm2) smoked one cigarette and collected specimens at specific intervals. After two days, Sm1 collected a second set of specimens after smoking another cigarette.

Table 2: Thiocyanate in Human SalivaCollected from Nonsmoking and Smoking Subjects

Saliva specimens	SCN, µmol/L	
	I-Az-SCN	GCMS[a]
Non-smokers		
Sp1-1	765	794
Sp1-2	448	442
Sp1-3	659	741
Sp1-4	508	531
Sp2-1	382	293
Sp3-1	449	463
Sp4-1	685	726
Sp4-2	743	771
Sp5-1	620	573
Average (N=9)	584	593
Std	141	175
CV%	24	30

Smokers			Nicotine[b]	Cotinine[b]
			ng/mL	ng/mL
Sm1-0 min	1542	1356	36	166
Sm-10 min	1104	994	1282	129
Sm1-15 min	2273	2093	1004	191
Sm1-1 h 20 min	2899	2768	291	177
Sm1-1 h 55 min	2650	2357	183	175
Sm1-0 min	1103	945	25	137
Sm1-12 min	889	723	1306	129
Sm1-30 min	1665	1591	595	145
Sm1-1 h	1618	1379	219	130
Sm1-1 h 45 min	1652	1595	151	130
Sm2-0 min	3229	3588	9	150
Sm2-15 min	1621	1515	4016	134
An2-30 min	2678	2748	315	146
Sm2-1 h 10 min	2707	2862	59	135
Sn2-2 h 25 min	1102	1073	8	98
Sm2-0 min	2332	2162	5	105
Sm2-10 min	858	949	2476	80
Sm2-25 min	2510	2487	858	117
Sm2-1 h 5 min	2647	2588	85	106
Sm2-2 h 30 min	3114	3344	21	102

[a] Ref [23]

[b]GCMS method linearity and R^2: 5-100 ng/mL and 0.9963 for nicotine, and 2.5-100 ng/mL and 0.9997 for cotinine, respectively

For Sm2, the second set was collected 24 hour after the initial collection. No information was available concerning the pattern of smoking between the collections. The specimens were also tested for nicotine and cotinine by a GCMS method. Both nicotine and cotinine were found to be present in all specimens [Table 2]. The nicotine values in specimens collected before smoking (Sm1-0 min or Sm2-0 min) initially increased and then gradually decreased over the period of 2 h. But the cotinine concentrations during this period remained within a range of 80-191 ng/mL (Av $\pm$ SD, 134 $\pm$ 29, N=20). This average is consistent with previously reported values of 166 $\pm$ 170 ng/mL (N=207) [30]. Generally, the presence of nicotine/cotinine in biological samples only indicates exposure to tobacco products (smoking, chewing, dermal), whereas the presence of any of these two compounds along with elevated SCN, is an indication of tobacco smoking. While actively smoking a cigarette, cyanogen and cyanide as pyrolytic products are also consumed and subsequently metabolized to SCN. Moreover, SCN is also a metabolic product of dietary cyanide [4, 5]. Therefore, an increase in SCN in smoker's saliva could be related to smoking. In the non-smoking specimens the average concentrations of SCN is 584 $\pm$ 141 µmol/L (34 $\pm$ 8 µg/mL) [Table 2]. The average is close to two previously reported values of 0.54 $\pm$ 0.41 mmol/L (31 $\pm$ 24 µg/mL, N=20) [3] and 0.51 $\pm$ 0.31 mmol/L (30 $\pm$ 18 µg/mL, N=10) [30]. Considering normal distribution of the nine non-smoking values, Grubbs' exclusion test was applied to find a value that may be considered as a potential outlier. The critical value for an upper one-tailed test is 2.18 (G_{Crit} = 2.18, N=10, α = 0.05). Using Grubbs' equation, a value of 1085 shows the G_{Cal} same as the $G_{Crit.}$ Therefore, any value greater than 1085 µmol/L may be considered as potential outlier, meaning not part of the non-smoking results. For convenience, a rounded value of SCN at 1100 µmol/L (64 µg/mL) was chosen as the threshold concentration (G_{Cal} = 2.21 > G_{Crit}). Therefore, any saliva of SCN concentration greater than the threshold could be related to smoking cigarette. The SCN concentrations in 18 out of 20 saliva specimens collected from smokers were above the threshold concentration, while others from non-smokers were below this level. The two smoking specimens below the threshold (Sm1-12 min, Sm2-10 min) were collected too early before cyanide could metabolized to SCN. Although number of samples (N=9) from five non-smoking subjects are small, the SCN values are in close conformity with two other studies [3, 31]. Analyte stability and result reproducibility have also been investigated. Sp1-4 was frozen at -18 ^{0}C tested again for SCN after 1y 9m using the same iodine-azide reaction. The result 609 µmol/L, was within 11% of the original result of 551 µmol/L, supporting a long-term stability and reproducibility of the compound.

