Determination of thiocyanate in saliva by headspace gas chromatography-mass spectrometry, following a single-step aqueous derivatization with triethyloxonium tetrafluoroborate

Sara Ammazzini [a,b], Massimo Onor [a], Enea Pagliano [c,#], Zoltán Mester [c], Beatrice Campanella [a,b], Emanuela Pitzalis [a], Emilia Bramanti [a], and Alessandro D'Ulivo [a]

[a] C.N.R., Institute of Chemistry of Organometallic Compounds, UOS of Pisa, Via Moruzzi, 1, 56124 Pisa, Italy
[b] University of Pisa, Department of Chemistry and Industrial Chemistry, Via Risorgimento, 35 56125 Pisa, Italy
[c] National Research Council of Canada, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada

[#] corresponding author's *e*-mail: enea.pagliano@nrc-cnrc.gc.ca and e.pagliano@libero.it

Abstract

A novel method for the determination of salivary thiocyanate is presented. Thiocyanate has been converted into ethyl thiocyanate by single-step aqueous derivatization based on triethyloxonium tetrafluoroborate and measured by headspace gas chromatography-mass spectrometry (15 min runtime). $S^{13}CN^{-}$ isotopic internal standard has been employed for quantitation purposes. Technical details and fundamental aspects relatively to derivatization chemistry and calibration strategy are here presented.

The method is fast, simple, safe, and sensitive; an instrumental detection limit of 25 ng/g is reported. This procedure has been applied for the determination of thiocyanate in saliva for the discrimination between smokers and nonsmokers.

Keywords: Thiocyanate, saliva, smokers and nonsmokers, triethyloxonium tetrafluoroborate derivatization, GCMS, isotope dilution

1. Introduction

The determination of thiocyanate in biological fluids is of biomedical and toxicological relevance [1]. Thiocyanate is the major metabolic detoxification product of cyanide and its generation is associated with the transsulfuration reaction between a sulfur-donor molecule – such as thiosulfate – and CN^-, catalyzed by the rhodanese enzyme [2-4]. Traces of cyanide can be found in almost all edible plants such as almonds, cabbages, and turnip [5]. However, the main pathway for human exposure to cyanide is represented by tobacco smoke which can yield up to 500 μg of HCN per cigarette [6]. HCN may enter the body via lungs, gastrointestinal tract and skin; chronic exposure to cyanide, even at low levels, can promote the development of serious health conditions, including myelin degeneration and abnormal thyroid activity [7]. Thiocyanate is regarded as a long-term biomarker for cyanide exposure [6]; it is the first biomarker for the assessment of the internal smoking dose [6] and its determination in saliva, serum and urine is still used to distinguish smokers from nonsmokers [8-9].

Historically, the first analytical approaches for the determination of this metabolite in biological fluids were based on the spectrophotometric detection of colored complexes associated with this analyte [10-11]; for example, Bowler's method exploited the reactivity of SCN^- with Fe(III) in acidic media to yield the reddish $[Fe(SCN)]^{2+}$ [8, 12]. The use of ion chromatography was proposed later, and the detection of thiocyanate was accomplished with UV at 210 nm [13], or suppressed conductivity [14]. The use of a chromatographic technique is surely an advantage with respect to the direct spectrometric detection in terms of control of the interferences, but the use of a non-analyte specific detector may give rise to specificity problems when complex matrices are investigated. In this vein, the introduction of mass spectrometry has been beneficial in addressing both specificity and sensitivity issues. Moreover, mass spectrometry allows for the use of isotopic enriched internal standards for quantitative purposes, thus making the analytical methods robust and adequate for high-level analytical performance in terms of accuracy and precision [15]. For the determination of thiocyanate in biological fluids, mass spectrometry has been proposed in combination with high performance liquid chromatography (HPLC) [16] and gas chromatography (GC) [17-19]. HPLC methods, in general, require a laborious sample preparation designed to remove the biological matrix and a derivatization [20]. Similarly, thiocyanate is a non-volatile anion that requires derivatization before its introduction in gas chromatography. For example, extractive alkylation with pentafluorobenzyl bromide has been employed to convert SCN^- into F_5Bz-SCN [17]. Despite F_5Bz-SCN being a volatile molecule suitable for gas chromatography, derivatization with F_5BzBr presents some limitations. For example, F_5Bz-Br derivatization is a non-aqueous reaction carried out at 70 °C. Furthermore, the GC analysis is performed on a solvent extract that may contain a number of hydrophobic compounds associated with the biological matrix. Finally, the detection limits reported for mass spectrometric detection in positive EI (0.2 µg/mL of SCN^-) [17] are not ideal for trace analysis, and only the use of chemical ionization in negative mode can bring the limit of detection into the low part-per-billion range [19]. To overcome the disadvantages of the F_5Bz-Br approach, we recently proposed an alternative carbon-based derivatization chemistry which employs triethyloxonium salts [21-22]. $Et_3O^+[BF_4]^-$ [23-25] is a water soluble reagent able to perform ethylation directly in aqueous media and at room temperature. The derivatization step is straightforward and allows for conversion of thiocyanate anion to the corresponding ethyl derivative Et-SCN. Ethyl thiocyanate is a semi-volatile molecule whose boiling point is 146 °C [26] and, therefore, it is suitable to be sampled from the headspace, providing for a first order separation of the analyte from the sample matrix. In this paper we describe this novel analytical method for the determination of thiocyanate by GCMS. The method has been implemented for the determination of salivary thiocyanate of smokers and nonsmokers.

