Application of Ion Chromatography for the Determination of Inorganic Ions, Especially Thiocyanates, in Human Semen Samples as Biomarkers of Environmental Tobacco Smoke Exposure

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Abstract

Tobacco smoking constitutes a significant source of indoor air pollution. Various chemical compounds that are emitted during tobacco smoking can have a direct cytotoxic effect on spermatozoa by damaging DNA. There is some evidence that tobacco smoking in men could affect male fertility. The goals of this study were to find relationships between thiocyanates (as biomarkers of environmental tobacco smoke exposure) and other inorganic ions in human semen samples and present the effectiveness of the proposed sample preparation procedure combined with ion chromatography technique for the determination of inorganic ions, especially thiocyanates, in human semen samples collected from heavy, moderate, and passive smokers, as well as nonsmoking individuals.

Introduction

Tobacco smoking is a major factor influencing indoor air quality. Various toxic, mutagenic and carcinogenic compounds emitted during tobacco smoking can adversely affect male fertility (1). Sperm function is highly dependent on ionic environment (2). The presence of abnormal levels of calcium, magnesium, and trace elements (zinc, copper) may affect spermatogenesis with regard to production, maturation, motility, and fertilizing capacity of spermatozoa (3–6).

One of the biomarkers of environmental tobacco smoke exposure is thiocyanate ions (cyanide metabolites). The level of thiocyanates in human biological fluids (saliva, urine) is considered a good probe for distinguishing between smokers and nonsmokers, and its determination is useful for the evaluation of smoking behavior (7–18).

Based on the published literature, there are no analytical approaches for the determination of thiocyanate concentration levels in human semen samples. Some of the research studies only focused on the relation between tobacco smoking and semen quality parameters, such as density, motility, and morphology (19–22). Additionally, there is still a lack of information regarding relationships between thiocyanates (as biomarkers of environmental tobacco smoke exposure) and other inorganic ions in human semen samples.

Two main goals of the analytical work described here are to 1. present the effectiveness of the proposed sample preparation procedure combined with ion chromatography technique for the determination of inorganic ions, especially thiocyanates in human semen samples (as biomarkers of environmental tobacco smoke exposure), collected from male heavy, moderate and passive smokers, as well as nonsmoking individuals and 2. determine whether tobacco smoking has a significant influence on the chemical composition of human semen.

To the best of our knowledge, this paper can be treated as the first contribution to studies in this area.

Materials and Methods

Chromatographic conditions

Chromatographic separations were performed using ion chromatograph Dionex DX500 (Dionex, Sunnyvale, CA) composed of a GP50 gradient pump and a CD20 conductivity detector. Anions were determined on an IonPac AS9-HC (2 mm × 250 mm) anion exchange column using 9.0 mM Na_2CO_3 eluent at 0.25 mL/min and suppressed conductivity detection with ASRC®-ULTRA suppressor (2 mm) in recycle mode (50

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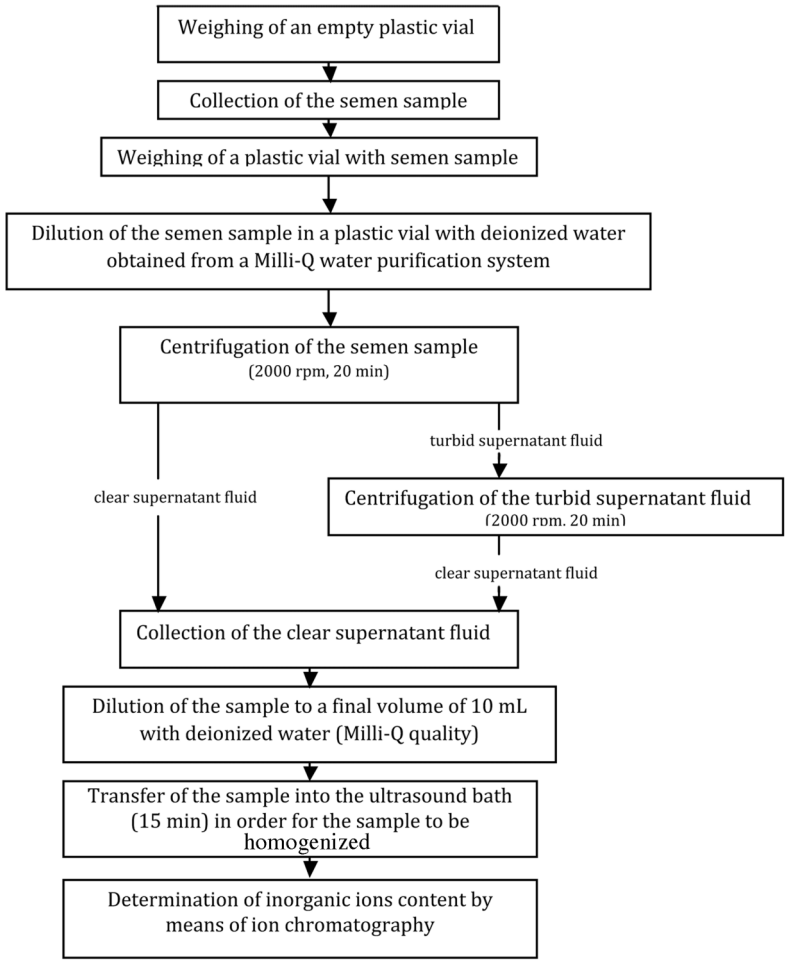