Lactoperoxidase (LPO) and myeloperoxidase (MPO), two oxidizing enzymes, are common constituents in human saliva. In presence of peroxides these two enzymes may oxidize iodide to iodine and interfere with the detection of SCN. However, the reactivity of LPO and MPO can be inhibited by the use of azide [32, 33]. In an experiment we tested a specimen with and without azide (50 mmol/L). The respective SCN values were 767 and 337 µmol/L. The decrease in concentration is due to decrease in delta-absorption, implying oxidation of iodide to iodine by the enzymes. To ensure complete inhibition, another specimen was tested with azide concentrations of 10, 15, 20, 30, 40, 50, 60, and 75 mmol/L. The SCN concentrations were 455, 422, 398, 386, 455, 433, 445, and 380 µmol/L, respectively (Av 422 $\pm$ 30.4, CV% 7.2). Similarity in results indicates that the LPO and MPO do not interfere with the SCN detection at azide concentration 10-75 mmol/L. Interfering substances other than oxidizing enzymes are not known. To evaluate specificity, the method was directly applied to test 29 saliva specimens and the SCN

concentrations were compared with that of a GCMS confirmatory method [Table 2]. Any interfering substances that may be present in saliva would be known from the direct comparison.

The results show a good correlation with slope and R^2 at 0.9595 and 0.9790, respectively. The average ± std (CV%) were 1567 ± 931 µmol/L (65%) for Iodine-azide method and 1533 ± 960 µmol/L (63%) for GCMS method, indicating specificity of the method. The t-test also indicates no significant difference between the two results (t_{cal} = 0.139 <t_{crit} = 1.960, N=29, α=0.05). However, in forensic investigation, the SCN determination by iodine-azide reaction should be considered as screening. All presumptive positive specimens with SCN ≥ 1100 µmol/L (64 µg/mL) and nicotine/cotinine ≥ 5/2.5 ng/mL, should be tested again for SCN by GCMS [23]. Nevertheless, the iodine-azide method is the choice for initial screening because it is cost-effective and relatively faster than the GCMS method. In a typical batch analysis, 25 samples can be tested in less than 25 min.

Mechanism of reaction

In the iodine-azide-SCN reaction, iodine appeared to oxidize both azide and SCN. There may be several reactions that are considered as intermediates to produce the final result. Some of the intermediate reactions are well documented [34-40]. In an approach, we investigated a few important reactions and then combined them to get the final reaction. Based on the published reports [references] and our experimental results (Expt.), following sequence of reactions are postulated:

azide in water was compared with that of iodine/iodide, azide, and SCN in the same proportions (pH 7.09 vs. 6.24).The decrease in pH is due to formation of hydrogen ion as presented in eq-1. The cyanide from eq-1 was then oxidized by iodine according to the reaction outlined in eq-2. In another experiment, solutions of cyanide (100 - 2000 µmol/L) were treated with a known amount of iodine. After the I-CN reaction, the unreacted iodine was determined by treating the solutions with ABTS and comparing the absorptions with that from a set of chromate standards (250 - 3000 µnormal/L) also treated with ABTS. In both I-CN-ABTS and Cr-ABTS reactions, the responses were linear over the concentration range mentioned (R^2 0.9979 for CN and R^2 0.9896 for Cr) [Table 3]. From the difference between the initial and unreacted iodine absorptions, the amount of iodine that reacted with CN was determined by the Cr-ABTS standard absorbance. The ratio of I:CN (average 1.36 + 0.00) over the CN concentration range of 100-2000 µmol/L, supports the molar amounts of iodine and cyanide in eq-2 (4:3 = 1.33). The same I-CN reaction in the presence of azide (I-CN-N3) resulted in almost the same I:CN ratio when the reaction was performed in two separate experiments (ratios 1.31 + 0.01, 1.38 + 0.00). The stoichiometric amounts of iodine and cyanide (4:3) support the reaction as proposed in eq-2. In a prior study, cyanogen-iodide (ICN) was the only compound mentioned in 77% yield when iodine was added in portions to sodium cyanide at 0 ^{0}C [36]. The molar amounts in this reaction were approximately 1:1.