2. Materials and methods

2.1 Reagents and standards

Isotopically enriched thiocyanate, $KS^{13}CN$ ($x(^{13}C) = 0.99$ mol/mol) was purchased from Aldrich, while a primary standard solution of thiocyanate of natural isotopic composition was obtained from Fluka (1000 ± 5 µg/mL of SCN^- in water, analytical standard). Triethyloxonium tetrafluoroborate (Fluka; $w(Et_3O^+[BF_4]^-) \geq 0.97$ g/g), sodium hydroxide solution (Fluka, *Trace***SELECT**®; $w(NaOH) \approx 0.3$ g/g), and sodium sulfate decahydrate (Carlo Erba; $w(Na_2SO_4{\cdot}10\ H_2O]) \geq 0.99$ g/g) were used. Preparation/dilution of samples and solutions was performed gravimetrically using ultrapure water (MilliQ; 18.2 MΩ·cm at 25 °C).

2.2 Samples of saliva

Saliva samples were collected from ten healthy volunteers by using STARSTED Salivette® for Cortisol Testing. The sampling was performed in the morning before breakfast, first cigarette, and teeth brushing by holding the Salivette's swab in the mouth for 10 min without chewing. Each sample was then kept frozen at –20 °C until the day of analysis. Among the volunteers, five were nonsmokers (samples C1 to C5) while five were smokers (samples C6 to C10). The volunteers were chosen regardless of age (from 20 to 60 years old) and sex.

2.3 Safety considerations

Triethyloxonium tetrafluoroborate is a strong alkylating agent and a water-soluble nonvolatile salt. To avoid exposure to its decomposition byproducts, it is recommended this chemical be handle in a fumehood. The solid $Et_3O^+[BF_4]^-$ should be kept in a refrigerator at –20 °C.

2.4 Analytical procedure

For the calibration of the instrumental response, aliquots of 50 µL, 100 µL, 200 µL, 400 µL, and 800 µL volume of a primary standard solution of thiocyanate (0.3000 µg/g of SCN^-) were introduced in 10 mL headspace vials used for a CombiPAL autosampler (CTC Analytics, Switzerland). On the other hand, for the preparation of the samples, the saliva was first diluted 1:10 with ultrapure water and then 50 µL of this solution was placed in a CombiPAL vial. The transfer of such volumes was accomplished using adjustable pipettes and, for better precision, all aliquots were weighted. At this point each of the vials containing standards/samples was spiked with 50 µL of the isotopic internal standard (100.0 µg/g of $S^{13}CN^-$) following by the addition of 1 g of $Na_2SO_4 \cdot 10\ H_2O$, 10 µL of 10 M NaOH, and enough ultrapure water to reach a final volume of 6 mL. All the above solutions were then reacted with 100 µL of an aqueous solution of triethyloxonium tetrafluoroborate. The latter solution was prepared by dissolving 1 g $Et_3O^+[BF_4]^-$ in 1 mL of cold water (4 °C). In such conditions, the reagent is not stable for a long time and must be added quickly to samples and standards. The vials were then sealed with holed screw-caps equipped with teflon/silicon septum for use with the CombiPAL and kept at room temperature in the dark for at least 30 min in order to allow the ethylation of the analytes to proceed.