Figure 1. Schematic presentation of the analytical protocol used at the step of semen samples preparation for analysis.

Table I. Metrological Characteristics of Analytical Procedure Used in Studies of Human Semen Samples

Analyte	Precision CV (%)	Linearity (Correlation Coefficient R^2)*	LOD (mg/L)	LOQ (mg/L)	LOD† (× 10^{-5} mg/g)	LOQ† (× 10^{-5} mg/g)	Expanded Uncertainty (%)
Na^+	1.04	0.999	0.01	0.03	1.09	3.27	10
NH_4^+	1.01	0.991	0.01	0.03	1.09	3.27	10
K^+	0.96	0.999	0.01	0.03	1.09	3.27	10
Mg^{2+}	1.17	0.996	0.01	0.03	1.09	3.27	10
Ca^{2+}	0.98	0.993	0.01	0.03	1.09	3.27	10
SCN^-	1.08	0.999	0.01	0.03	1.09	3.27	10
F^-	1.03	0.998	0.01	0.03	1.09	3.27	10
Cl^-	0.98	0.998	0.01	0.03	1.09	3.27	10
Br^-	1.06	0.989	0.01	0.03	1.09	3.27	10
SO_4^{2-}	0.93	0.999	0.01	0.03	1.09	3.27	10
NO_2^-	1.54	0.989	0.03	0.09	3.26	9.78	10
NO_3^-	0.97	0.998	0.01	0.03	1.09	3.27	10
PO_4^{3-}	1.13	0.997	0.02	0.06	2.17	6.51	10

* Linearity determined over the concentration range of 0.2–20 mg/L.
† Calculated on the basis of experimental value of average semen density = 0.92 g/cm^3.

mA). Cations were determined on an IonPac CS14 (2 mm × 250 mm) cation exchange column using 4 mM methanesulfonic acid/5.45 mM pyrophosphoric acid eluent at 0.25 mL/min and suppressed conductivity detection with CSRS®-ULTRA suppressor (2 mm) in recycle mode (50 mA). The chromatographic separation was obtained within 42 min for anions and 18 min for cations. The sample injection volume was 7.5 µL for anions and 2.5 µL for cations.

Chemicals

All standards (Mg^{2+}, Ca^{2+}, NH_4^+, Na^+, K^+, SO_4^{2-}, NO_2^-, NO_3^-, F^-, Cl^-, Br^-, PO_4^{3-} 1000 mg/L, SCN^- 0.1 M) , pyrophosphoric acid and anhydrous sodium carbonate were obtained from Merck (Darmstadt, Germany). Methanesulfonic acid was purchased from Sigma-Aldrich (Schnelldorf, Germany). Deionized water was obtained from a Millipore Gradient A10 (resistivity 18.2 MΩcm at 25°C) water purification system (Millipore, Bedford, MA).

Analytical procedure

Human semen samples were collected by self-stimulation of the genitals into sterile plastic bottles after sexual abstinence, allowed to liquefy at 25°C, and transported to the laboratory for analysis. A schematic presentation of the analytical protocol is shown in Figure 1.

