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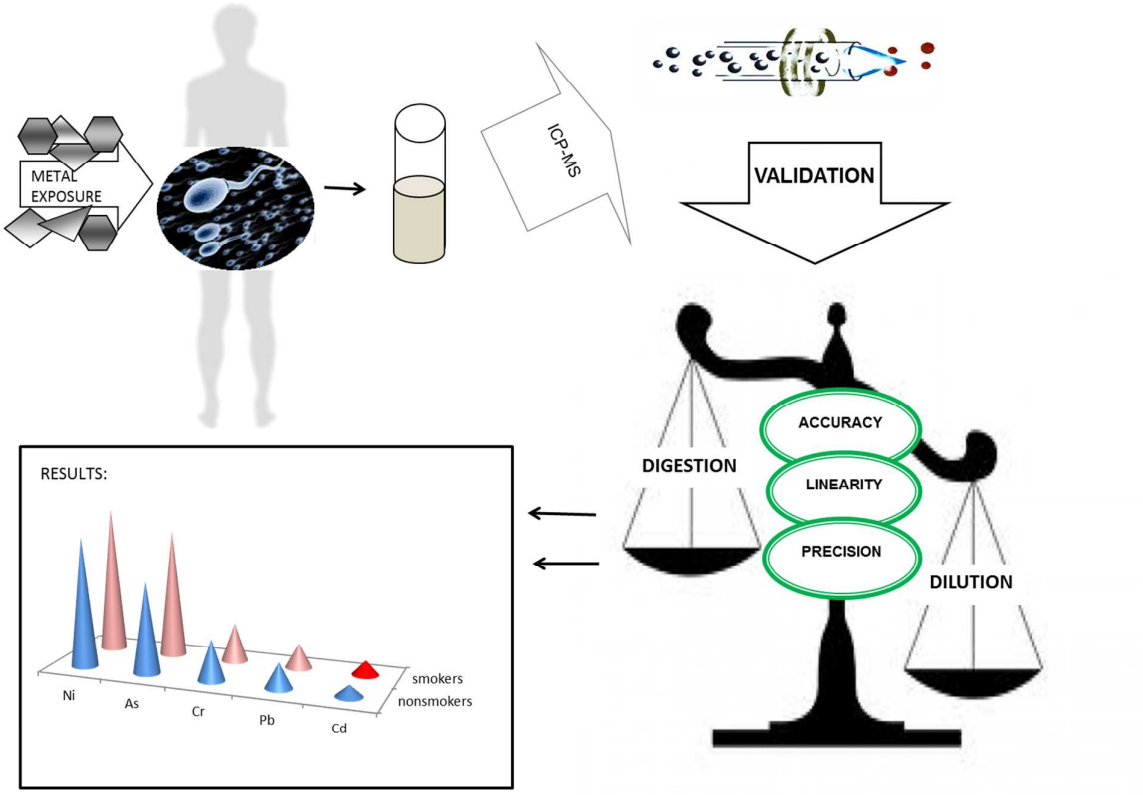
GRAPHICAL ABSTRACT

The method of simple dilution of seminal plasma allowed sufficiently sensitive and reproducible simultaneous measurement of 20 elements in seminal plasma by ICP-MS.



Multielement analysis of human seminal plasma by octopole reaction cell ICP-MS

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This study describes optimisation and validation processes for the simultaneous determination of Ag, As, B, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, Pb, Se, Sn, and Zn in seminal plasma samples by inductively coupled plasma mass spectrometry (ICP-MS) using the octopole reaction system (ORS) collision/reaction cell. Two sample pre-treatment procedures, i.e. digestion and dilution, were explored and compared with regard to linearity, detection and quantification limits obtained on a real sample, and precision. To verify the accuracy of these methods, serum and urine QC materials were used, while spiked seminal plasma pooled samples were used to assess precision and recovery. Although both pre-treatment methods allowed sensitive and reproducible analyses, the method of simple dilution of seminal plasma 1+19 with aqueous solution containing 0.01 mmol L^{-1} EDTA, 0.004% v/v NH_3, and 0.07% v/v TritonX-100 was chosen due to the reduced time of analysis and risks of sample contamination. The proposed method was applied for the simultaneous determination of 20 elements in seminal plasma of 76 adult men with suspected infertility, and the results were compared against recent data from the literature. We investigated the element distribution depending on smoking habits and the results of the comparison between 37 smokers and 39 non-smokers showed significantly higher concentrations of Cd in seminal plasma of smokers as well as dependence on increases in the number of cigarettes smoked per day (P=0.004). In addition, our results are the first to provide data for Ag and B concentrations in human seminal plasma.

Key-words: trace elements, heavy metals, optimization, validation, pre-treatment method

1 Introduction

Seminal plasma is composed of secretions from the male accessory glands, including prostate, seminal vesicles, and epididymis. It provides a safe surrounding and nutrition for spermatozoa, influencing spermatozoa survival and overall fertilization success. Therefore, imbalances in its physiological composition can be related to infertility.[1, 2]

Alongside a wide variety of both organic and inorganic chemical constituents such as sugars, lipids, hormones, enzymes, and vitamins, seminal plasma also contains various chemical elements that can influence male semen quality. The adverse effects of certain elements on human male reproduction have been reviewed recently.[3, 4] It has been indicated that even low-level exposure to lead (Pb) and cadmium (Cd) could adversely affect reproductive health. On the other hand, elements such as copper (Cu), selenium (Se) and zinc (Zn) are cofactors of many enzymes and metalloproteins involved in a wide range of biological processes and are necessary for spermatogenesis, DNA metabolism and repair, and gene expression.[5, 6] Moreover, these elements can reduce the adverse effects of toxic elements.[5, 7-10] One of the most important non-occupational source of potentially harmful elements is tobacco smoke. It contains approximately 30 metal ions, including Cd, Pb, nickel (Ni), and chromium (Cr) and could considerably contribute to increasing body burden.[11] Their concentration could also be increased in seminal plasma.[12-14] Therefore, determinations of the presence and level of various elements in seminal plasma have an important role in the evaluation of reproductive health in men as well as in exposure prevention plans.

In the last twenty years, an increasing number of publications have reported the concentration of some elements in human seminal plasma, mostly with respect to semen quality and

infertility problems.[15-19] Although inductively coupled plasma-mass spectrometry (ICP-MS) is one of the most suitable techniques for the simultaneous determination of trace elements in biological fluids because of its rapidity, low limits of detection, wide linear dynamic range, and minimum sample quantity needed for analysis, only a few studies have used ICP-MS for multi-element determination in human seminal plasma.[20-22] Furthermore, all of these studies provided little or no information about analytical procedures including sample preparation and validity of results, which makes it difficult to compare the published data. In addition, there has been no recent study on multielement status in seminal plasma from European men. Moreover, data for silver (Ag) and boron (B) are still lacking.

The aim of this paper was to: a) determine the most suitable preparation method for the simultaneous determination of Ag, arsenic (As), B, calcium (Ca), Cd, cobalt (Co), Cr, Cu, iron (Fe), potassium (K), lithium (Li), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), Ni, Pb, Se, tin (Sn), and Zn in human seminal plasma by ICP-MS; b) optimize and validate this method; c) determine the concentrations of these 20 elements in 76 men with no-occupational exposure to metals; d) assess the influence of smoking habits on multielement seminal plasma content.

The complexity of the matrix, limited sample volume, very low concentrations of many elements in seminal plasma and the risk of contamination during sample collection and handling, as well as the wide concentration range of elements of interest can cause problems in their reliable quantification. Therefore, the development of a sensitive, easily applicable and accurate method for multi-element analysis of seminal plasma could present an analytical challenge.