$3I_2 + SCN^- + 4H_2O \longrightarrow 6I^- + SO_4^{2-} + HCN + 7H^+$ (eq-1) [34-36] Expt. (pH)

Eq-1 multiplied by 3

$9I_2 + 3SCN^- + 12H_2O \longrightarrow 18I^- + 3SO_4^{2-} + 3HCN + 21H^+$ (eq-1x)

$2I_2 + 3HCN \longrightarrow 3I^- + ICN + (CN)_2 + 3H^+$ (eq-2) Expt. I:CN =1.33

$ICN + 2N_3^- + H^+ \longrightarrow I^- + H(N_3\text{-}CN\text{-}N_3)$ (eq-3) [39]

$(CN)_2 + 2N_3^- + 2H^+ \longrightarrow H_2(N_3\text{-}CN)_2$ (eq-4) [40] Expt. $(CN)_2$+N_3^- GCMS

$H(N_3\text{-}CN\text{-}N_3) \longrightarrow HCN + 3N_2$ (eq-5) [39]

$H_2(N_3\text{-}CN)_2 \longrightarrow 2HCN + 3N_2$ (eq-6) [39]

Adding equations (eq-2) to (eq-6)

$2I_2 + 4N_3^- \longrightarrow 4I^- + 6N_2$ (eq-7)

Adding equations (eq-1x) and (eq-7)

$9I_2 + 3SCN^- + 12H_2O \longrightarrow 18I^- + 3SO_4^{2-} + 3HCN + 21H^+$

$2I_2 + 4N_3^- \longrightarrow 4I^- + 6N_2$

$11I_2 + 3SCN^- + 4N_3^- + 12H_2O \longrightarrow 22I^- + 3SO_4^{2-} + 3HCN + 6N_2 + 21H^+$ (eq-8)

With the same amount of SCN, and increasing amounts iodine and azide, eq-8 could be rewritten as

$(9 + 2n)I_2 + 3SCN^- + (4n)N_3^- + 12H_2O \longrightarrow 2(9 + 2n)I^- + 3SO_4^{2-} + 3HCN + (6n)N_2 + 21H^+$ ($n \geq 1$) (eq-9)

The oxidation of SCN to sulfate and cyanide (eq-1) is similar to that mentioned by several authors [34-36]. The reaction in eq-1 was also favored considering the reduction potentials of I_2/I^- and($SO4^{2-}$ +CN^-)/SCN^- (E0 0.536 v vs. 0.399 v) [36]. In our experiment, an increase in acidity was observed when the pH of iodine/iodide and ICN was reported to be sensitive to heat with the formation of iodine and cyanogen [38]. In our study, cyanide was added to an excess amount of iodine at room temperature. The excess iodine and the elevated reaction temperatures appeared to be the foundation for the reaction described in eq-2. Even though ICN was the main reaction product,

as suggested in the report [37], part of the ICN may have broken down to cyanogen under the condition used in our experiment. The breakdown is likely represented by the following equations:

GCMS. A strong cyanogen response from the molecular ion (M^+ 52) was observed. When the toluene solution was treated with an aqueous solution of azide, a gradual decrease in cyanogen response was observed,

$$3x[I_2 + CN^- \longrightarrow I^- + ICN] \quad \text{(eq-10)}$$

$$2ICN \longrightarrow I_2 + (CN)_2 \quad \text{(eq-11)}$$

$$2I_2 + 3CN^- \longrightarrow 3I^- + ICN + (CN)_2 \quad \text{(eq-12)}$$

Table 3: Stoichiometric Amounts of Reactants (Ratio I:CN) in Iodine-Cyanide Reaction

Cyanide µmol/L	Unreacted I ABTS abs	Cyanide found µmol/L[a]	Unreacted I by Cr^{6+} µmol/L (µN)[b]	Reacted I by Cr^{6+} µmol/L (µN) (ex.)	I:CN ratio (ex.)
0	1.687		3363[c]		
100	1.635	121	3199	164 (3363 – 3199)	1.36 (164/121)
200	1.595	178	3120	243	1.36
500	1.401	460	2736	627	1.36
1000	1.050	968	2041	1322	1.37
2000	0.321	2025	597	2766	1.37
				Average	1.36 ± 0.00

[a] Calculated from slope = -0.00069 and intercept = 1.7181 Abs, R^2 0.9979; slope interval (-0.00074, -0.00064) Abs/CN µmol/L and intercept interval (1.7031, 1.7332) Abs at 95% confidence