Performance of the analytical procedure used in the studies of human semen samples are presented in Table I.

Results and Discussion

Semen samples were collected from a selected population of male passive, moderate and heavy smokers in order to demonstrate the effectiveness of this method as a means of evaluating smoking behavior. Nonsmoking individuals were also selected. The subjects were classified into the following groups by using their responses from the smoking habits questionnaire. All individuals gave written consent for participation in the research study, which was approved by the independent bioethics committee of the Medical University of Gdańsk (80/2010). The range of analyte concentrations determined in semen samples collected from

heavy, moderate, and passive smokers, as well as nonsmoking individuals is presented in Table II.

Thiocyanate ions as biomarkers of tobacco smoke exposure

In Figure 2, the concentrations of thiocyanate, phosphate, and magnesium ions determined in semen samples corresponding to the different tobacco consumption categories established from the self-reports are shown.

The average concentration values of sodium, potassium, magnesium, and calcium ions were significantly lower in semen samples collected from active and passive smokers compared to semen samples collected from nonsmoking individuals.

Calcium ions regulate a variety of processes occurring in the human body and have the influence on the fertility and pregnancy preservation (2–6). The low concentrations of calcium ion determined in semen samples collected from active and passive smokers (compared to semen samples collected from nonsmoking individuals) can have the influence on the fertility of these individuals. Additionally, low concentrations of sodium, potassium, magnesium, and calcium ions in semen samples can be a result of loss of these analytes from the organism of smoking individuals; the quality of semen also decreases.

The highest concentration levels of nitrate, phosphate, sulfate, and thiocyanate ions (as biomarkers of environmental tobacco smoke exposure) were observed in semen samples collected from active smokers (compared to semen samples collected from nonsmoking individuals), what confirms the influence of tobacco smoking on the concentration levels of these constituents.

Table II. The Range of Analyte Concentrations Determined in Semen Samples Collected from Male Heavy, Moderate, and Passive Smokers and Nonsmoking Individuals*

Analyte	Heavy Smokers†	Moderate Smokers‡	Passive Smokers	Nonsmoking Individuals
Na^+	< LOD–1.67 (0.345)	< LOD–1.52 (0.326)	< LOD–0.999 (0.303)	< LOD–1.52 (0.593)
NH_4^+	< LOD–0.559 (0.127)	0.00005–16.2 (1.84)	< LOD–11.9 (2.04)	< LOD–0.721 (0.231)
K^+	< LOD–1.99 (0.257)	< LOD–1.56 (0.267)	< LOD–1.21 (0.259)	< LOD–1.28 (0.516)
Mg^{2+}	< LOD–0.088 (0.027)	< LOD–0.054 (0.041)	< LOD–0.112 (0.070)	< LOD–0.236 (0.092)
Ca^{2+}	< LOD–1.89 (0.211)	< LOD–0.975 (0.129)	< LOD–1.47 (0.259)	< LOD–2.71 (0.584)
SCN^-	0.00004–0.058 (0.043)	0.00008–0.043 (0.022)	0.00004–0.028 (0.012)	0.00003–0.0020 (0.0004)
F^-	0.041–10.7 (2.40)	0.095–5.12 (0.629)	0.0007–2.94 (1.00)	0.0319–4.06 (0.990)
Cl^-	< LOD–43.3 (7.74)	< LOD–22.9 (5.04)	0.032–20.6 (8.26)	0.107–8.79 (2.94)
Br^-	< LOD–0.591 (0.055)	< LOD–0.474 (0.110)	< LOD–0.414 (0.092)	< LOD–0.100 (0.034)
NO_2^-	< LOD–39.7 (5.90)	< LOD–76.0 (14.0)	< LOD–48.4 (8.07)	< LOD–59.4) (11.9)
NO_3^-	< LOD–0.597 (0.083)	< LOD–0.617 (0.083)	< LOD–0.164 (0.027)	< LOD–0.0360 (0.014)
PO_4^{3-}	$7.67 \cdot 10^{-3}$–178 (44.1)	< LOD–143 (32.7)	$8.22 \cdot 10^{-3}$–46.1 (11.3)	< LOD–5.68 (1.08)
SO_4^{2-}	< LOD–17.7 (1.78)	< LOD–0.917 (0.061)	< LOD–2.36 (0.393)	< LOD (< LOD)

* Each sample was injected in triplicate; the average concentration value of a given analyte calculated for a certain type of the individual is in parentheses.
† More than 10 cigarettes smoked per day.
‡ 1–10 cigarettes smoked per day.