2 Experimental

2.1 Instrumentation

The development of the method was carried out using an Agilent 7500cx ICP-MS (Agilent Technologies, Waldbronn, Germany) with an Octopole Reaction System (ORS) collision/reaction

cell in order to minimize the influence of possible interferences. The ORS was operated in one of 3 different modes: no-gas for measurement of Ag (m/z=107), B (m/z=11), and Li (m/z=7), hydrogen for Cr (m/z=52) and Se (m/z=78), and helium mode for As (m/z=75), Ca (m/z=43), Cd (m/z=114), Co (m/z=59), Cu (m/z=63), Fe (m/z=56), K (m/z=39), Mg (m/z=24), Mn (m/z=55), Mo (m/z=95), Na (m/z=23), Ni (m/z=60), Pb (m/z=208), Sn (m/z=118) and Zn (m/z=68). High purity (99.999% V/V) argon, helium and hydrogen were used as cell gases in order to minimize the potential problems caused by unidentified reactive contaminant species in the cell. The 7500cx ICP-MS was equipped with an integrated auto sampler, MicroMist glass concentric nebulizer, a Scott Quartz spray chamber and Ni sampler and skimmer cones, all from Agilent Technologies. Instrumental settings were optimised daily using the software auto-tune function to obtain good sensitivity, low production of oxides (<1%) and doubly charged ions (<2%). Typical optimized instrumental conditions and measurement parameters are listed in Table 1. For internal standardisation, scandium (Sc, m/z=45), germanium (Ge, m/z=72, m/z=74), rhodium (Rh, m/z=103), terbium (Tb, m/z=159), iridium (Ir, m/z=193), and lutetium (Lu, m/z=175) were used.

An UltraCLAVE IV (Milestone Srl, Sorisole, Italy) with integrated software was used for microwave assisted digestion of samples. Details of the procedure are provided in Table 2. The instruments operate under positive pressure maintained by the HVAC (heating, ventilating, and an air-conditioning) system with HEPA filters.

2.2 Reagents and standards

Individual standard solutions (1000±7 mg L^{-1} in 4-10% HNO_3 or 20% HCl) for the determination of total Ag, As, B, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, Pb, Se, Sn, and Zn and internal standard elements, i.e. Sc, Ge, Rh, Tb, Ir, and Lu were obtained from SCP SCIENCE (SCP Science, Quebec, Canada).

The following commercial chemicals were used: suprapur nitric acid (65% HNO_3) and hydrochloric acid (30% HCl) from Merck (Merck, Darmstadt, Germany); ammonia solution (35%

NH_3) and Triton X-100 from BDH Chemicals Ltd (BDH Chemicals Ltd, Poole, England); ethylenediaminetetraacetic acid diammonium salt hydrate (97% EDTA) from Sigma-Aldrich (Sigma-Aldrich, Tokyo, Japan). Nitric acid was purified using the MILESTONE SubPUR/DuoPUR sub-boiling distillation system (Milestone Srl, Sorisole, Italy). The water used was purified with the TKA GenPure Ultra pure water system (TKA Niederelbert, Germany) with specific resistivity of 18.2 MΩ*cm.

Since no suitable reference material for seminal plasma was available, we used standard reference material for serum and urine. Control materials were ClinChek® Serum Controls and ClinChek® Urine Controls, lyophilised for Trace Elements, Levels I and II (Recipe, Munich, Germany), and Seronorm™ Trace Elements Serum L-1 and L-2 (Sero AS, Billingstad, Norway).

Particular attention was paid to precautions against external contamination. All equipment and laboratory glassware were soaked in 10% v/v HNO_3 for 24 h, then rinsed with ultrapure water and dried in a clean box until use.

2.3 Analytical procedures

Initial investigations on the optimisation of the method were conducted with pooled seminal plasma and quality control materials to determine the most suitable sample preparation for the analysis. To obtain optimal analytical characteristics for most analysed elements in seminal plasma, several steps were performed and optimised.

Calibration curves were constructed from calibration standards added in: A, aqueous solution containing 0.01 mmol L^{-1} EDTA, 0.004% v/v NH_3, and 0.07% v/v TritonX-100; AN, aqueous solution containing 1% v/v HNO_3; AP, pool of seminal plasma diluted with aqueous solution A; and DP, deproteinised seminal plasma diluted with aqueous solution AN. Calibration standards were chosen to ensure all samples fell within the expected range. Internal standardisation was achieved by adding Sc, Ge, Rh, Tb, Ir, and Lu single element standards to diluents. The final concentration of internal standards in all sample and calibration solutions was 1 µg L^{-1}.

This study took simple dilution and digestion of samples into consideration as possible sample preparation methods. In the procedure of simple dilution, an aliquot of 150 µL of well mixed seminal plasma was diluted 1+19 with aqueous solution A. For the microwave-assisted digestion procedure, an aliquot of 250 µL of seminal plasma was diluted 1+4 with 50% v/v HNO_3 in quartz glass vessels and digested according to the programme described in Table 2. Colourless digestion solutions without visible precipitate and high recovery results for the most of measured elements in pooled seminal plasma indicated that the samples were completely digested. After cooling, the digested solution of seminal plasma was diluted up to 5 mL with ultrapure water. In addition, an aliquot of 300 µL was further diluted 1+9 with 1% v/v HNO_3.

Each sample was prepared and analysed in duplicate. Blanks, reference materials, and calibration standards were prepared in the same way as the samples and analysed accordingly. The calibration and instrument sensitivity control was performed by re-analysing selected calibration standard every 30 analyses.

For Ca, K, Li, Mg, and Na only aqueous calibrations were utilised. For B, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Ag, Cd, Sn, and Pb, both aqueous and matrix-matched calibrations were utilised.

2.4 Study population

The seminal plasma samples for multi-element concentration determination were obtained from 76 men with suspected infertility, recruited at the Department of Endocrinology and Reproductive Medicine, University Clinic "Vuk Vrhovac" in Zagreb, Croatia. A questionnaire including data on age, place of residence, occupation, dietary habits, smoking habits, alcohol consumption, and medical and occupational history was completed by a physician for each subject. The participants, aged 32 (23-42) years, were not occupationally exposed to metals. The mean and SD of body mass index was 26.1±3.5 kg m^{-2}. The mean semen volume of the participants was 3.2 mL, mean sperm concentration was 61.6x10^6 mL^{-1}, and the average percentage of progressively motile sperm was

21.4%. In addition to seminal plasma Zn, other parameters of prostate secretory function such as acid phosphatase and citric acid were also measured, and the mean±SD values were 2189±1276 U per ejaculate and 81±39 mmol per ejaculate, respectively. Out of the 76 men, 37 were cigarette smokers and 39 non-smokers. Participants in the group of smokers smoked 14±9 cigarettes per day.

All of the participants were asked to fast in the preceding 10 h and abstain from alcohol in the preceding 24 h. They all gave their informed consent before inclusion in the study. The study was performed in compliance with the ethical standards of the Helsinki Declaration, and was approved by the ethics committees of both institutions.

2.5 Sample collection

The ejaculate was collected by masturbation in a metal-free glass container after abstinence from sexual activity in the preceding 4 days. After the ejaculate liquefaction (approximately 15 min), the seminal plasma was obtained by centrifugation at 1,500*g* for 10 min at room temperature. An aliquot of approximately 500 µL of seminal plasma was decanted into a metal-free polypropylene microtube. The samples were frozen and stored at -20 °C until analysed by ICP-MS. Before analysis, seminal plasma samples were thawed at room temperature and then mixed gently for homogenisation.

For the development of analytical method and matrix-matched calibration, a pool of seminal plasma samples was prepared using seminal plasma collected from healthy participants, non-smokers with no occupational exposure to metals.

2.6 Statistics

Statistical analyses were performed using Statistica 12 for Windows (StatSoft Inc., Tulsa, OK, USA). The data were analysed using Shapiro-Wilks test for normality. Because of the skewed distribution of most of the analysed elements, their values were transformed by logarithm. The results relating to the verification of our method are expressed as mean±SD, whereas the results of

elements concentration in seminal plasma in our participants are expressed as mean±SE, median and 5^{th}-95^{th} range. The difference between the groups was assessed by Student's t-test. Correlation analysis between variables was performed by Pearson's test. The level of statistical significance was set at $P<0.05$.