[b] Calculated from Cr^{6+} slope = 0.000505 Abs/Cr^{6+} µN and intercept = 0.01945 Abs.Example for CN 100 µmol/L: (1.635 – 0.01945)/0.000505

[c] Unreacted iodine (I) was based on CN intercept (1.7181 µmol/L) when CN=0 µmol/L. Example for CN intercept: (1.7181 – 0.01945)/0.000505

Cr^{6+} µnormal/L (µN)	ABST abs	Cr^{6+} found µnormal/L (µN)[a]
0	-0.051	
250	0.150	258
500	0.285	526
1000	0.541	1033
1500	0.867	1678
3000	1.482	2896

[a] Calculated from slope = 0.00051Abs/Cr^{6+} µN and intercept = 0.01945 Abs, R^2 0.9896; slope interval (0.00042, 0.00059) Abs/Cr^{6+} µN and intercept interval (-0.01965, 0.05865) Abs at 95% confidence

Both ICN and cyanogen react with azide according to reactions described in eq-3 and eq-4. Formation of $H(N_3\text{-}CN\text{-}N_3)$ [Figure 2a] from a similar reaction starting with ClCN and azide has been reported previously [39]. Azide also reacts with cyanogen to produce a compound $H_2(N3\text{-}CN)_2$ [Figure 2b] [40]. In our experiment cyanogen was prepared by copper sulfate mediated oxidation of sodium cyanide [40]. The cyanogen gas was trapped in toluene and analyzed by indicating a competing cyanogen-azide reaction. The two cyanogenic azides produced are inherently unstable and ultimately break down to hydrogen cyanide and nitrogen (eq-5 and eq-6). Decomposition of cyanogen-azide, with loss of nitrogen, was previously reported [38]. The net result of reactions in eq-2 through eq-6 results in an iodine-azide reaction with the formation of iodide and nitrogen (eq-7). The HCN produced from

the reaction described in eq-1x appears to be responsible for the iodine-azide reaction defined by eq-7 and recycles back to the reaction seen in eq-2 for all the reactions mentioned in eq-2 to eq-8. Although the reaction time for the iodine-azide-SCN method was 5 min, the reaction appears to continue even after that period. Delta absorption (reacted iodine) for SCN 100 µmol/L was 0.559 at 5 min but increased to 0.753 after 30 min of reaction. Recycled HCN appeared to be responsible for the continued reaction of iodine and azide to form iodide and nitrogen. The complete reaction starting with SCN appeared to be the sum of the reactions described in eq-1x and eq-7 (eq-8). A general formula (eq-9) was established to account for the reactions of SCN with increasing amounts of azide.

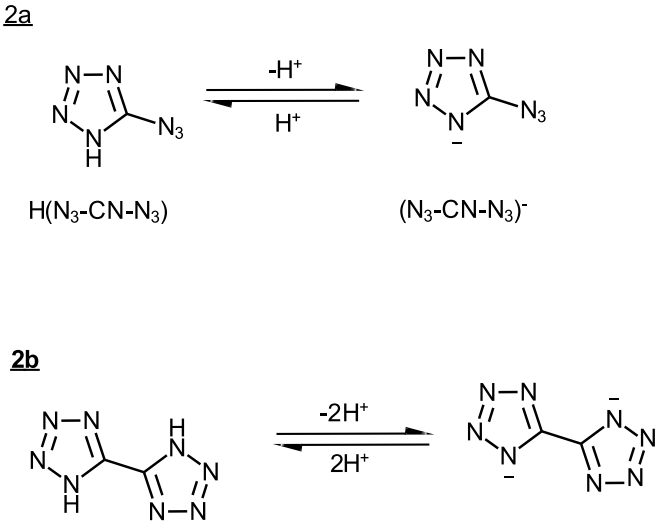

Figure 2: Chemical structures of two cyanogen-azide compounds (2a, 2b) and their ionic forms