Correlations between ions under investigation determined in semen samples collected from active and passive smokers

The relationships between inorganic ions were calculated and presented in the form of correlation matrices for ion pairs in Table III. The greatest number of the strongest correlation coefficients between inorganic ions was noticed in a group of passive smokers. The number of the strongest correlation coefficients significantly decreased with increasing amount of cigarettes smoked per day, which can be connected with the influence of the intensity of tobacco smoking on the chemical composition of this biological fluid.

Statistical ANOVA test application

In order to compare the concentration levels of ions present in semen samples collected from passive smokers and moderate smokers (A) and nonsmoking individuals and heavy smokers (B), the statistical ANOVA test was conducted. The null hypothesis that average concentration values are equal ($\mu_1 = \mu_2$) against the alternative hypothesis that average concentration values are different ($\mu_1 \neq \mu_2$) in two investigated groups of smokers was

verified. All necessary calculations were made on the basis of the appropriate mathematical model.

If the calculated F_{sample} value is greater than the critical value of F, then the null hypothesis was rejected in favor of the alternative one at the 95% confidence level. In this case, the analyte concentration levels differed statistically significantly in two investigated groups of smokers. If the calculated F_{sample} value is lower than the critical value of F, then the null hypothesis was not rejected in favor of the alternative one at the 95% confidence level. In this case, the analyte concentration