3 Results and discussion

3.1 Pre-treatment method optimization

During the daily instrument optimisation, instrumental parameters such as the gas flow rate, radio frequency (RF) power, and lens setting were chosen to reduce the doubly charged ions or oxide formation. Moreover, to reduce possible plasma-based or matrix interferences, [23, 24] ORS with helium collision mode was used for the measurement of most elements. In helium collision mode, separation between analyte and polyatomic ions is based on Kinetic Energy Discrimination (KED). To be precise, all polyatomic ions are larger than analyte ions of the same mass. Due to more collisions with the He cell gas, polyatomics lose more energy than analyte ions preventing them to pass through to the analyser. This separation allows the multi-element measurement within a wide range of concentrations using constant operating conditions for As, Ca, Cd, Co, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Sn, and Zn in a complex matrix such as seminal plasma. In hydrogen mode, simple reactions with hydrogen remove argon-based polyatomic ions by charge, proton or atom transfer.[25] This mode was used to measure Cr and Se. Standard ("no-gas") mode was used for Ag, B, and Li. Further correction for instrumental drifts and matrix effects included the addition of internal standards to all samples and standards. Memory effects were reduced by adjusting the speed of peristaltic pump during rinse time and by increasing the number of rinsing cycles. Therefore, no interferences were observed at *m/z* values selected in this study.

Seminal plasma samples are very complex due to the high matrix protein content and it was necessary to perform sample treatment prior to analysis. The usual sample preparation methods for determining elements in seminal plasma found in literature are sample digestion or direct dilution.

We included both preparation methods in our initial investigation to find the most suitable method which should be simple, sensitive, accurate and precise, with minimal volume and manipulation of the sample required, and to be useful for the simultaneous measurement of macro- and microelements in the same sample. Sample digestion method is usually the method of choice because of the elimination of organic matter which reduces the matrix effect. However, this method increases the analysis time and risk of sample loss or contamination. The direct dilution method is faster and simpler, and requires minimal manipulation of samples. The disadvantage of this method could be its possible matrix effects.

3.2 Comparison between analytical parameters obtained by dilution and digestion method

To investigate the matrix influence on determination of selected elements in seminal plasma, the slopes for aqueous and matrix-matched calibration graphs were compared by Wilcoxon signed-rank test. Significant difference was found between the slopes of calibration graphs A and AP for As (P=0.003), B (P=0.003), Cd (P=0.041), Pb (P=0.004), Se (P=0.003), Sn (P=0.008) and Zn (P=0.035), and between AN and DP for B (P=0.028), Sn (P=0.028) and Zn (P=0.043). The results indicate that there is a matrix effect on these elements and calibration needs to be prepared on pooled seminal plasma samples (matrix-matched calibration). For simplicity, matrix-matched calibration with pooled seminal plasma was utilised for most elements. However, due to high endogenous concentrations of Ca, K, Li, Mg, Na, and Zn in seminal plasma, aqueous calibration was chosen for these elements.

The limit of detection (LOD) was calculated as 3-times the SD of ten measurements of pool seminal plasma sample and was equivalent to the element concentration in seminal plasma. Comparative results for LOD and the limit of quantification (LOQ=10 SD) between dilution and digestion methods are shown in Table 3. For most measured elements, the obtained LOD and LOQ values were lower for diluted than for digested samples. The only exception was Ni with LOQ of 0.037 μg L^{-1} and 0.018 μg L^{-1} for diluted and digested samples, respectively. Several authors have

previously reported LOD or LOQ values for the measured elements, and only a few used ICP-MS methods. Values obtained for Ca, Mg, and Zn[26] and for Co and Cr[27] in diluted seminal plasma were 5 to 10 times higher than our LOD values, although reported values were obtained on blank samples. Contrary to our results, Li et al.[20] obtained lower LOD values for As (0.01 μg L^{-1}), Cu (0.01 μg L^{-1}), Mn (0.01 μg L^{-1}), Pb (0.001 μg L^{-1}), and Se (0.1 μg L^{-1}) in microwave digested samples. The authors did not specify the method for LOD calculation; however, these values are more similar to our LOD values obtained using blank samples (results not shown).

Using the appropriate calibration approach, different methods of sample preparation were evaluated with serum reference material ClinChek for As, Cd, Co, Cr, Cu, Fe, Li, Mg, Mn, Mo, Ni, Se, Sn, and Zn, which had available certified values. The results based on six measurements of each sample (Table 4) showed good agreement between certified and measured concentrations for most elements measured by both preparation methods. Measured values for Cd, Fe, Mg, Ni, and Sn, obtained by digestion method, were significantly different ($P<0.05$, Student's t-test) from certified values. Using the dilution method, only the values for Fe and Sn were significantly different from the certified values. It should be stressed, however, that all of these measured values were within the control range and therefore would be considered satisfactory.

In addition, recovery tests were performed by adding adequate amounts of analyte to diluted as well as digested solution of pooled seminal plasma. Samples were spiked with concentrations of 0.01 μg L^{-1} for Ag, 0.05 μg L^{-1} for Cd, Co, Cr, Mo, and Sn, 0.1 μg L^{-1} for B, Mn, and Ni, 0.2 μg L^{-1} for As and Se, 7 μg L^{-1} for Cu and Fe, 150 μg L^{-1} for Li and Zn, 1 mg L^{-1} for Ca and Mg, 4 mg L^{-1} for K and 7 mg L^{-1} for Na. Each measurement was performed in duplicate. Recoveries obtained for diluted samples ranged from 92% for B to 110% for K, whereas recoveries for digested samples ranged from 81% for Ni to 123% for B (Figure 1).

Comparison between different sample preparation methods was reported by only one group of authors. Results for the determination of Ca, Cu, Fe, Mg, Zn, Mn, and Se in bovine semen samples by quadrupole-ICP-MS showed good agreement between the method of dilution 1:50 with

a solution containing 0.01% v/v Triton X-100 and 0.5% v/v nitric acid and microwave digestion.[28] Authors also compared aqueous and matrix-matching calibration for dilution method and found no interference for measured elements.

Although both pre-treatment methods resulted in acceptable results, we chose the method of dilution for further investigation. Our results showed that the method of simple dilution of seminal plasma 1+19 with aqueous solution A (containing 0.01 mmol L^{-1} EDTA, 0.004% v/v NH_3, and 0.07% v/v TritonX-100) was adequate to satisfy the majority of crucial requirements for most elements. Moreover, the selected dilution factor was sufficient to overcome accumulation of seminal plasma residue on the nebulizer.

3.3 Analytical parameters

In the concentration ranges used for calibration, the linearity of calibration curves resulted in correlation coefficients ≥ 0.999 for all elements. The LODs and LOQs obtained in this study (Table 3) are satisfactory and suitable for seminal plasma element determination by selected dilution method.

The accuracy of the chosen method was controlled daily using ClinChek® Serum Controls with two levels of element concentrations and the obtained results were within the control range (Table 4). However, ClinChek® Serum Controls is certified only for As, Cd, Co, Cr, Cu, Fe, Li, Mg, Mn, Mo, Ni, Se, Sn, and Zn, whereas there is no certified values for Ag, B, Ca, K, Na, and Pb. Therefore, Seronorm™ Trace Elements Serum with two levels of element concentrations was also used, although it provides certified values for Ca, Co, Cr, Cu, Fe, Li, Mg, Mn, Ni, Se, and Zn, and approximate values for Ag, As, B, Cd, K, Mo, Na, Pb, and Sn. The obtained results (Table 5) were in good agreement with certified or approximate values for the most of measured elements. Table 5 also shows results of the analyses of ClinChek® Urine Control. Precision was controlled by analysing an aliquot of pooled seminal plasma. The relative standard deviation (RSD) of five replicate measurements ranged between 0.11% for Co and 1.83% for Ag when measured on the

same day and between 0.39% for Ca and 7.27% for Ag when measured on different days. In addition, precision (RSD) and trueness, presented as the recovery of added concentration, were calculated on the basis of eight replicate measurements of spiked pooled seminal plasma samples. Samples were spiked with three levels of added element and the results are shown in Table 6. Precision was in the range from 0.26% (Na, low level) to 7.61% (Cr, low level). Recovery ranged from 90% to 110% for almost all analytes except for B (88%), and Zn (89%) at a middle level of added concentration and for B (88%) at a high level.