2.5 Gas chromatography mass spectrometry

$Et_3O^+[BF_4]^-$ converts thiocyanate to ethyl thiocyanate which can be analyzed by headspace GCMS. An Agilent 6850 gas chromatograph, equipped with a split/splitless injector, was used in combination with an Agilent 5975c mass spectrometer. A CTC CombiPAL autosampler was employed for the headspace sampling. The vials were incubated at 70 °C for 10 min. A 1 mL headspace volume was then sampled (gas-tight syringe held at 80 °C) and injected in the gas chromatograph. The syringe was then flushed with helium. The inlet liner (internal diameter of 1 mm) was held at 200 °C and the injection was performed in split mode (split ratio 8:1; helium flow rate: 1 mL/min). Compounds were then separated on a mid-polarity column (DB-624; length: 60 m; stationary phase: 6%-cyanopropyl-phenyl-94%-dimethyl polysiloxane; 0.25 mm inner diameter; 1.40 µm coating) using the following temperature program: 2 min at 60 °C, then 15 °C/min to 200 °C followed by 30 °C/min to 250 °C held for 2 min (total runtime: 15

min). The temperature of the transfer line was set at 260 °C. After GC separation, ethyl thiocyanate was ionized in positive EI. The acquisition was performed in total ion chromatography (TIC) and in single ion monitoring (SIM). TIC allowed for the identification of the derivative, while SIM was implemented for quantitative purposes by monitoring *m*/*z* of 59, 60, 87 and 88 (100 ms dwell time).

3. Results and discussion

3.1 Fundamental aspects of triethyloxonium derivatization

The use of triethyloxonium tetrafluoroborate has been recently introduced in the field of analytical chemistry as a reagent for derivatization of several inorganic anions of environmental and biomedical interest [21-22]. Such inorganic anions cannot be analyzed directly by gas chromatography and are converted into volatile ethyl-derivatives by reaction with triethyloxonium. For example, thiocyanate is reacted with $Et_3O^+(aq)$ according to the following reaction:

$$SCN^-(aq) + Et_3O^+(aq) \rightarrow Et{-}SCN(l) + Et_2O \tag{1}$$

$$Et{-}SCN(l) \;\square\; Et{-}SCN(g) \tag{2}$$

Triethyloxonium salts can perform ethylation in aqueous media at room temperature. An aqueous solution of $Et_3O^+[BF_4]^-$ is readily prepared by dissolving the oxonium reagent in water and can be employed directly for derivatization of an aqueous sample.

Aqueous solutions of triethyloxonium, however, are not stable because of their disposition to hydrolysis. At room temperature complete hydrolysis can occur after 3 hours:

$$H_2O + Et_3O^+ \rightarrow Et{-}OH + Et_2O + H^+ \tag{3}$$

therefore aqueous $Et_3O^+[BF_4]^-$ is being typically used within 10 minutes from its preparation. In general, the reactivity of triethyloxonium tetrafluoroborate toward the analytes of interest and, consequently, the efficiency of the derivatization, depend on experimental conditions such as the amount of reagent used for derivatization, and the acidity of the reaction media.

By increasing the amount of triethyloxonium, derivatization yield increases, but the pH of the sample turns acidic (3). For certain analytes, such as thiocyanate, strong acidic conditions are undesirable because of the potential protonation of the analyte:

$$SCN^- + H^+ \;\square\; HSCN \; (pKa = 0.9) \tag{4}$$

The protonation of the analyte is responsible for the deactivation of the analyte toward ethylation, resulting decreased derivatization yield.

In order to optimize the experimental conditions, the use of an alkaline buffer is therefore suggested for limiting reaction (4). For the determination of thiocyanate we found that the pre-treatment of an aqueous sample with 10 µL of 10 M NaOH followed by the addition of 100 µL of aqueous 50% (w/w) $Et_3O^+[BF_4]^-$ is suitable to keep the pH of the reaction medium above 1.5, ideal for the derivatization of thiocyanate.

3.2 Quantification model

The use of an isotopic internal standard in analytical chemistry allows for isotope dilution calibration which is regarded as the best method of quantitation for accurate and precise measurements in mass spectrometry. Over the years the formulation of isotope dilution has witnessed many variations [15]. The easiest isotope dilution quantitation is based on a linear calibration plot wherein the analyst adds varying amounts of a primary standard solution of the analyte ($m_{A*(A*B)}$) to different vials, spiking them with the same amount of isotopic internal standard ($m_{B(A*B)}$) and, after homogenization of the resulting blend (A*B), proceeds with the GCMS. The mass spectrometric analysis will provide a response under the form of an isotope ratio (r_{A*B}).