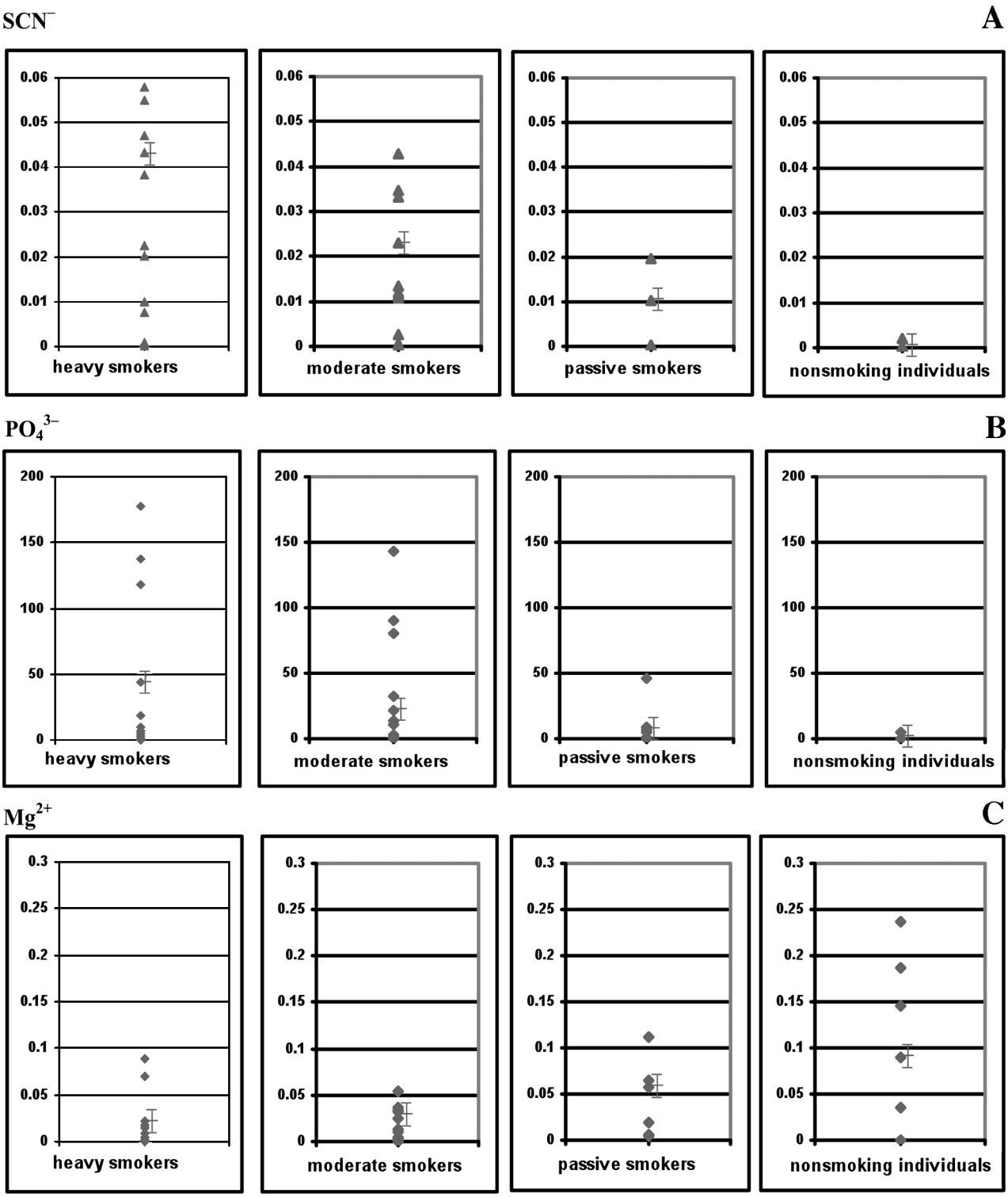

Figure 2. Concentration levels of thiocyanate (A), phosphate (B), and magnesium (C) ions determined in semen samples corresponding to the different tobacco consumption categories.

levels did not differ statistically significantly in two investigated groups of smokers. The calculated F_{sample} values for analytes determined in semen samples collected from passive and moderate smokers (A) and nonsmoking individuals and heavy smokers (B) are presented in Table IV.

The statistical parameters calculated as a result of ANOVA test application can be the basis for statement that analytes determined in semen samples collected from moderate smokers (compared to semen samples collected from passive smokers) did not differ statistically significantly. However, analytes determined in semen samples collected from heavy smokers (compared to semen samples collected from nonsmoking individuals) differed statistically significantly in the case of magnesium ion, which can influence the quality of semen as a genetic material.

Table III. Correlation Matrices for Ion Pairs*

	F^-	Cl^-	PO_4^{3-}	SCN^-	Na^+	NH_4^+	K^+	Mg^{2+}	Ca^{2+}
Nonsmoking individuals									
F^-	1.00								
Cl^-	**0.87**	1.00							
SCN^-	**0.99**	**0.85**		1.00					
Na^+	–0.33	–0.09		–0.36	1.00				
NH_4^+	–0.12	–0.15		–0.18	**0.70**	1.00			
K^+	–0.16	0.19		–0.23	**0.84**	**0.67**	1.00		
Mg^{2+}	–0.51	–0.28		–0.52	**0.97**	**0.61**	**0.72**	1.00	
Ca^{2+}	–0.24	0.27		–0.28	**0.57**	0.07	**0.78**	**0.52**	1.00
Passive smokers									
F^-	1.00								
Cl^-	**0.75**	1.00							
PO_4^{3-}	**0.95**	**0.51**	1.00						
SCN^-	–0.38	–0.50	–0.23	1.00					
Na^+	–0.39	–0.13	–0.41	**0.84**	1.00				
NH_4^+	**0.87**	0.35	**0.98**	–0.08	–0.35	1.00			
K^+	**0.80**	0.25	**0.93**	0.10	–0.20	**0.98**	1.00		
Mg^{2+}	**0.86**	0.34	**0.98**	–0.07	–0.35	**1.00**	**0.99**	1.00	
Ca^{2+}	**0.86**	0.34	**0.97**	–0.06	–0.34	**1.00**	**0.99**	**1.00**	1.00
Moderate smokers									
F^-	1.00								
Cl^-	–0.06	1.00							
PO_4^{3-}	**0.98**	–0.16	1.00						
SCN^-	–0.03	–0.23	–0.02	1.00					
Na^+	–0.23	**0.77**	–0.30	–0.19	1.00				
NH_4^+	0.35	–0.10	0.47	0.01	–0.27	1.00			
K^+	0.08	0.10	0.15	–0.19	0.04	0.15	1.00		
Mg^{2+}	0.14	–0.13	0.30	–0.06	–0.28	**0.69**	**0.57**	1.00	
Ca^{2+}	0.13	–0.13	0.27	–0.06	–0.22	**0.95**	0.15	**0.72**	1.00
Heavy smokers									
F^-	1.00								
Cl^-	0.06	1.00							
PO_4^{3-}	**0.68**	–0.30	1.00						
SCN^-	0.19	–0.20	**0.74**	1.00					
Na^+	**0.59**	–0.17	0.23	–0.15	1.00				
NH_4^+	0.41	–0.33	0.28	–0.03	**0.91**	1.00			
K^+	–0.21	–0.27	–0.03	0.07	–0.05	0.02	1.00		
Mg^{2+}	–0.09	–0.23	0.12	0.32	–0.02	–0.09	–0.12	1.00	
Ca^{2+}	–0.15	–0.20	–0.16	–0.11	0.05	–0.05	–0.16	**0.89**	1.00