3.4 Element concentration in human seminal plasma

The developed method was applied for the determination of 20 essential and non-essential elements in 76 men with suspected infertility and no-occupational exposure to metals. The results are shown in Table 7. Concentrations measured in seminal plasma were one or more orders of magnitude higher than LOQ values for all elements except for Li. Only Li concentrations were below LOD of 0.017 mg L^{-1} in 31% (24/76) of samples. Therefore, for the statistical analysis, the measured concentrations below the LOD were assigned to LOD/2. A very low concentration of <1 μg L^{-1} was found for Ag, Cd and Sn, whereas a concentration of several hundred mg L^{-1} was found for Ca, K, Zn and Na.

Besides us, only Katayama et al.[22] reported Li values in seminal plasma and their results were within a narrower range of values than ours (Table 8). No published results for Ag or B were found. Our values for Ca, Co, Na, Ni, and Se were more or less similar to those found in other studies (Table 8), whereas the results for As, Pb and Sn were lower than those of other authors.[21, 22]

For some elements, however, literature data varies over a wide range. For example, the reported values for Zn ranged from $<10^{-2}$ mg L^{-1} in men referred for fertility treatment from India[29] to about 10^{3} mg L^{-1} in normozoospermic, oligospermic and azoospermic men from Ghana.[30] Both authors used atomic absorption spectrometry (AAS) for the measurement of Zn in several groups of men divided according to semen quality parameters. The reported AAS value for Cu in

normozoospermic men was 15.5±5.0 μg L^{-1} in men from India[29], but (173.29±19.3)*10^3 μg L^{-1} in men from Iraq.[31] Considerably different Fe values were reported for oligozoospermic men from Lucknow, India by Shukla et al.[29] (59.7±5.7 μg L^{-1}) and by Pant and Srivastava[32] (46.67±15.95)*10^3μg L^{-1}, but the reason behind the difference in the two findings is hard to explain. Elevated levels of Pb in seminal plasma were found in IVF patients from the US[16], and of Cd in Nigeria[17], although these studies were performed in occupationally unexposed subjects. Because analytical or validation parameters were reported only occasionally, it is possible that the observed discrepancies between the reported concentrations were the result of analytical difficulties due to different techniques or preparation methods used; however, they could also be the result of different geographical origin, dietary habits or differences in physiological state among the participants.

In addition, lifestyle such as smoking habits and alcohol consumption could also have a considerable impact. Tobacco smoking is one of the most important non-occupational sources of metal exposure. Among several thousands of potentially toxic chemicals, elements such as Cd, As, Cr, Pb, and Ni are identified in tobacco smoke and accumulate in tissues and fluids through smoking. However, only several studies (Table 8b) have reported element concentration in seminal plasma with regard to smoking habits and their results are inconsistent. For example, a significant difference was found for Zn in seminal plasma between smokers and non-smokers among infertile patients from China[33] and fertile men from Egypt,[34] whereas no significant difference for Ca, Mg, Zn, Na, and K was found between smokers and non-smokers among the groups of fertile and infertile subjects from Iran.[18] In the group of infertile men, seminal plasma levels of Pb and Cd were higher in smokers than in non-smokers.[14] Highly significant[12, 35] or no significant[36] difference in seminal plasma Cd between smokers and non-smokers was found. The results of our study showed significantly higher Cd in seminal plasma of smokers than non-smokers (P=0.02), whereas there was no significant difference in other measured elements between the groups (Table 7). In addition, Cd in seminal plasma significantly increased with a higher number of cigarettes smoked per day (r=0.393, P=0.004). Figure 2 shows seminal plasma Cd values with regard to the number of

cigarettes smoked per day. It is important to note that these results were obtained from the group of 39 non-smokers and 37 smokers, of whom only 9 smoked 20 or more cigarettes per day.

4 Conclusions

The method of simple dilution of seminal plasma 1+19 with aqueous solution A (containing 0.01 mmol L^{-1} EDTA, 0.004% v/v NH_3, and 0.07% v/v TritonX-100) allowed sufficiently sensitive and reproducible simultaneous measurement of 20 elements in seminal plasma by ICP-MS. The use of ORS collision/reaction cell minimized the influence of interferences. The method of dilution was selected due to reduced time of analysis and risks of sample contamination and was applied for the simultaneous determination of 20 elements in seminal plasma of 76 adult men with suspected infertility. The first data for Ag and B concentration in human seminal plasma were proposed, although these results have not been verified. Our results also showed a significantly higher concentration of Cd in seminal plasma of smokers than in non-smokers and this value depended on the increasing number of cigarettes smoked per day ($p=0.004$).

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Table 1 Optimized instrumental conditions and measurement parameters used in different gas modes

ICP-MS Agilent 7500cx			
Gas mode	No-gas	Hydrogen	Helium
Parameter		Value	
RF Power / W	1550	1550	1550
Rf Matching / V	1.72	1.73	1.72
Smpl depth / mm	8.3	8.3	8.3
Torch-H / mm	0,3	0.3	0.3
Torch-V / mm	0	0	0
Carrier Gas / L min^{-1}	0.92	0.92	0.92
Makeup Gas / L min^{-1}	0.15	0.15	0.15
Nebulizer pump / Rps	0.08	0.08	0.08
SC Temp / °C	2	2	2
Extract lens 1 / V	0.5	0.5	0
Extract lens 2 / V	-126	-120	-120
Omega bias / V	-24	-24	-24
Omega lens / V	-0.6	-0.6	-0.6
Cell entrance / V	-26	-26	-26
Cell exit / V	-30	-48	-44
Quadrupole bias / V	-3	-14	-14.5
Octopole bias / V	-6	-18	-18
Gas flow / mL min^{-1}	Not used	3.8	3.1

Table 2 Program of the MILESTONE UltraCLAVE IV used for digestion of seminal plasma samples analysed by ICP-MS

Step	t (min)	E (W)	T1 (°C)	T2 (°C)	P (bar)
1	10	1000	80	60	100
2	10	500	130	60	100
3	4.5	1000	180	60	120
4	6.5	1000	220	60	130
5	20	1000	220	70	130
6	40	0	20	20	10

Table 3 Comparison of limit of detection (LOD) and quantification (LOQ) determined after dilution or digestion of the seminal plasma pooled sample

Element	LOD		LOQ	
	Dilution method	Digestion method	Dilution method	Digestion method
Ag (μg L^{-1})	0.001	0.006	0.004	0.021
As (μg L^{-1})	0.019	0.059	0.064	0.198
B (μg L^{-1})	0.127	0.889	0.423	2.964
Ca (mg L^{-1})	0.193	0.538	0.644	1.795
Cd (μg L^{-1})	0.003	0.013	0.012	0.044
Co (μg L^{-1})	0.006	0.016	0.022	0.053
Cr (μg L^{-1})	0.004	0.044	0.014	0.148
Cu (μg L^{-1})	0.192	1.491	0.639	4.969
Fe (μg L^{-1})	0.278	2.103	0.926	7.009
K (mg L^{-1})	0.445	1.578	1.484	5.259
Li (mg L^{-1})	0.017	0.130	0.056	0.434
Mg (mg L^{-1})	0.203	0.266	0.678	0.886
Mn (μg L^{-1})	0.027	0.040	0.091	0.132
Mo (μg L^{-1})	0.008	0.080	0.027	0.265
Na (mg L^{-1})	2.832	4.681	9.440	15.60
Ni (μg L^{-1})	0.011	0.005	0.037	0.018
Pb (μg L^{-1})	0.006	0.114	0.020	0.379
Se (μg L^{-1})	0.263	0.837	0.878	2.790
Sn (μg L^{-1})	0.007	0.057	0.024	0.191
Zn (mg L^{-1})	0.218	0.325	0.726	1.082