In our case, the isotope ratio r_{A*B} is the ratio between the area of the chromatographic peak extracted at $m/z = 87$ ($CH_3CH_2S^{12}CN^{+\cdot}$, the most abundant natural isotope) and the area of the chromatographic peak extracted at $m/z = 88$ ($CH_3CH_2S^{13}CN^{+\cdot}$, the most abundant isotope in the isotopic internal standard).

The construction is constructed by plotting the ratio $m_{A*(A*B)}/\ m_{B*(A*B)}$ (x-axis) versus the measured isotopic ratio r_{A*B}. The dependence of these two quantities is described by a hyperbolic relation [27]. In our case, however, the deviations of the calibration plot from linearity are modest; therefore a linear fitting may be accepted:

$$r_{A*B} = a_0 + a_1 \cdot \left(\frac{m_{A*(A*B)}}{m_{B(A*B)}} \right) \qquad (5)$$

For the utmost precision and accuracy a higher model for quantitation could be chosen [15]. Figure 5 shows the calibration graph obtained applying Eq. 5 (the raw data for the construction of the plot are presented in the supplementary information). The mass fraction of the analyte in the sample under investigation (w_A) could readily be obtained by the following equation:

$$w_{A} = d \cdot w_{A^*} \cdot \frac{r_{AB} - a_0}{a_1} \cdot \frac{m_{B(AB)}}{m_{A(AB)}} \qquad (6)$$

where d is the dilution factor (in this case 10 because the saliva samples where initially diluted 1:10), w_{A^*} is the mass fraction of thiocyanate in the primary standard used for the preparation of the calibration blends (in this case 3.05 µg/g), a_0, and a_1 are the fitting parameters of equation (5), $m_{A(AB)}$ and $m_{B(AB)}$ are respectively the mass of the diluted (1:10) sample and the mass of the isotopic internal standard used to prepare the sample blend (AB). Finally r_{AB} is the isotopic ratio of the blend AB measured in the same way as r_{A^*B}. An example calculation is reported in the supplementary information.

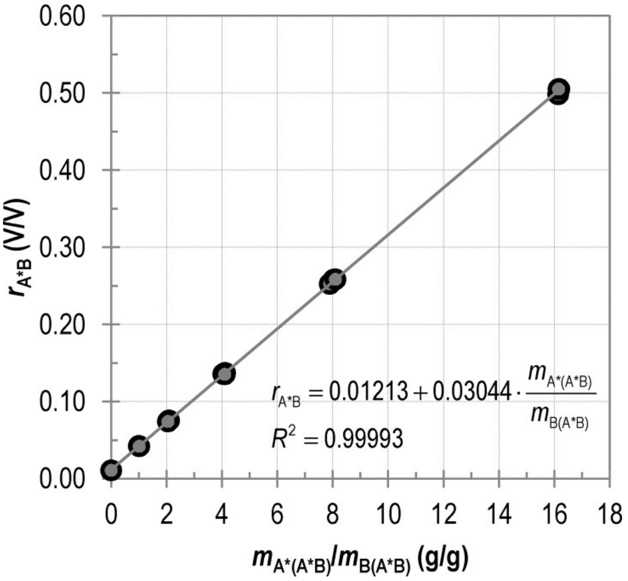

Figure 1. Calibration plot used to quantitate thiocyanate in the saliva sample. Despite the intrinsic nonlinearity of the isotope ratio versus mass fraction, the linear approximation produces satisfactory results in this limited concentration range

3.3 Reproducibility of the isotope ratios in time

For isotope dilution calibration is a prerequisite that the blend of sample and isotopic internal standard is fully equilibrated and there is no formation of analyte during the analytical process [28]. Each of these possible events would result in variations during time of the measured isotopic ratio of the blend of sample and isotopic standard, leading to systematic errors. In order probe the equilibration assumption, the method has been applied to measure the isotope ratio of a blend of saliva sample and isotopic standard over time. Table 1 shows that the equilibration between sample and internal standard occurs instantly.

Isotope dilution, however, can account for analyte losses due for example to sample handling or storage. It was observed that a saliva sample not properly stored can suffer a loss of thiocyanate up to 10% within a week. In this regards, it is suggested the isotopic internal standard to be added within a short period of time after the sampling with Salivette's swab.