* Positive significant correlation coefficients are bold, and negative significant correlations are underlined.

Conclusions

A new, successful approach for human semen sample preparation was presented in this research. Ion chromatography was applied for the first time for the determination of inorganic ions, especially thiocyanates (as biomarkers of environmental tobacco smoke exposure) in human semen samples. It should be underlined that the proposed sample preparation procedure combined with ion chromatography analysis is undoubtedly a valuable tool for studies of concentration level of a wide spectrum of inorganic ions in human semen samples.

Taking the obtained results into consideration, lower concentration values of sodium, potassium, magnesium, and calcium ions were observed in semen samples collected from active and passive smokers compared to semen samples collected from nonsmoking individuals. It can be a result of loss of these analytes from the organism of smoking individuals, the quality of semen also decreases. The highest concentration levels of nitrate, phosphate, sulfate, and thiocyanate ions (as biomarkers of environmental tobacco smoke exposure) were noticed in semen samples collected from active smokers (compared to semen samples collected from nonsmoking individuals), what confirms the influence of tobacco smoking on the concentration levels of these constituents.

The analytical data presented in this research study may be treated as a source of information on the influence of intensity of smoking on the chemical composition of human semen samples.

Table IV. The Calculated F_{sample} Values for Analytes Determined in Semen Samples Collected from Passive and Moderate Smokers (A) and Nonsmoking Individuals and Heavy Smokers (B)

Analyte	Calculated F_{sample} Value	Critical Value of F	Verification of the Null Hypothesis Against the Alternative One
Passive and moderate smokers (A)			
F^-	0.382	4.38	$\mu_1 = \mu_2$
Cl^-	0.537	4.41	$\mu_1 = \mu_2$
Br^-	0.157	4.84	$\mu_1 = \mu_2$
PO_4^{3-}	0.818	4.49	$\mu_1 = \mu_2$
SCN^-	0.194	4.38	$\mu_1 = \mu_2$
Na^+	0.0340	4.60	$\mu_1 = \mu_2$
NH_4^+	0.228	4.45	$\mu_1 = \mu_2$
K^+	0	4.49	$\mu_1 = \mu_2$
Mg^{2+}	0.172	4.54	$\mu_1 = \mu_2$
Ca^{2+}	1.28	4.49	$\mu_1 = \mu_2$
Nonsmoking individuals and heavy smokers (B)			
F^-	1.07	4.45	$\mu_1 = \mu_2$
Cl^-	0.961	4.54	$\mu_1 = \mu_2$
Br^-	0.266	5.59	$\mu_1 = \mu_2$
PO_4^{3-}	0.794	4.54	$\mu_1 = \mu_2$
SCN^-	0.578	4.45	$\mu_1 = \mu_2$
Na^+	2.54	4.67	$\mu_1 = \mu_2$
NH_4^+	1.02	4.67	$\mu_1 = \mu_2$
K^+	1.14	4.60	$\mu_1 = \mu_2$
Mg^{2+}	6.63	4.84	$\mu_1 \neq \mu_2$
Ca^{2+}	0.760	4.75	$\mu_1 = \mu_2$

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