Table 4 Comparison of dilution and digestion methods for multielement determination using ClinChek serum control samples

	ClinChek Serum Control, Level 1			ClinChek Serum Control, Level 2		
Element	Certified value Mean (Range)	Measured value Mean ± SD		Certified value Mean (Range)	Measured value Mean ± SD	
		Diluted samples	Digested samples		Diluted samples	Digested samples
As (μg L^{-1})	14.3 (11.4-17.2)	14.2±0.3	13.7±0.8	27.5 (22.0-33.0)	26.8±0.6	26.3±1.6
Cd (μg L^{-1})	0.233 (0.163-0.303)	0.20±0.01	0.21±0.02	4.42 (3.32-5.53)	4.91±0.27	5.09±0.28
Co (μg L^{-1})	0.380 (0.228-0.532)	0.33±0.03	0.46±0.11	1.75 (1.23-2.28)	1.90±0.05	1.61±0.16
Cr (μg L^{-1})	1.15 (0.863-1.44)	1.28±0.04	1.18±0.23	6.54 (5.23-7.85)	6.29±0.12	6.6±0.45
Cu (μg L^{-1})	817 (654-980)	858±5	832±17	1625 (1300-1950)	1703±24	1569±28
Fe (μg L^{-1})	866 (779-953)	941±4	862±7	1698 (1528-1868)	1699±9	1589±2
Li (mg L^{-1})	NA	--	--	8.63 (7.77-9.49)	7.94±1.3*	8.9±0.87*
Mg (mg L^{-1})	12.5 (11.3-13.8)	12.9±0.4*	13.4±0.1*	26.1 (23.5-28.7)	25.7±0.9*	27.7±0.3*
Mn (μg L^{-1})	23.7 (19.0-28.4)	23.5±0.3	24.9±1.8	23.7 (19.0-28.4)	23.4±0.9	24.5±0.7
Mo (μg L^{-1})	1.08 (0.756-1.40)	1.19±0.17	0.96±0.26	5.22 (3.92-6.53)	5.70±0.26	5.62±0.35
Ni (μg L^{-1})	3.95 (2.96-4.94)	4.29±0.18	4.80±0.78	10.1 (8.08-12.1)	10.16±0.37	11.75±0.09
Se (μg L^{-1})	83.3 (62.5-104)	86.3±2.5	83.0±5.3	129 (103-155)	139±1.6	134.8±4.9
Sn (μg L^{-1})	0.980 (0.686-1.27)	0.74±0.06	0.77±0.06	77.2 (61.8-92.6)	78.63±1.61	84.00±0.80
Zn (μg L^{-1})	943 (754-1132)	937±11*	1029±13*	1884 (1601-2167)	1850±100*	1819±88*

*Measured according to aqueous calibration.

Table 5 Results of the analyses of reference materials Seronorm™ Trace Elements and ClinChek® Urine Control with respect to certified or approximate values

	Seronorm™ Trace Elements Serum			
	L-1		L-2	
Element	Certified value Approximate value[a] Mean (Range)	Measured value Mean ± SD	Certified value Approximate value[a] Mean (Range)	Measured value Mean ± SD
Ag (μg L^{-1})	0.16[a]	0.17±0.01	0.23[a]	0.24±0.02
As (μg L^{-1})	0.47[a]	1.60±0.08	0.67[a]	1.02±0.09
B (μg L^{-1})	154[a]	196±2	203[a]	200±1
Ca (mg L^{-1})	94.2 (88.5-99.9)	93.3±1.2*	145 (136-154)	135±1*
Cd (μg L^{-1})	0.126[a]	0.134±0.018	0.13[a]	0.13±0.01
Co (μg L^{-1})	1.2 (0.9-1.5)	1.1±0.1	3.2 (2.8 – 3.6)	2.9±0.3
Cr (μg L^{-1})	1.5 (1.1-1.9)	2.1±0.1	4.8 (4-5.6)	4.8±0.3
Cu (μg L^{-1})	1691 (1523-1859)	1666±80	2887 (2689-3085)	2794±195
Fe (μg L^{-1})	1.39 (1.23-1.55)	1.38±0.13	2.03 (1.77-2.29)	1.93±0.13
K (mg L^{-1})	135[a]	133±1*	240[a]	242±1*
Li (mg L^{-1})	5741 (5397-6085)	4959±33*	10950 (10293 - 11607)	9205±46*
Mg (mg L^{-1})	20.1 (18.6-21.6)	19.4±0.8*	40.8 (37.7-43.9)	38.8±0.4*
Mn (μg L^{-1})	15 (13.2-16.8)	14.4±1.0	19.9 (17.7-22.1)	19.0±1.0
Mo (μg L^{-1})	0.7[a]	0.6±0.1	0.9[a]	0.9±0.1
Na (mg L^{-1})	2998[a]	2957±143*	3736[a]	3823±5*
Ni (μg L^{-1})	5.8 (4.5-7.1)	5.9±0.5	9.8 (8.6-11)	10.0±0.3
Pb (μg L^{-1})	1.02[a]	1.01±0.1	1.11[a]	0.9±0.1
Se (μg L^{-1})	107 (93-121)	101±5	163(143-183)	140±2
Sn (μg L^{-1})	0.52[a]	0.51±0.1	0.51[a]	0.50±0.1
Zn (μg L^{-1})	1738 (1596-1880)	1647±8*	2520 (2108-2932)	2469±2*
	ClinChek® Urine Control			
	Level 1		Level 2	
As (μg L^{-1})	44.5 (35.7-53.5)	41.0±1.7	84.5 (67.6-101)	71.9±3.9
Cd (μg L^{-1})	2.42 (1.94-2.90)	2.5±0.1	14.7 (11.8-17.6)	15.2±0.7
Co (μg L^{-1})	2.25 (1.8-2.7)	2.3±0.07	35.6 (28.5-42.7)	34.5±1.7
Cr (μg L^{-1})	4.23 (3.30-5.08)	4.34±0.35	20.3 (16.2-24.4)	19.6±1.0
Cu (μg L^{-1})	58.6 (46.9-70.3)	60.6±2.0	111 (88.8-133)	108.2±5.7
Fe (μg L^{-1})	40.9 (32.7-49.1)	45.4±2.6	225 (180-270)	230.7±11
Mg (mg L^{-1})	20.5 (16.4-24.6)	19.1±0.6*	46.9 (39.9-53.9)	42.2±2.0*

Mn ($\mu g\ L^{-1}$)	4.18 (3.34-5.02)	4.35±0.15	19.7 (18.8-23.6)	19.5±1.0
Ni ($\mu g\ L^{-1}$)	6.22 (4.98-7.46)	7.3±1.3	44.7 (35.8-53.6)	43.3±0.1
Pb ($\mu g\ L^{-1}$)	24.0 (19.2-28.8)	25.3±1.0	64.3 (51.4-77.2)	62.4±3.1
Se ($\mu g\ L^{-1}$)	29.3 (23.4-35.2)	24.6±0.6	79.0 (63.2-94.8)	61.1±3.6
Sn ($\mu g\ L^{-1}$)	4.81 (3.85-5.77)	4.51±0.1	9.50 (7.60-11.4)	9.7±0.5
Zn ($\mu g\ L^{-1}$)	215 (161-269)	222±11	536 (429-643)	480±32

*Measured according to aqueous calibration

Table 6 Precision (RSD) and trueness (presented as the recovery) of spiked pooled seminal plasma samples spiked with 3 levels of added element concentrations, calculated on the basis of 8 replicate measurements