3.4 Analytical figures of merit

Figure 2 shows a typical extracted ion chromatogram of thiocyanate in a saliva sample. Despite the complexity of this matrix, it is noteworthy the chromatogram interference free, likely because of the intrinsic advantage of headspace analysis.

The thiocyanate elutes under the form of the ethyl-derivative which is readily detected with mass spectrometry in EI+ mode. The EtSCN mass spectrum is shown in Figure 3. The mass spectrum of the ethyl thiocyanate generated from a standard of natural isotopic composition reveals main signals at *m*/*z* = 87 $[\mathrm{Et\text{-}S^{12}CN}]^+$, 72 $[\mathrm{CH_2\text{-}S^{12}CN}]^+$, 59 $[\mathrm{HS^{12}CN}]^+$, 45 $[\mathrm{CH\text{-}S}]^+$, and 29 $[\mathrm{CH_3CH_2}]^+$. The mass spectrum of the isotopic internal standard, enriched in ^{13}C, exhibits the expected +1 shift.

The instrumental limit of detection for thiocyanate in saliva is 25 ng/g. The estimation of the detection limit is based on the signal-to-noise ratio calculated from the standard deviation of the baseline in proximity to the analytical peak (i.e., detection limit is the concentration which produces a signal-to-noise-ratio of 3). Moreover, thanks to the use of the isotopic internal standard, the relative standard deviation of the method is below 2% based on three independent measurements.

Table 1. Variation of the $S^{12}CN^{-}/S^{13}CN^{-}$ isotope ratio in a saliva sample

Time /h	**Isotope ratio /(V/V)**
0	0.4544
1	0.4508
2	0.4513
5	0.4529
24	0.4560
48	0.4555
49	0.4517
50	0.4533
51	0.4591
52	0.4519
Average	0.4537 ± 0.0026
RSD	0.57% (*a*)

(*a*). A blend of saliva sample was prepared according to the procedure describe herein and measured at different times. No significant variations could be observed.

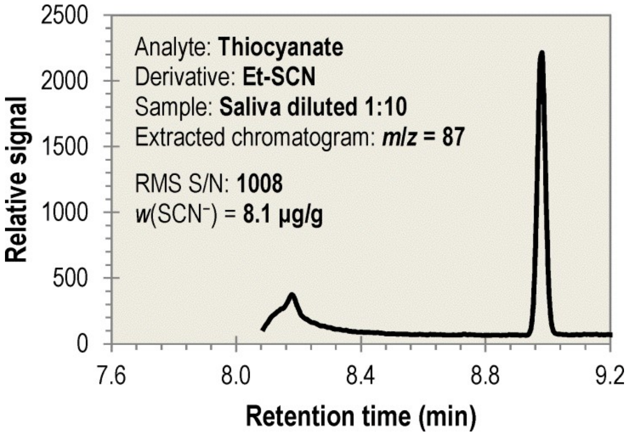

Figure 2. SIM gas chromatogram (extracted at *m*/*z* = 87) of a sample of derivatized saliva (diluted 1:10). The thiocyanate retention time is 8.97 min.