Method optimization was based on the amounts of iodine, azide, and SCN as reactants with a reaction time of 5 min. Although reaction time of 5, 10, 20, and 30 min gave similar results (R^2>0.99), the 5 min reaction time was found to be convenient for testing a batch with large number of samples. For a SCN range of 25-150 µmol/L, an iodine concentration of 3 mmol/L was found to be optimal. While higher iodine concentrations cause optical saturation from iodine-ABTS absorption (Abs > 1.5), lower concentrations limit the sensitivity of the method. Azide was used in excess to induce the reaction with a concentration of 50 mmol/L found to be suitable. At an azide concentration more than 75 mmol/L and SCN of 150 µmol/L most of the iodine was consumed (Abs <0.1), however, SCN reactions were found to be linear at any azide concentration of 10-75 mmol/L (R^2 > 0.9898) [Figure 3]. Graphical examination indicates the consumption of iodine increased with increasing amount of azide within the range of 10-60 mmol/L. This indicates that azide stimulated the iodine-azide-SCN reaction at any concentration of SCN in 12.5-150 µmol/L. Using chromate-ABTS standard absorbance, the amount of iodine reacted in the iodine-azide-SCN reaction was calculated. Increasing amount of azide correlates well with the increasing amount of iodine consumption for the same amount of SCN, indicating iodine-azide reaction catalyzed by SCN. The iodine:SCN ratios (I:SCN) were calculated at each azide concentration (10-60 mmol/L) and compared with those from eq-9 [Table 4]. When the ratios were plotted against the azide

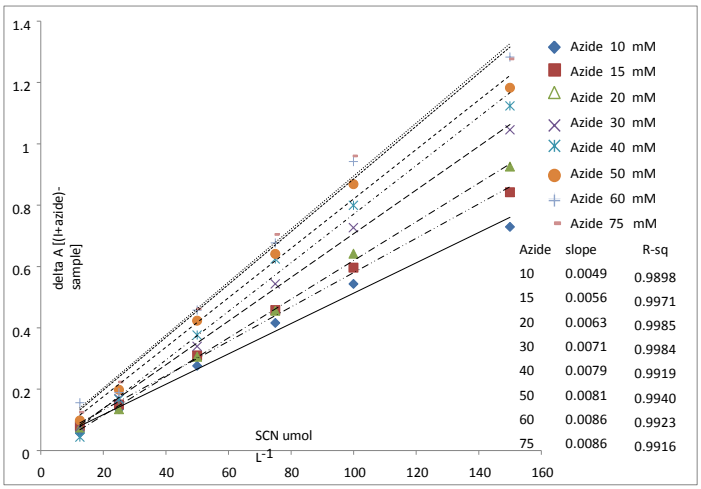

Figure 3: Iodine consumption (delta A) with increasing amount of SCN (12.5 – 150 µmol/L) and azide (10 – 75 mmol/L) in an iodine-azide-SCN reaction

concentrations, the relation followed a quadratic equation with R^2 0.9981 (y = -0.0009x^2 + 0.2022x + 7.8626). The I:SCN intercept (azide=0 µmol/L) shows a value similar to that derived from eq-9 (found 7.8626 vs. 7.3333 when n=1), supporting the reaction in eq-9. The I:SCN found from azide concentration of 50 mmol/L compares well with the value obtained from eq-9 using n=7 (15.5 vs. 15.3). Substituting n=7 in eq-9, the final reaction of SCN in saliva is as follows:

$$23I_2 + 3SCN^- + 28N_3^- + 12H_2O \longrightarrow 46I^- + 3SO_4^{2-} + 3HCN + 42N_2 + 21H^+$$

(eq-13) Exp. (Table 4, I:SCN found 15.5, theory 15.3)

Table 4: Relation Between Azide Concentrations and Iodine:SCN Ratios

Azide, mM (x)	I:SCN found (y) Av[a]	Std	General reaction Eq-9, n=	I:SCN Theory
			1	7.3
10	9.7	0.1	3	10
15	10.8	0.1	3.5	10.7
20	11.5	0.4	4	11.3
30	13.2	0.4	5	13.3
40	14.5	0.5	6.5	14.7
50	15.5	0.2	7	15.3
60	16.8	0.1	8	16.7

[a] Average values of I:SCN at SCN concentrations of 25, 50, 75, 100, and 150 µmol/L Azide (x) and I:SCN (y) relation expressed in equation, y = -0.0009x^2+0.2022x +7.8626; R^2 0.9981

Summary

Salivary SCN can be accurately determined by employing an iodine-azide-SCN reaction. In the reaction, the amounts of SCN reacted correlated well with the amounts of iodine consumed. The SCN is linear over the concentrations ranging from 12.5 to 150 µmol/L (0.725-8.70 µg/mL). When applied to investigate SCN in saliva specimens, the results of the method compared well with that from a GCMS method. The reaction was successfully applied to distinguish

smokers from non-smokers with a SCN threshold concentration of 1100 µmol/L (64 µg/mL). The specific reactions involved in the iodine-azide-SCN reaction are difficult to elucidate; however, based on previous reports and few important predictive reactions presented here, a plausible mechanism of the complete reaction is presented.

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