	Spike (Added concentration) (μg L^{-1})					
Element	Precision (%)	Trueness (%)	Precision (%)	Trueness (%)	Precision (%)	Trueness (%)
	0.05		0.1		0.4	
Ag	3.00	91	1,41	98	2.21	98
As	0.95	101	1.13	99	0.85	99
Cd	5.69	98	1.47	101	1.94	99
Co	3.23	101	0.64	100	1.39	101
Cr	7.61	101	6.75	99	1.55	99
Mn	0.71	103	1.01	96	1.50	97
Ni	1.64	103	0.78	99	1.23	99
Pb	2.29	97	6.85	107	1.43	101
Sn	4.60	96	5.89	105	1.95	98
	0.3		2		4	
B	0.54	92	0.87	88	2.74	88
Mo	0.36	99	1.28	98	1.23	100
Se	1.65	103	0.98	96	1.83	100
	1		7		10	
Cu	0.77	104	0.66	101	0.65	97
Fe	1.40	103	0.74	101	0.58	97
	30		300		400	
Li	1.42	103	0.93	100	0.29	100
	5000		10000		20000	
Ca	0.85	105	0.73	101	0.36	100
Mg	0.85	105	0.73	101	0.36	100
Zn	0.72	96	0.68	89	0.91	103
K	0.34	105	0.56	101	1.33	101
Na	0.26	104	0.50	102	1.62	100

Table 7 Mean±SE, median and 5th-95th percentile range for the element concentration in seminal plasma in 76 men, including 37 smokers and 39 non-smokers, and significance of the difference between the groups regarding smoking habits (p; t-test)

Element	Total (N=76)	Smokers (N=37)	Non-smokers (N=39)	p
Ag (μg L^{-1})	0.089±0.004	0.089±0.006	0.090±0.007	0.926
	0.086 (0.038-0.163)	0.087 (0.038-0.148)	0.081 (0.033-0.177)	
As (μg L^{-1})	2.235±0.344	2.389±0.520	2.088±0.459	0.605
	1.029 (0.249-8.754)	1.308 (0.249-10.829)	0.931 (0.246-6.318)	
B (μg L^{-1})	52.62±3.12	50.71±3.95	54.38±4.80	0.938
	50.44 (21.09-110.90)	45.90 (21.98-88.99)	53.79 (20.29-123.73)	
Ca (mg L^{-1})	260.2±9.7	254.8±12.8	260.3±12.2	0.653
	237.0 (155.8-425.9)	238.1 (130.3-385.7)	237.2 (158.3-414.2)	
Cd (μg L^{-1})	0.169±0.013	0.197±0.021	0.142±0.013	0.020
	0.127 (0.057-0.399)	0.148 (0.065-0.502)	0.118 (0.051-0.317)	
Co (μg L^{-1})	0.574±0.093	0.487±0.065	0.656±0.171	0.443
	0.415 (0.222-1.340)	0.427 (0.188-0.676)	0.413 (0.245-1.549)	
Cr (μg L^{-1})	0.517±0.045	0.434±0.035	0.535±0.069	0.133
	0.411 (0.216-1.138)	0.392 (0.216-0.962)	0.418 (0.208-1.482)	
Cu (μg L^{-1})	86.02±3.32	82.04±4.68	89.88±4.69	0.123
	83.21 (42.26-143.14)	79.76 (36.10-143.14)	85.68 (50.41-156.54)	
Fe (μg L^{-1})	101.89±3.79	104.63±5.09	99.36±5.61	0.296
	94.74 (62.31-156.41)	99.87 (55.88-156.41)	93.58 (62.31-197.91)	
K (mg L^{-1})	908.8±28.2	884.3±37.1	916.2±35.4	0.237
	866.9 (525.6-1388.5)	848.5 (506.2-1304.3)	866.9 (575.4-1363.0)	
Li (mg L^{-1})	5.404±1.168	4.454±1.581	6.280±1.716	0.971
	1.041 (<0.017-29.065)	0.939 (<0.017-30.20)	1.484 <0.017-29.06)	
Mg (mg L^{-1})	93.29±4.81	93.02±6.95	93.55±6.76	0.707
	83.21 (39.24-168.80)	85.22 (28.33-168.80)	82.33 (39.24-169.35)	
Mn (μg L^{-1})	4.026±0.252	3.873±0.307	4.175±0.400	0.439
	3.639 (1.424-9.160)	3.363 (1.191-6.768)	3.715 (1.424-10.975)	
Mo (μg L^{-1})	1.322±0.065	1.283±0.115	1.359±0.066	0.720
	1.210 (0.792-2.120)	1.082 (0.748-2.037)	1.264 (0.829-2.142)	
Na (mg L^{-1})	2663±20	2670±31	2657±25	0.769
	2674 (2354-2956)	2666 (2323-3002)	2681 (2397-2895)	
Ni (μg L^{-1})	1.709±0.121	1.656±0.121	1.762±0.210	0.686
	1.374 (0.942-3.916)	1.495 (0.985-3.916)	1.323 (0.902-6.492	
Pb (μg L^{-1})	0.363±0.041	0.313±0.037	0.410±0.070	0.653
	0.259 (0.071-1.161)	0.252 (0.015-0.565)	0.259 (0.071-1.619)	
Se (μg L^{-1})	46.41±1.99	46.97±4.20	45.43±2.42	0.926

	44.82 (23.17-85.28)	41.22 (19.74-94.70)	44.32 (26.34-82.32)	
Sn ($\mu g\ L^{-1}$)	0.221±0.022	0.207±0.027	0.234±0.034	0.482
	0.173 (0.050-0.625)	0.156 (0.044-0.464)	0.200 (0.050-0.649)	
Zn ($mg\ L^{-1}$)	132.03±7.88	134.61±12.00	129.58±10.44	0.953
	120.37 (38.34-265.82)	125.51 (30.94-278.92)	119.04 (44.40-256.80)	

Table 8a Summary data of element concentration in seminal plasma reported in the literature

Element	Technique	Subjects	N		Concentration	Country	Reference
As ($\mu g\ L^{-1}$)	ICP-MS	Normal	28	Mean±SD	53.16±16.39	China	21
		Abnormal	21		68.62±41.66		
	ICP-MS	Normozoospermic	28	Mean±SD	17.1±12.7	Japan	22
		Oligozoospermic	28		13.6±8.8		
		Azoospermic	28		11.4±8.0		
Ca ($mg\ L^{-1}$)	ICP-MS	Control / normal semen analysis	15	Mean (Range)	158 (89-219)	Italy	20
	ICP-MS	Normozoospermic	28	Mean±SD	230.41±78.33	Japan	22
		Oligozoospermic	28		403.81±82.68		
		Azoospermic	28		179.24±30.81		
	F-AAS	Fertile men	50	Median (IQR)		Denmark	26
		S-TTP			533 (450-672)		
		L-TTP			470 (391-541)		
	Colorimetric	Subfertile	103	Mean±SD	244.49±64.13	The Netherlands	7
		Fertile	107		240.48±56.11		
Cd ($\mu g\ L^{-1}$)	ICP-MS	Control / normal semen analysis	15	Mean (Range)	1 (<LOD-3)	Italy	20
	ICP-MS	Normozoospermic	28	Mean±SD	0.2±0.6	Japan	22
		Oligozoospermic	28		0.8±0.4		
		Azoospermic	28		0.5±0.3		
	AAS	Men with proven fertility	12	Mean±SD	0.38±0.64	Germany	35
		Normozoospermic	44		0.43±0.69		
		Unselected patients	118		0.44±0.73		
	AAS	IVF patients	91	Mean±SD	0.294±0.091	USA	16
				Median	0.280 (0.091-0.692)		