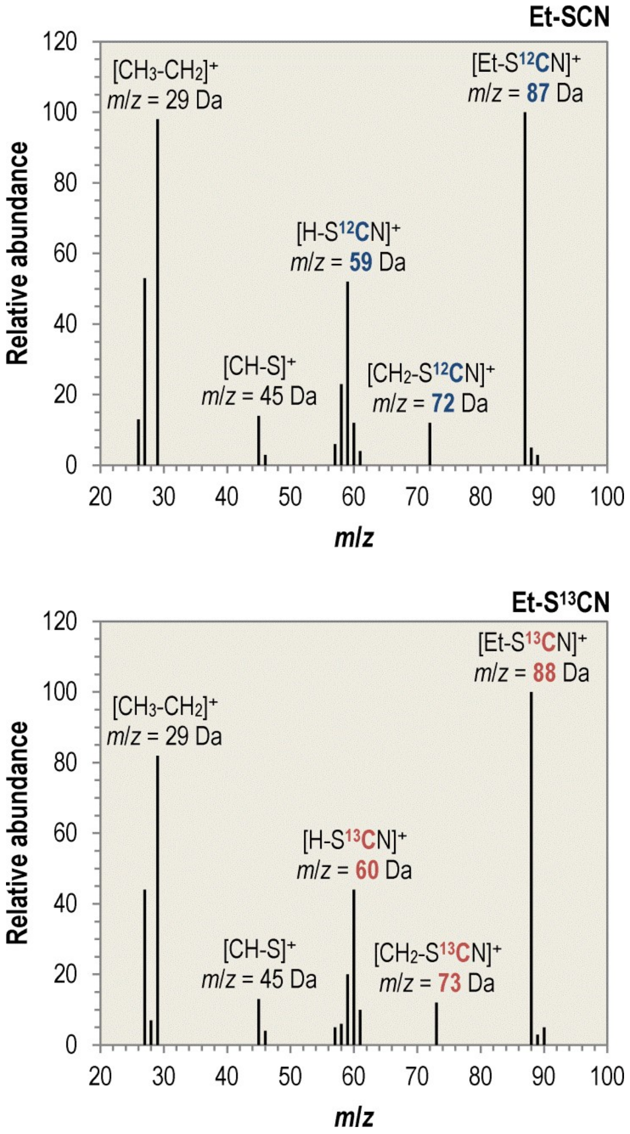

Figure 3. Experimental EI+ mass spectra of the ethyl thiocyanate of natural isotopic composition (top) and of the ^{13}C enriched ethyl thiocyanate (bottom)

3.5 Determination of salivary thiocyanate of smokers and nonsmokers

The method was applied for the quantitation of thiocyanate in some saliva samples. Among ten healthy volunteers five of them were nonsmokers (C1 to C5) and five smokers (C6 to C10). Within the smokers the sampling was performed after at least 12 hours' time from the last cigarette (the half-life of thiocyanate in saliva range from 10 to 14 days [6]). The levels of thiocyanate observed in the saliva of these volunteers are reported in Figure 4. In a grand average the smoking group shows a mass fraction of thiocyanate in their saliva of 145.0 ± 49.3 μg/g while the nonsmoking group is 36.1 ± 21.7 μg/g. The analytical results obtained are consistent with the data reported in the literature [9, 29] and can effectively be used to distinguish smokers from nonsmokers [8].

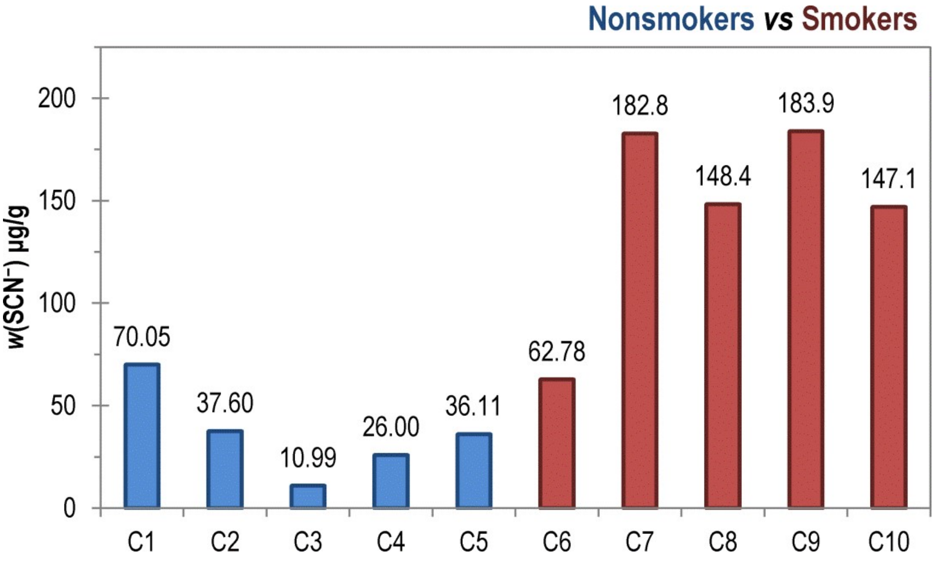

Figure 4. Salivary thiocyanate levels from ten healthy volunteers. The bars from C1 to C5 report the mass fraction of thiocyanate recovered from the saliva of nonsmokers (on average 36.1 ± 21.7 µg/g), while the bars from C6 to C10 present the mass fraction of thiocyanate recovered from the saliva of smokers (on average 145.0 ± 49.3 µg/g)

4. Conclusion

Since the end of the 19th century [6], thiocyanate has been regarded as a biomarker for the internal smoking dose, and its determination in biological fluids serves to discriminate between smokers and nonsmokers. The GCMS analytical method proposed here for the determination of this analyte is sensitive, specific, robust, and can be applied to the analysis of complex biological matrices such as saliva. The derivatization is a safe single-step process, performed with aqueous chemistry which allows for headspace sampling. Furthermore, the use of an isotopic internal standard enhances the precision and accuracy of the generated analytical results.

References

---

[1] D.M. Prue, J.E. Martin, A.S. Hume, Behav. Ther. 11 (1980) 368.

[2] S.I. Baskin, D.W. Porter, G.A. Rockwood, J.A. Romano Jr., H.C. Patel, R.C. Kiser, C.M. Cook, A.L. Ternay Jr., J. Appl. Toxicol. 19 (1999) 173.

[3] L. Frankenberg, Arch. Toxicol. 45 (1980) 315.

[4] H. Fiedler, J.L. Wood, J. Biol. Chem. 222 (1956) 387.

[5] R.G.H. Baumeister, H. Schievelbein, G. Zickgraf-Rüdel, Arzneim.-Forsch. Drug Res. 25 (1975) 1056.

[6] G. Scherer, Exp. Toxicol. Pathol. 58 (2006) 101.

[7] S.L. Youso, G.A. Rockwood, B.A. Logue, J. Anal. Toxicol. 36 (2012) 265.

[8] W.C. Butts, M. Kuehneman, G.M. Widdowson, Clin. Chem. 20 (1974) 1344.

[9] M.T. Jafari, M. Javaheri, Anal. Chem. 82 (2010), 6721.

[10] A.R. Pettigrew, G.S. Fell, Clin. Chem. 18 (1972) 996.

[11] W.N. Aldridge, Analyst 69 (1944) 262.

[12] R.G. Bowler, Biochem. J. 38 (1944) 385.

[13] Y. Michigami, K. Fujii, K. Ueda, Y. Yamamoto, Analyst 117 (1992) 1855.

[14] I. Demkowska, Ż. Polkowska, J. Namieśnik, J. Chromatogr., B 875 (2008) 419.

[15] E. Pagliano, Z. Mester, J. Meija, Anal. Bioanal. Chem. 405 (2013) 2879.

[16] R.K. Bhandari, E. Manandhar, R.P. Oda, G.A. Rockwood, B.A. Logue, Anal. Bioanal. Chem. 406 (2014) 727.

[17] S. Kage, T. Nagata, K. Kudo, J. Chromatogr., B 675 (1996) 27.

[18] B.D. Paul, M.L. Smith, J. Anal. Toxicol. 30 (2006) 511.

[19] R.K. Bhandari, R.P. Oda, S.L. Youso, I. Petrikovics, V.S. Bebarta, G.A. Rockwood, B.A. Logue, Anal. Bioanal. Chem. 404 (2012) 2287.

[20] K. Minakata, H. Nozawa, I. Yamagishi, K. Gonmori, K. Hasegawa, M. Suzuki, K. Watanabe, O. Suzuki, Forensic Toxicol. 30 (2012) 45.

---

[21] A. D’Ulivo, E. Pagliano, M. Onor, E. Pitzalis, R. Zamboni, Anal. Chem. 81 (2009) 6399.

[22] E. Pagliano, J. Meija, J. Ding, R.E. Sturgeon, A. D’Ulivo, Z. Mester, Anal. Chem. 85 (2013) 877.

[23] H. Meerwein, E. Battenberg, H. Gold, E. Pfeil, G. Willfang, J. Prakt. Chem. 154 (1939) 83.

[24] H. Perst, Oxonium Ions in Organic Chemistry, Verlag Chemie GmbH, Weinheim, 1971.

[25] G.A. Olah, K.K. Laali, Q. Wang, G.K. Surya Prakash, Onium Ions, Wiley, New York, 1998.

[26] R.C. Weast, J.G. Grasselli, CRC Handbook of Data on Organic Compounds, 2nd Editon, CRC Press, Inc., Boca Raton, FL, 1989, 1.

[27] J.F. Pickup, K. McPherson, Anal. Chem. 48 (1976) 1885.

[28] J. Meija, Z. Mester, Anal. Chim. Acta 607 (2008) 115.

[29] Z.-F. Chen, B.W. Darvell, V.W.-H. Leung, Arch. Oral. Biol. 49 (2004) 863.