				(Range)			
	AAS	Normozoospermic	40	Mean (CI)	1100 (1010-1180)	Nigeria	17
		Oligozoospermic	40		650 (590-710)		
		Azoospermic	20		1570 (1440-1830)		
Co ($\mu g\ L^{-1}$)	ICP-MS	Normozoospermic	28	Mean±SD	1.4±1.3	Japan	22
		Oligozoospermic	28		7.2±9.5		
		Azoospermic	28		5.3±8.2		
	ICP-MS	Patients with metal-on-metal total hip arthroplasty	11	Median (Range)	2.89 (1.15-6.1)	Canada	27
		Control	5		1.12 (0.71-1.94)		
Cr ($\mu g\ L^{-1}$)	ICP-MS	Control / normal semen analysis	15	Mean (Range)	1.2 (0.2-2)	Italy	20
	ICP-MS	Normozoospermic	28	Mean±SD	32.2±16.2	Japan	22
		Oligozoospermic	28		19.9±4.8		
		Azoospermic	28		19.4±8.3		
	ICP-MS	Patients with metal-on-metal total hip arthroplasty	11	Median (Range)	0.57 (0.06-1.995)	Canada	27
		Control	5		0.06 (0.06-1.35)		
Cu ($\mu g\ L^{-1}$)	ICP-MS	Control / normal semen analysis	15	Mean (Range)	75 (8-135)	Italy	20
	ICP-MS	Normal	28	Mean±SD	195.00±45.24	China	21
		Abnormal	21		222.76±0.91		
	ICP-MS	Normozoospermic	28	Mean±SD	570.3±214.2	Japan	22
		Oligozoospermic	28		760.1±685.4		
		Azoospermic	28		443.2±268.1		
	F-AAS	Oligozoospermic	17	Mean±SD	566.2±325.2	India	32
		Oligoasthenozoo-	14		604.7±218.4		

		spermic					
		Asthenozoospemic	12		622.9±424.4		
		Azoospermic	7		387.6±81.3		
	AAS	Subfertile	103	Mean±SD	349.52±247.84	The Netherlands	7
		Fertile	107		374.94±235.13		
	AAS	Normozoospermic	41	Mean±SD	$(173.29\pm19.3)*10^3$	Iraq	31
		Oligozoospermic	43		$(169.52\pm15.77)*10^3$		
		Azoospermic	35		$(128.34\pm21.83)*10^3$		
	AAS	Fertile	75	Mean±SD	28.7±10.7	India	29
		Normozoospermic	25		15.5±5.0		
		Oligozoospermic	25		10.5±4.9		
		Asthenozoospermic	25		11.4±4.5		
Fe ($\mu g\ L^{-1}$)	ICP-MS	Normozoospermic	28	Mean±SD	$(13.88\pm8.44)*10^3$	Japan	22
		Oligozoospermic	28		$(17.74\pm8.23)*10^3$		
		Azoospermic	28		$(16.21\pm8.27)*10^3$		
	F-AAS	Oligozoospermic	17	Mean±SD	$(46.67\pm15.95)*10^3$	India	32
		Oligoasthenozoo-spermic	14		$(35.73\pm16.52)*10^3$		
		Asthenozoospemic	12		$(43.10\pm18.42)*10^3$		
		Azoospermic	7		$(46.35\pm15.87)*10^3$		
	AAS	Fertile	75	Mean±SD	68.0±17.8	India	29
		Normozoospermic	25		60.6±10.4		
		Oligozoospermic	25		59.7±5.7		
		Asthenozoospermic	25		51.5±5.9		
K ($mg\ L^{-1}$)	ICP-MS	Normozoospermic	28	Mean±SD	>3000	Japan	22
		Oligozoospermic	28		>3000		

		Azoospermic	28		>3000		
Li (mg L^{-1})	ICP-MS	Normozoospermic	28	Mean±SD	0.479±0.213	Japan	22
		Oligozoospermic	28		0.372±0.072		
		Azoospermic	28		0.316±0.038		
Mg (mg L^{-1})	ICP-MS	Control / normal semen analysis	15	Mean (Range)	75 (37-151)	Italy	20
	ICP-MS	Fertile men	50	Median (IQR)		Denmark	26
		S-TTP			86 (57-134)		
		L-TTP			100 (56-118)		
	ICP-MS	Normozoospermic	28	Mean±SD	90.22±19.48	Japan	22
		Oligozoospermic	28		20.09±6.56		
		Azoospermic	28		78.30±18.03		
	F-AAS	Oligozoospermic	17	Mean±SD	14.34±4.60	India	32
		Oligoasthenozoospermic	14		12.29±6.84		
		Asthenozoospemic	12		13.63±0.68		
		Azoospermic	7		8.55±2.72		
	AAS	Normozoospermic	41	Mean±SD	126.2±22.8	Iraq	31
		Oligozoospermic	43		57.0±6.5		
		Azoospermic	35		55.9±6.9		
	Colorimetric	Subfertile	103	Mean±SD	87.52±26.74	The Netherlands	7
		Fertile	107		89.95±24.31		
Mn (μg L^{-1})	ICP-MS	Control / normal semen analysis	15	Mean (Range)	5 (2-10)	Italy	20
	ICP-MS	Normal	28	Mean±SD	103.96±25.00	China	21
		Abnormal	21		127.57±33.69		

	ICP-MS	Normozoospermic	28	Mean±SD	7.4±4.6	Japan	22
		Oligozoospermic	28		6.5±1.3		
		Azoospermic	28		5.8±2.2		
Mo (μg L^{-1})	ICP-MS	Normozoospermic	28	Mean±SD	11.2±23.0	Japan	22
		Oligozoospermic	28		3.1±1.4		
		Azoospermic	28		3.2±1.2		
	ICP-MS	Patients with metal-on-metal total hip arthroplasty	11	Median	1.44	Canada	27
		Control	5		1.41		
Na (mg L^{-1})	ICP-MS	Normozoospermic	28	Mean±SD	>3000	Japan	22
		Oligozoospermic	28		>3000		
		Azoospermic	28		>3000		
Ni (μg L^{-1})	ICP-MS	Control / normal semen analysis	15	Mean (Range)	6 (2-11)	Italy	20
Pb (μg L^{-1})	ICP-MS	Control / normal semen analysis	15	Mean (Range)	3 (<LOD-12)	Italy	20
	ICP-MS	Normal	28	Mean±SD	74.84±22.41	China	21
		Abnormal	21		84.00±5.56		
	ICP-MS	Normozoospermic	28	Mean±SD	36.1±15.3	Japan	22
		Oligozoospermic	28		79.2±32.2		
		Azoospermic	28		47.4±13.1		
	AAS	Control subjects / no occupational exposure to metals	35	Median (Range)	8.6 (4.2-16.6)	Croatia	13
	AAS	IVF patients	74	Mean±SD	395.0±359.7	USA	16
				Median (Range)	282.8 (<10-1650)		
	AAS	Male partners of infertile couples	341	Mean±SD	2.19±1.45	Taiwan	19
				(Range)	(0.08-9.50)		

Se ($\mu g\ L^{-1}$)	ICP-MS	Control / normal semen analysis	15	Mean (Range)	77 (40-123)	Italy	20
	ICP-MS	Normal	28	Mean±SD	73.16±20.35	China	21
		Abnormal	21		103.79±37.89		
	ICP-MS	Normozoospermic	28	Mean±SD	61.8±65.4	Japan	22
		Oligozoospermic	28		51.4±46.4		
		Azoospermic	28		39.1±53.0		
Sn ($\mu g\ L^{-1}$)	ICP-MS	Normozoospermic	28	Mean±SD	119.5±8.2	Japan	22
		Oligozoospermic	28		208.4±221.8		
		Azoospermic	28		102.6±84.3		
Zn ($mg\ L^{-1}$)	ICP-MS	Control / normal semen analysis	15	Mean (Range)	97 (80-172)	Italy	20
	ICP-MS	Fertile men	50	Median (IQR)		Denmark	26
		S-TTP			106 (72-183)		
		L-TTP			113 (68-212)		
	ICP-MS	Normozoospermic	28	Mean±SD	6.798±5.012	Japan	22
		Oligozoospermic	28		10.87±7.712		
		Azoospermic	28		9.300±6.480		
	AAS	Referred for fertility treatment	1178	Mean (IQR)	117.6 (75.19-176.53)	Canada	15
	AAS	IVF patients	83	Mean±SD	50.800±24.485	USA	16
				Median (Range)	43.74 (10.53-102.97)		
	AAS	Fertile	75	Mean±SD	$(17.8\pm4.4)*10^{-3}$	India	29
		Normozoospermic	25		$(10.9\pm4.7)*10^{-3}$		
		Oligozoospermic	25		$(9.5\pm4.5)*10^{-3}$		
		Asthenozoospermic	25		$(8.8\pm3.9)*10^{-3}$		

	AAS	Normozoospermic	37	Mean±SD	1012.0±22.4	Ghana	30
		Oligozoospermic	46		977.5±21.7		
		Azoospermic	7		946.2±67.4		
	F-AAS	Subfertile	103	Mean±SD	91.53±45.77	The Netherlands	7
		Fertile	107		91.53±45.77		
	F-AAS	Oligozoospermic	17	Mean±SD	4.62±1.78	India	32
		Oligoasthenozoospermic	14		10.56±42.8		
		Asthenozoospermic	12		6.60±33.05		
		Azoospermic	7		5.02±15.48		

S-TTP = short time-to-pregnancy (1-2 months); L-TTP = long time-to-pregnancy (7.5-11.5 months); IQR = inter-quartile range; CI = confidence interval

Table 8b Summary data of element concentration in seminal plasma reported in the literature regarding smoking habits

Element	Technique	Subjects	N		Concentration	Country	Reference
Ca (mg L^{-1})	AAS	Fertile non-smokers	19	Mean±SD	244.9±55	Iran	18
		Fertile smokers	17		233.1±79.1		
		Infertile non-smokers	21		219.6±68.1		
		Infertile smokers	15		198.1±54.6		
Cd ($\mu g\ L^{-1}$)	AAS	Normozoospermic non-smokers		Mean±SD	0.42±0.67	Germany	35
		Normozoospermic smokers			0.55±0.81		

	AAS	Nonsmokers	42	Median (Range)	0.54 (0.17-1.67)	Croatia	12
		Never smoked	33		0.48 (0.17-1.09)		
		Former smokers	9		0.62 (0.30-1.67)		
		Smokers	78		0.85 (0.29-3.56)		
	AAS	Control smokers	23	Mean±SD	18.7±5.0	Turkey	14
		Control non-smokers	22		12.4±2.7		
		Infertile smokers	26		33.2±12.8		
		Infertile non-smokers	24		17.9±5.4		
	AAS	Infertile smokers	16	Median (IQR)	0.279 (0.257-0.356)	USA	36
		Infertile non-smokers	107		0.285 (0.243-0.347)		
		General population - smokers	10		0.092 (0.082-0.118)		
		General population – non-smokers	21		0.086 (0.073-0.105)		
Mg (mg L^{-1})	AAS	Fertile non-smokers	19	Mean±SD	68.5±18.9	Iran	18
		Fertile smokers	17		55.8±22.5		
		Infertile non-smokers	21		58.3±21.5		
		Infertile smokers	15		50.7±21.3		
Na (mg L^{-1})	AAS	Fertile non-smokers	19	Mean±SD	2227.8±434.9	Iran	18
		Fertile smokers	17		2103.9±363.6		
		Infertile non-	21		2086±339		

		smokers					
		Infertile smokers	15		2065.5±313.6		
Pb (μg L^{-1})	AAS	Control smokers	23	Mean±SD	293.0±38.3	Turkey	14
		Control non-smokers	22		230.9±46.4		
		Infertile smokers	26		473.8±76.0		
		Infertile non-smokers	24		283.3±38.7		
Zn (mg L^{-1})	AAS	Fertile non-smokers	19	Mean±SD	140.8±20.1	Iran	18
		Fertile smokers	17		124.3±26.4		
		Infertile non-smokers	21		103.2±29.8		
		Infertile smokers	15		80.7±26.5		
	F-AAS	Fertile non-smokers	80	Mean±SD (Range)	139.51±7.78 (127.4-153.6)	Egypt	34
		Fertile smokers	80		101.21±11.03 (80.4-121.4)		
	Spectro-photometry	Men with suspected infertility		Mean±SD		China	33
		Non-smokers	79		155.6±68.0		
		Smokers	68		120.3±55.6		

IQR = inter-quartile range

Figure 1 Recovery of seminal plasma samples spiked with 0.01 μg L^{-1} for Ag, 0.05 μg L^{-1} for Cd, Co, Cr, Mo and Sn, 0.1 μg L^{-1} for B, Mn and Ni, 0.2 μg L^{-1} for As and Se, 7 μg L^{-1} for Cu and Fe, 150 μg L^{-1} for Li and Zn, 1 mg L^{-1} for Ca and Mg, 4 mg L^{-1} for K and 7 mg L^{-1} for Na.

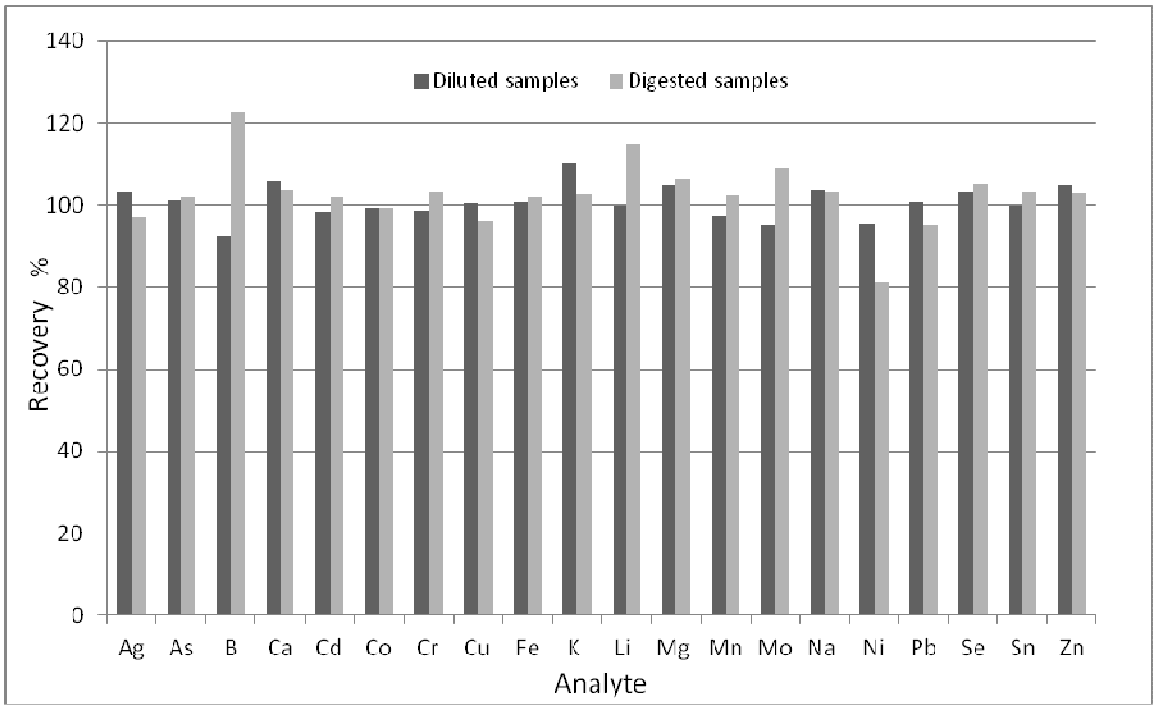

Figure 2 Seminal plasma Cd values with regard to the number of cigarettes smoked per day in 76 men (37 smokers and 39 non-smokers)